

1 COMPARATIVE FLORAL ECOLOGY OF BICOLOUR AND CONCOLOUR 2 MORPHS OF *VIOLA PEDATA* L. (VIOLACEAE) FOLLOWING CONTROLLED 3 BURNS

4 Peter Bernhardt¹, Retha Edens-Meier², Downen Jocson¹, Justin Zweck¹, Zong-Xin Ren³, Gerardo R.
5 Camilo¹, Michael Arduser⁴

6 ¹Department of Biology, Saint Louis University, St. Louis, MO, USA 63103

7 ²School of Education, Saint Louis University, St. Louis, MO, USA 63103

8 ³Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, 132 Lanhei
9 Road, Kunming, Yunnan 650201, P. R. China

10 ⁴325 Atalanta Ave., Webster Groves, MO, USA 63119

11 *Abstract*—We compared pollinators, pollination rates and seed set of bicolour and concolour morphs in self-
12 incompatible, *Viola pedata* over two seasons. The two populations grew on a wooded slope (CR) vs. an exposed
13 glade (SNR) and were of unequal sizes. Both were burned in 2014. The number of flowers produced by concolour
14 plants at SNR was higher in 2014 while the number of flowering bicolour plants increased significantly at CR in
15 2015. Petal temperatures, regardless of site, showed that the dark purple, posterior petals of bicolours were
16 consistently warmer than their own mauve-lilac, anterior (lip) petals and the all mauve petals of concolours. Major
17 pollen vectors were polylectic/polyphagic bees (Andrenidae, Apidae and Halictidae) but females of *Andrena carlinii*
18 dominated at both sites. Bees foraged on flowers upside down or right side up but neither mode correlated with
19 either morph. Bees foraged preferentially on concolour at both sites. Pollen tube counts were higher in concolours at
20 both sites with a marginally greater number of pollen tubes penetrating concolour ovules regardless of site or year.
21 While both populations produced more seeds in 2014 SNR plants always produced more seeds than CR plants.
22 The increasing numbers of bicolour plants at CR in 2015 suggested that bicolours may equal or outnumber
23 concolours as dark petals offer additional warmth to ecto-thermic pollinators foraging in a cooler, shady forest vs.
24 an open, sunny glade. Subtle environmental factors may give a floral trait a selective advantage influencing fitness
25 when an unbalanced polymorphism persists in discrete and localized populations.

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

27 INTRODUCTION

28 Colour polymorphisms have been well documented in
29 flowers of unrelated species. Unlike plants with heterostylous
30 flowers (e.g. *Linum*, see Armbruster et al. 2006) most colour
31 morphs are interpreted as unbalanced polymorphisms (sensu
32 Futuyma 2013) as the frequencies of 2-4 colour morphs vary
33 broadly with natural distribution over time (Irwin & Strauss
34 2005; Pellegrino et al. 2008). A population's shift in colour
35 morph frequencies may have more than one explanation
36 (Rausher 2008). While the population's response to the
37 selective foraging of its dominant pollinators is anticipated
38 (Epperson & Clegg 1987; Irwin & Strauss 2005; Malberla &
39 Nattero 2011; Russell et al. 2016) there are other factors.
40 These may include florivory/herbivory (Carlson &
41 Holsinger 2013; de Jager & Ellis 2014; Sobral et al. 2016),
42 differential rates of self-pollination in discrete morphs (Fehr
43 & Rausher 2004), variation in inflorescence display (Gomez
44 2000), genetic trends that cause unidirectional changes in

45 pigments (Rausher 2008) and/or an indirect response to
46 selection related to pleiotropic, non-floral traits (Armbruster
47 2002).

48 Curiously, *Viola* species have not been used to study
49 variation in frequencies of floral colours although they are
50 recorded throughout the genus in North America and
51 Europe (McKinney 1992; Hildebrandt et al 2006; Mereda
52 et al. 2008; Pellegrino et al 2008; Marcussen & Borgen
53 2011). Instead flowers of *Viola* species were more likely to
54 be used as model systems in genetic variation (Clausen 1926;
55 Culley 2002), systematic variation (Nieuwland & Kaczmarek
56 1914; McKinney 1992), developmental morphology (Johri
57 et al 1992; Weberling, 1989), molecular development
58 (Wang 2008) and reproductive ecology (Gurevitch et al.
59 2006; Winn & Moriuchi 2009). In some *Viola* species
60 endemic to Europe petal colour frequency is predictable
61 according to whether populations grow on old soils polluted
62 by zinc or lead (Hildebrandt et al. 2006). Gradations in
63 petal colour in the "zinc" violets are also results of a past
64 history of interspecific introgression (Migdalek et al. 2013).

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

Received 26 July 2016, accepted 03 October 2016

*Corresponding author: bernhap2@slu.edu

67 their pollination ecology as interpretations of floral
68 adaptations in their chasmogamous flowers started in the
69 19th century (Darwin 1876; Müller 1883). However the
70 much later work of Beattie (1969; 1971a; 1972; 1974;
71 1976) remains the definitive introduction to pollination
72 mechanisms and breeding systems in this genus. Specifically,
73 Beattie's observations showed that *Viola* petals and pedicels
74 changed positions and angles over their respective lifespans
75 encouraging visits by many pollinators representing at least
76 three insect Orders (Diptera, Hymenoptera, Lepidoptera).
77 Some insects were more likely to forage for nectar in an
78 inverted position acquiring ventral depositions of pollen
79 (sternotribic) while others foraged after landing prone on the
80 liplike anterior petal receiving dorsal depositions
81 (nototribic). Beattie (1974) attributed these foraging
82 behaviors to the evolution of two overlap-ping syndromes
83 based on floral architecture, petal ornamentation and
84 modifications of the terminal surfaces of anthers and pistils.

85 With more than 400 species in the genus *Viola*
86 (Mabberley, 1997) it is not surprising that a few recent
87 studies challenge the earlier descriptions of generalist
88 entomophily reported by Davidse (1968) and Beattie (1969;
89 1971; 1974). Herrera (1990; 1993) concluded that floral
90 traits of *V. cazorensis* most probably evolved under
91 disruptive selection as only one hawkmoth, *Macroglossum*
92 *stellatarum* was the primary pollinator. Freitas & Sazima
93 (2003) found that flowers of two, Neotropical, high
94 elevation species produced little or no nectar and depended
95 primarily on pollen harvesting bees (*Anthenoides*,
96 Andrenidae). This conflicts with the generalization that
97 insects forage on *Viola* flowers for nectar exclusively, and
98 this ends in the passive deposition of pollen onto the vector's
99 body (sensu Bernhardt 1996).

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

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

126 the pollen of the host flower. Gibson & Davie (1901)
127 proposed that the species was pollinated by a combination of
128 Lepidoptera and long tongue bees. Beattie (1974) was far
129 more specific recording 63% of all visits to the flower by
130 bees in the family, Andrenidae (species unidentified) and
131 21% to Lepidoptera including day-flying hawkmoths
132 (*Hemaris*; Sphingidae). In contrast, Carroll & Goldman
133 (1994) described a more specialized system in Missouri
134 based on small Lepidoptera. For Beattie (1974), floral
135 presentation in *V. pedata* expressed intermediate floral
136 characters based on its floral architecture and observations of
137 how insects foraged on the flower. About 36% of all flowers
138 of *V. pedata* received insects landing on the anterior (lip-
139 like) petal leading to dorsal (nototribic) depositions of
140 pollen while 64% foraged in an inverted manner leading to
141 ventral (sternotribic) depositions.

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

166 MATERIALS AND METHODS

167 *Study sites and field states*

168 The first site used from 5/2/13 – 5/7/13, from
169 4/14/14 – 5/27/14 and from 4/2/15 – 6/3/15 was on
170 Oak Ridge, adjacent to the 11 km Sugar Creek trail within
171 Cuivre River State Park (CR), Lin-coln County, Missouri.
172 Less than 200 flowering plants of *V. pedata* were found on
173 the slope under mixed hardwood (*Quercus* dominated)
174 forest annually for all three seasons (Fig. 1). The site is
175 usually burned in late winter in alternate years by park staff
176 and was burned in 2014 but not in 2013 or 2015. Voucher
177 specimens were deposited in the herbarium of the Missouri
178 Botanical Garden (MO).

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



183

184

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



185

186

187

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).

188 highly drained, sandy soil lower edges of the dolomite glade
 189 (Crescent Glade) dominated by grasses and forbs with some
 190 shrubs (*Rhus* species). In 2014 and 2015 this site produced
 191 > 1,000 flowering, basal rosettes of *Viola pedata*. The site
 192 was burned in 2014 by Shaw employees but not in 2015.
 193 Burns are conducted in the winter at 3-year intervals,
 194 coinciding with the burning of surrounding woodlands.
 195 However, some years do not burn well due to the sparseness
 196 of fuel in a true glade flora. Prior to 2014 this site was
 197 burned last in 2010 (James Trager, personal
 198 communication). Voucher specimens were deposited as
 199 above. Pooled field observations, bagging and collection

200 hours (see below) at both sites by co-authors from 2013 –
 201 2015 totalled approximately 112 hours.

202 *Morphs frequency and flowers per morph*

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

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

217 *Attractants, rewards and petal temperatures*

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

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

251 *Floral foragers*

252 Insect visitors were observed *in situ* at both sites. Videos
 253 of floral foragers were made by R. Edens-Meier using a Sony
 254 Full HD 1080 Handycam, HDR-CX760V, 24.1
 255 Megapixels at the CR site. Three of these videos are shown
 256 on Youtube; [https://www.youtube.com/results?](https://www.youtube.com/results?search_query=viola+pedata)
 257 [search_query=viola+pedata](https://www.youtube.com/results?search_query=viola+pedata). Insect foragers were netted only
 258 when they were observed visiting one or more flowers of *V.*
 259 *pedata* and could be observed extending proboscides down
 260 the floral tube or manipulating anthers. From 4/6 –
 261 4/7/2013 at CR and from 2014 -2015 all insects collected
 262 were euthanized using fumes of ethyl acetate and they were
 263 pinned, labelled and identified. From 2014-2015 insects
 264 were always euthanized in separate jars according to the
 265 morph on which they were captured.

266 *Pollen load analyses*

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



269
 270
 271
 272
 273
 274
 275
 276
 277
 278
 279
 280
 281
 282
 283
 284
 285
 286
 287
 288
 289
 290
 291
 292
 FIGURE 3. Bicolour flower of *Viola pedata*, with thermocoupler probe inserted through the posterior petals (R. Edens-Meier, photographer).



273
 274
 275
 276
 277
 278
 279
 280
 281
 282
 283
 284
 285
 286
 287
 288
 289
 290
 291
 292
 FIGURE 4. Concolour flower of *Viola pedata* (R. Edens-Meier, photographer).

293
 294
 295
 296
 297
 298
 299
 300
 301
 302
 303
 304
 305
 306
 307
 308
 309
 310
 311
 312
 313
 314
 315
 316
 317
 318
 319
 320
 321
 322
 323
 324
 325
 326
 327
 328
 329
 330
 331
 332
 333
 334
 335
 336
 337
 338
 339
 340
 341
 342
 343
 344
 345
 346
 347
 348
 349
 350
 351
 352
 353
 354
 355
 356
 357
 358
 359
 360
 361
 362
 363
 364
 365
 366
 367
 368
 369
 370
 371
 372
 373
 374
 375
 376
 377
 378
 379
 380
 381
 382
 383
 384
 385
 386
 387
 388
 389
 390
 391
 392
 393
 394
 395
 396
 397
 398
 399
 400
 401
 402
 403
 404
 405
 406
 407
 408
 409
 410
 411
 412
 413
 414
 415
 416
 417
 418
 419
 420
 421
 422
 423
 424
 425
 426
 427
 428
 429
 430
 431
 432
 433
 434
 435
 436
 437
 438
 439
 440
 441
 442
 443
 444
 445
 446
 447
 448
 449
 450
 451
 452
 453
 454
 455
 456
 457
 458
 459
 460
 461
 462
 463
 464
 465
 466
 467
 468
 469
 470
 471
 472
 473
 474
 475
 476
 477
 478
 479
 480
 481
 482
 483
 484
 485
 486
 487
 488
 489
 490
 491
 492
 493
 494
 495
 496
 497
 498
 499
 500
 501
 502
 503
 504
 505
 506
 507
 508
 509
 510
 511
 512
 513
 514
 515
 516
 517
 518
 519
 520
 521
 522
 523
 524
 525
 526
 527
 528
 529
 530
 531
 532
 533
 534
 535
 536
 537
 538
 539
 540
 541
 542
 543
 544
 545
 546
 547
 548
 549
 550
 551
 552
 553
 554
 555
 556
 557
 558
 559
 560
 561
 562
 563
 564
 565
 566
 567
 568
 569
 570
 571
 572
 573
 574
 575
 576
 577
 578
 579
 580
 581
 582
 583
 584
 585
 586
 587
 588
 589
 590
 591
 592
 593
 594
 595
 596
 597
 598
 599
 600
 601
 602
 603
 604
 605
 606
 607
 608
 609
 610
 611
 612
 613
 614
 615
 616
 617
 618
 619
 620
 621
 622
 623
 624
 625
 626
 627
 628
 629
 630
 631
 632
 633
 634
 635
 636
 637
 638
 639
 640
 641
 642
 643
 644
 645
 646
 647
 648
 649
 650
 651
 652
 653
 654
 655
 656
 657
 658
 659
 660
 661
 662
 663
 664
 665
 666
 667
 668
 669
 670
 671
 672
 673
 674
 675
 676
 677
 678
 679
 680
 681
 682
 683
 684
 685
 686
 687
 688
 689
 690
 691
 692
 693
 694
 695
 696
 697
 698
 699
 700
 701
 702
 703
 704
 705
 706
 707
 708
 709
 710
 711
 712
 713
 714
 715
 716
 717
 718
 719
 720
 721
 722
 723
 724
 725
 726
 727
 728
 729
 730
 731
 732
 733
 734
 735
 736
 737
 738
 739
 740
 741
 742
 743
 744
 745
 746
 747
 748
 749
 750
 751
 752
 753
 754
 755
 756
 757
 758
 759
 760
 761
 762
 763
 764
 765
 766
 767
 768
 769
 770
 771
 772
 773
 774
 775
 776
 777
 778
 779
 780
 781
 782
 783
 784
 785
 786
 787
 788
 789
 790
 791
 792
 793
 794
 795
 796
 797
 798
 799
 800
 801
 802
 803
 804
 805
 806
 807
 808
 809
 810
 811
 812
 813
 814
 815
 816
 817
 818
 819
 820
 821
 822
 823
 824
 825
 826
 827
 828
 829
 830
 831
 832
 833
 834
 835
 836
 837
 838
 839
 840
 841
 842
 843
 844
 845
 846
 847
 848
 849
 850
 851
 852
 853
 854
 855
 856
 857
 858
 859
 860
 861
 862
 863
 864
 865
 866
 867
 868
 869
 870
 871
 872
 873
 874
 875
 876
 877
 878
 879
 880
 881
 882
 883
 884
 885
 886
 887
 888
 889
 890
 891
 892
 893
 894
 895
 896
 897
 898
 899
 900
 901
 902
 903
 904
 905
 906
 907
 908
 909
 910
 911
 912
 913
 914
 915
 916
 917
 918
 919
 920
 921
 922
 923
 924
 925
 926
 927
 928
 929
 930
 931
 932
 933
 934
 935
 936
 937
 938
 939
 940
 941
 942
 943
 944
 945
 946
 947
 948
 949
 950
 951
 952
 953
 954
 955
 956
 957
 958
 959
 960
 961
 962
 963
 964
 965
 966
 967
 968
 969
 970
 971
 972
 973
 974
 975
 976
 977
 978
 979
 980
 981
 982
 983
 984
 985
 986
 987
 988
 989
 990
 991
 992
 993
 994
 995
 996
 997
 998
 999
 1000

293 *Landing orientation and foraging bouts on* 294 *morphs by bees*

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

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

322 *Natural rates of ovule number, pollination (tubes* 323 *penetrating pistil tissue) and seed set*

324 Pedicels of both morphs in both populations were
325 selected at random and tagged with jeweller's tags while
326 flowers were in bud each week. As the populations at each
327 site remained in flower less than three weeks each year
328 tagging occurred over a two-week period at both sites in
329 2014 and 2015. Only one pedicel/basal rosette was tagged
330 and the perianth of the tagged bud was allowed to complete
331 its floral lifespan. Each bicour or concolour flower was
332 collected one to three days after we observed the wilting of
333 petals. The wilted flower was fixed in 3:1 95%
334 ethanol:glacial acetic acid for 2-6 hours then preserved in
335 70% ethanol. Pistils were excised, softened in sodium sulfite
336 solution under incubation and squashed in decolourized
337 aniline blue prior to viewing under epifluorescence using the
338 Zeiss Axioskop 40 as described in Edens-Meier et al. (2010)
339 to view pollen tubes germinating on the stigmas and
340 penetrating styles, ovaries and ovules. Due to the rigidity of
341 the style each pistil had to be softened for 60 minutes at
342 42°C but, as the stigma and style are so small, it was not
343 necessary to split them lengthwise to view pollen tube
344 progress. The ovary was butterflied with a scalpel, prior to
345 squashing to permit a count of the number of ovules and
346 observe pollen tube penetrations of micropyles.

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

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

356 *Statistical analyses*

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

367 RESULTS

368 *Morphs' frequency and the number of flowers per* 369 *morph*

370 At the CR (Cuivre River) site in 2014 (burn year) we
371 counted a total of 28 bicour morph plants and 32 plants
372 with concolour flowers (approximately 1:1.02 morph ratio).
373 The difference in morph ratios was not significant (binomial
374 test, $P = 0.5654$). In 2015 we counted 161 plants
375 producing bicour flowers and 88 producing concolours,
376 resulting in a ratio of 1.8: 1.0 respectively. This ratio was a
377 significant deviation from one to one ($P < 0.0001$). At the
378 SNR (Shaw Nature Reserve) in 2014 and 2015, we stopped
379 counting flowering basal rosettes after 1000 and morph
380 ratios remained self-consistent in both seasons (bicour
381 40.0: concolour 1.0). In 2015, the main area where bicour
382 was most common, produced 25 bicour plants and 71
383 concolour plants.

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

393 *Floral phenology and presentation (attractants,* 394 *rewards and petal warmth)*

395 Populations at both sites remained in bloom for 14 to
396 20 days each year, flowering from mid-April to early May.
397 Once the corolla of either morph opened at each site it
398 wilted within seven days or less. Melanism in the bicour
399 flower varied at the CR site where they were most common
400 in 2015. In most plants only the two, top posterior flowers
401 were dark purple but we found some other specimens in
402 which melanism extended to the tips of the lateral petals as
403 described and illustrated in the two species of Eurasian,
404 melanism pansies by Clausen (1926). We also found one
405 bicour plant in which all five petals showed some degree of
406 melanism. In the absence of lateral beards the anther cone

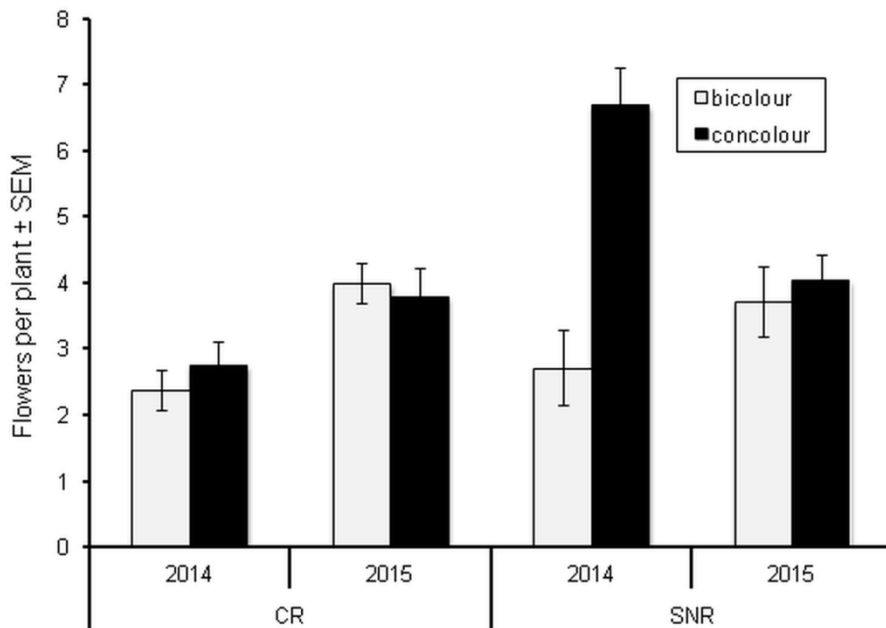


FIGURE 5. Mean number of flowers per plant of both morphs of *Viola pedata* at two sites and two years (2014 and 2015), Missouri, USA.

407

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

412 No discernible scent was detected in the flowers of either
413 morph at either site and that includes sampling flowers in
414 capped vials. Dissections of both morphs showed that each
415 of the two, lower, anther connectives in each flower wore a
416 large, elongated, green gland that protruded up to 66% the
417 length of each narrowly, keeled spur. Spur lengths of
418 bicolour and concolour morphs at SNR were greater than
419 5.0mm, but differences between morphs were not statistically
420 significant ($t = 0.81$, $P = 0.427$); (concolour, 5.2 ± 0.70 ; $N = 18$) and (bicolour 5.4 ± 0.71 ; $N = 0.71$).

422 Petal temperature data at both sites was pooled.
423 Regardless of colour morph the two, top (posterior) petals
424 were anywhere between 1 – 3 degrees warmer than the lower,
425 nodding, and often shaded, anterior (lip) petal ($F = 6.36$,
426 $P = 0.0179$). For the concolour morph, though, there was no
427 statistically significant difference between either of the two,
428 petal temperatures. In contrast, the deep purple, posterior
429 petals were always ~3 degrees warmer than the flower's
430 anterior petal and 2 degrees warmer than the posterior petals
431 of any concolour flower at either site ($t = 2.26$, $P =$
432 0.0317). Thus, it seems likely that the overall temperature
433 differences we recorded were driven by the bicolour morph.
434 Under shady conditions (e.g., under CR tree canopy or
435 cloudy periods at SNR) the differential between the
436 posterior and anterior petals decreased in magnitude but
437 remained consistent.

438 Forager and foraging diversity, and pollen load 439 analyses

440 Collections of insects at CR began on 5/2/13 when we
441 observed bees on both morphs. We netted and euthanized
442 12 specimens that day and all were identified as females of
443 *Andrena carlinii* but we did not record the morphs on which

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

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

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

481

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

482

*NA = Not assessed

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

485 We observed a total of 39 foraging bouts at the CR site
486 by females of *A. carlinii* from 4/10 – 5/2/2015. On
487 4/14/15 we observed that one, female, *A. carlinii* visited 31
488 flowers on 27 basal rosettes over a 20 minute period. It
489 visited 4 bicolor flowers and 10 concolor flowers before
490 resting on an inflorescence of an *Antennaria* species and
491 probing the florets ending first bout. It then began a
492 second bout returning to *V. pedata*, less than 30 seconds
493 later, to forage on 10 bicolor flowers and seven concolor
494 flowers before leaving the site.

495 In 2014 the ratio of concolor to bicolor flowers was
496 almost equal at the CR site (see above). In that season, we
497 observed that bees of four species and varying sizes visited
498 17 flowers of bicolor and 25 flowers of concolor. At the
499 same site in 2015, when bicolor morphs greatly
500 outnumbered concolours (see above), we observed bees of
501 different species and sizes visiting 51 flowers of bicolor and
502 59 concolor flowers. Over a two-year period of observation
503 at SNR, where the ratio of bicolor to concolor was always
504 40:1, only six observations of bee foraging observed were to
505 bicolor morphs.

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

510 nectar, they were observed to clasp the cone of anthers
511 around the style repeatedly while they scraped out pollen
512 with their forelegs. These grains were transferred to their
513 scopae (Figs.6, 7; and see
514 <https://www.youtube.com/watch?v=190rBkdVvXE>).

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



518
519
520 FIGURE 6. Female of *Andrena carlinii* foraging upside down on a bicolor flower. (Zong-Xin Ren, photographer).

521

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%)

522

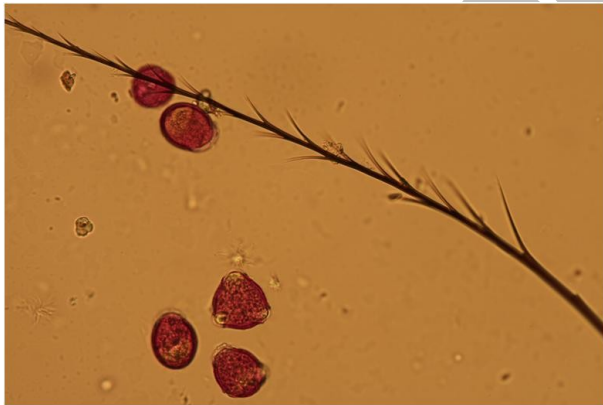
523

524

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)

525



526

527

528

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

529

530

531

532

533

534

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.

535

536

537

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

538

539

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

540

541

542

543

544

545

546

547

548

549

550

551

552

553

554

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.

555

Orientation of bees on morphs

556

557

558

559

560

561

562

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

563 2015 we counted 104 visits to these flowers in which (0.27)
564 of the landing orientations were right side up.

565 Therefore, when observations of the first visit of a bee to
566 a *V. pedata* at the CR site are combined for 2014 and 2015
567 the bee was more likely to land on the anterior (lip) petal
568 right side up, and insert its proboscis under the anther cone,
569 than it was to land on the posterior petals first and then
570 forage upside down when that foraging bout began.
571 However, as the same bee visited additional flowers during
572 the same foraging bout in 2015 at CR the more likely it
573 would change its foraging pattern from right side up to
574 upside down. As these bouts progressed and ended in 2015 a
575 total of 76 (0.74) of these orientations were made upside
576 down. When a bee foraged upside down we did observe and
577 record infrequent cases in which its third pair of legs clung
578 to one or both of the two lateral petals. This usually
579 occurred when posterior petals were askew (see,
580 <https://www.youtube.com/watch?v=qNAcOkcl9Ak>). In
581 the majority of observations, a female of *Andrena carlinii*
582 clutched the posterior petals with its third pair of legs and
583 the bee's abdomen also appeared to contact these petals (Fig.
584 6; <https://www.youtube.com/watch?v=190rBkdvVXE>)
585 regardless of colour morph.

586 In 2014 and 2015 there were no statistically significant
587 differences in bee orientation to bicolor vs. concolor
588 morphs at the Cuivre River site. All but four bee orientations
589 observed at SNR from 2014-2015 were always to concolor
590 morphs and were always made upside down with one
591 exception. The collection of the male *Anthophora ursina* in
592 2014 was made after the bee landed right side up on the
593 bicolor flowers. As related above, we were not able to
594 determine when bees began and ended foraging bouts on *V.*
595 *pedata* at SNR. Regardless of morph, bees foraging upside
596 down at CR and SNR continued to cling to the two
597 posterior petals via their third pair of legs.

598 *Ovule number and rates of pollination*

599 The number of ovules in ovaries (Tab. 3) was consistent
600 between sites ($F = 0.0158$, $P = 0.9972$), but not years ($F =$
601 35.3 , $P < 0.0001$). In 2015 ovule production was
602 consistently lower at SNR compared to SNR in 2014 and at
603 CR in 2014 and 2015 (Fig. 8). There was no difference
604 between the numbers of ovules inside an ovary between
605 morphs at either site in either year ($F = 0.7863$, $P =$
606 0.3831).

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

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

624 However, the mean number of pollen tubes actually
625 germinating and penetrating a pistil did not vary between
626 sites ($F = 0.04$, $P = 0.8345$) or years ($F = 1.75$, $P =$
627 0.1957). We did detect weak interaction effects between
628 morphs and years ($F = 3.87$, $P = 0.0519$). This effect seems
629 to be driven by the increased number of tubes in the style in
630 the concolor morph at SNR in 2015 (Fig. 9).

631 As this is a species with late-acting self-incompatibility
632 the number of pollen tubes that actually entered the ovary
633 and penetrated ovule micropyles within 7 days after the
634 flowers opened, required comparison. At both sites, ovule
635 penetration was higher in 2014 (burn year) compared to
636 2015 ($F = 35.3$, $P < 0.0001$; see also Tab. 3). The average
637 number of tubes penetrating ovules in 2014 and 2015 was
638 marginally higher in concolor pistils regardless of site ($F =$
639 3.9143 , $P = 0.0581$; Fig. 10).

640 *Seed set*

641 Seed production varied greatly between sites, morphs and
642 years (Tab. 3). There were no main effects in seed set among
643 years ($F = 0.6592$, $P = 0.6877$), location ($F = 0.3571$, $P =$
644 0.6910), or flower morph ($F = 0.2404$, $P = 0.4818$). There
645 was a significant interaction between year and location ($F =$
646 5.9292 , $P = 0.0161$), with seed set at SNR in 2014 being
647 significantly higher than in 2015 and it was significantly
648 higher compared to either year at CR.

649 DISCUSSION

650 *Variation in colour morph frequencies*

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

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

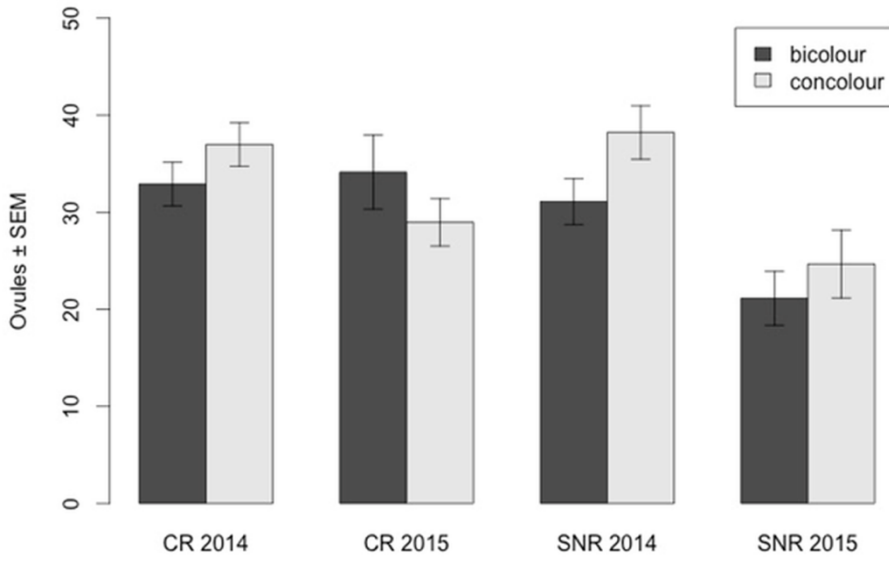


FIGURE 8. Mean number of ovules per flower in two morphs of *Viola pedata* at two sites and two years in Missouri, USA.

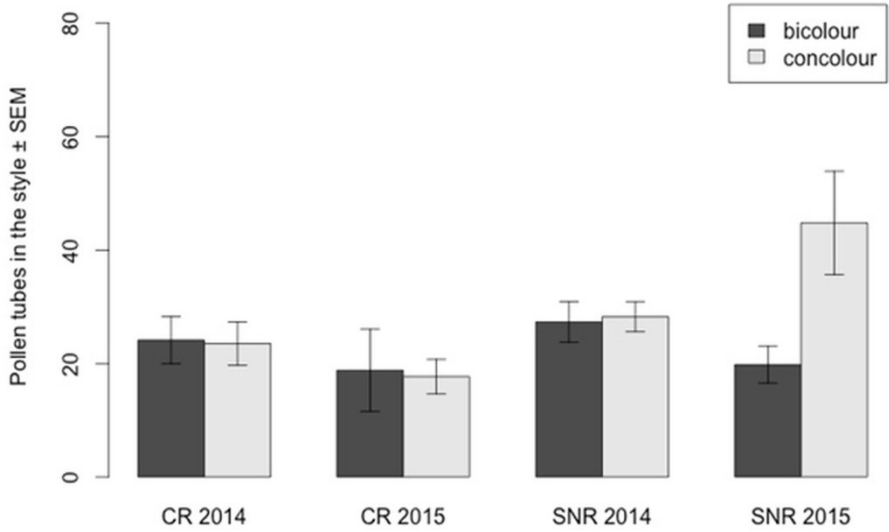


FIGURE 9. Mean number of pollen tubes germinating and penetrating styles in two morphs of *Viola pedata* at two sites and two years in Missouri, USA.

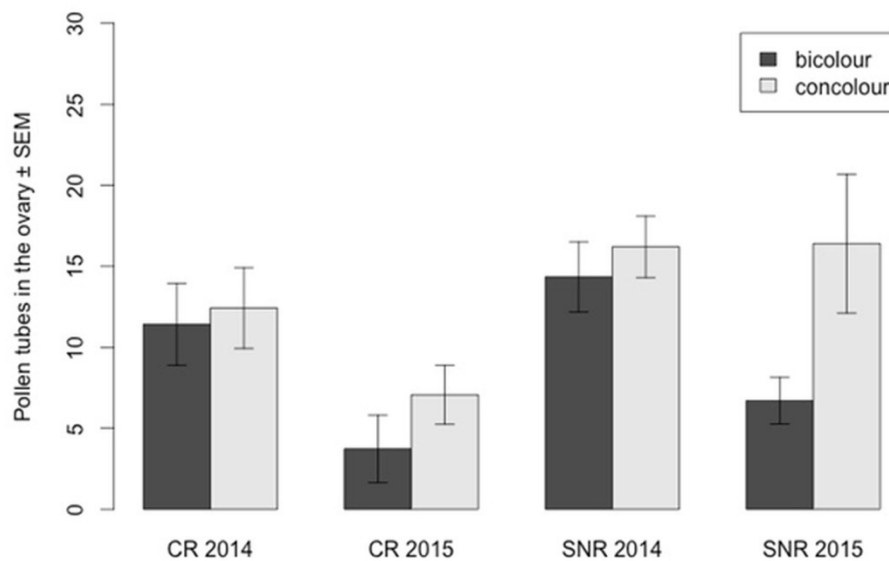


FIGURE 10. Mean number of pollen tubes penetrating ovules in two morphs of *Viola pedata* at two sites and two years in Missouri, USA.

677

678

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

682 *Comparative lack of pollinator diversity and* 683 *foraging preferences between sites*

684 We now have a third record of active (vector mediated)
685 pollen collection (sensu Bernhardt, 1996) in a *Viola* species.
686 The difference between this study and Freitas & Sazima
687 (2003) was that their Neotropical species offered pollen as
688 their primary (only?) reward. This did not appear to be the
689 case in *V. pedata*. It maintained prominent nectar glands and
690 spurs in both morphs. Our populations reflected the
691 potential plasticity of generalist pollination systems found in
692 *Viola* species in general. In other parts of its range
693 Lepidoptera and male bees visit *V. pedata* exclusively for
694 nectar (Beattie 1974; Carroll & Goldman 1994). While the
695 apices of anthers of *V. pedata* wear the modified “snow
696 shovels” (sensu Beattie 1974), associated with maximization
697 of passive release of pollen onto nectar foraging insects, our
698 females of *A. carlinii* remained active pollen collectors
699 regardless of site. Bisexual flowers pollinated by a
700 combination of nectar-drinkers and active pollen-collectors
701 are not unique. Some Neotropical *Fuchsia* species are
702 pollinated by a combination of hummingbirds and *Bombus*
703 species. The birds consume only nectar (passive anther
704 contact) while *Bombus* species drink nectar but also make
705 active pollen collections (Bernhardt & Montalvo 1979;
706 Breedlove 1969).

707 Within our two sites studied over three seasons the
708 dominant pollinators were female, short-tongued bees in the
709 family, Andrenidae as noted previously by Beattie (1974).
710 We note that, at both sites over two years, females of *A.*
711 *carlinii* dominated visits despite obvious differences in
712 habitat landscapes and differences in prescribed burn cycles
713 (see above). This is not surprising when the distribution of
714 this bee is reviewed. Its North American range far exceeds
715 the distribution of *V. pedata*. *Andrena carlinii* is polylectic
716 also foraging on flowers of *Vaccinium* species and ephemeral
717 woodland herbs. It nests in woodlands but is also native to
718 open sites, not obscured by vegetation (Schrader & LaBerge
719 1978). This explains its presence in a mixed hardwood forest
720 and in an exposed glade. In general, our insect collections
721 failed to show that pollen dispersal of *V. pedata* at either site
722 depended either on oligolectic bees (e.g. *Andrena violae*) or
723 on insects foraging exclusively for nectar.

724 Beattie (1969; 1971b; 1972; 1974) provided ample
725 evidence that many temperate zone, *Viola* species have
726 generalist pollination systems. However, we should also
727 consider the possibility that *V. pedata* may have a regionally
728 narrow spectrum of pollinators when its populations are
729 discontinuous, discrete and disjunctive. In our case we were
730 disappointed repeatedly by the comparative lack of
731 participation by native Lepidoptera at both of our sites
732 compared to the observations of Carroll and Goldman
733 (1994) in Missouri. Over three seasons at two sites we failed
734 to observe the diurnal sphingid moths first described visiting
735 *V. pedata* in West Virginia by Beattie (1974). Potential
736 variation in guilds of anthophilous insects, based on the
737 broad distribution of *V. pedata*, must be expected as this

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

743 *Variation in bee orientation on morphs vs. morph* 744 *preference*

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

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

789 *Floral warmth vs. foraging preferences*

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

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

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

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

832 *Variation in ovule production pollination rates* 833 *and seed set*

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

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

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

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

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

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

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

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

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

926 ACKNOWLEDGEMENTS

927 We would like to thank the staff of the Shaw Nature Reserve
 928 and Cuivre River State Park for allowing us to tag and bag
 929 populations. We are especially grateful to James Trager (SNR) and
 930 Bruce Schuette (CR) for showing us the larger populations at
 931 respective sites and informing us as to burned sites and burn cycles.
 932 We are grateful to Larry Meier for maintaining equipment and
 933 helping with temperature measurements and videography and to
 934 Ms. Courtney Dvorsky and Ms. Kelli Frye for their lab work. Dr
 935 Zong-Xin Ren's work at St. Louis University was funded by the
 936 Chinese Academy of Sciences.

937 REFERENCES

938 Armbruster WS (2002) Can indirect selection and genetic context
 939 contribute to trait diversification? A transition-probability study
 940 of blossom-colour evolution in two genera. *Journal of*
 941 *Evolutionary Biology* 15: 468-486.
 942 ----, Perez-Barrales RM, Arroyo J, Edwards ME, Vargas P (2006)
 943 Three-dimensional reciprocity of floral morphs in wild flax
 944 (*Linum suffuticosum*): a new twist on heterostyly. *New*
 945 *Phytologist* 171:581-590.
 946 Beattie AJ (1969) Pollination ecology of *Viola*. I. Contents of
 947 stigmatic cavities. *Watsonia* 7:142-156.
 948 ---- (1971a) Itinerant pollinators in a forest. *Madrono* 21:120-
 949 124.
 950 ---- (1971b) Pollination mechanisms in *Viola*. *New Phytologist*
 951 70:343-360.
 952 ---- (1972) The pollination of *Viola*. 2. Pollen loads of insect-
 953 visitors. *Watsonia* 9:13-25
 954 ---- (1974) Floral evolution in *Viola*. *Annals of the Missouri*
 955 *Botanical Garden* 61:781-793.
 956 ---- (1976) Plant dispersion, pollination and gene flow in *Viola*.
 957 *Oecologia* 25:291-300.
 958 Becker RE, Ewart LC (1990) Pollination, seed set and pollen tube
 959 growth investigations in *Viola pedata* L. *Acta Horticulturae* 272:
 960 33-36.
 961 Bernhardt P (1996) Anther adaptations in animal-pollination. In:
 962 D'Arcy WG, Keating RC (eds) *The Anther: Form, function and*
 963 *phylogeny*. Cambridge University Press, Cambridge, pp 192-220.
 964 ----, Edens-Meier R, Westhus EJ, Vance, N (2014) Bee-mediated
 965 pollen transfer in two populations of *Cypripedium montanum*
 966 Douglas ex Lindley. *Journal of Pollination Ecology* 13:188-202.
 967 ----, Montalvo EA (1979) The pollination of *Echeandia*
 968 *macrocarpa* (Liliaceae). *Brittonia* 31:64-71.

969 Breedlove DE (1969) The systematics of *Fuchsia* section
 970 *Enclandra* (Onagraceae). University of California Publications
 971 in Botany 53:1-69.
 972 Davidse G (1968) A biosystematic investigation of the
 973 intermountain yellow violets. MSc Thesis, Utah State University,
 974 Logan, Utah.
 975 Carlson JE, Holsinger KE (2013) Direct and indirect selection on
 976 floral pigmentation by pollina-tors and seed predators in a colour
 977 polymorphic South African shrub. *Oecologia* 171: 905-919.
 978 Carroll SB, Goldman P (1994) Analysis of a flower colour
 979 polymorphism in *Viola pedata* (birdfoot violet). *Proceedings of*
 980 *the North American Conference on Savannas and Barrens*.
 981 <https://archive.epa.gov/ecopage/web/html/carroll.html>.
 982 Clausen J (1926) Genetical and cytological investigations on *Viola*
 983 *tricolor* L. and *V. arvensis* Murr. *Hereditas* 8:1-156.
 984 Culley TM (2002) Reproductive Biology and delayed selfing in
 985 *Viola pubescens* (Violaceae), an understory herb with
 986 chasmogamous and cleistogamous flowers. *International Journal*
 987 *of Plant Sciences* 163:113-122.
 988 De Jager ML, Ellis AG (2014) Floral polymorphism and the fitness
 989 implications of attracting pollinating and florivorous insects.
 990 *Annals of Botany* 113: 213-222.
 991 Darwin C (1876) *The effects of cross and self fertilization in the*
 992 *vegetable kingdom* John Murray, London.
 993 Edens-Meier RM, Vance N, Luo YB, Li P, Bernhardt P (2010)
 994 Pollen-pistil interactions in North American and Chinese
 995 *Cypripedium* L. (Orchidaceae). *International Journal of Plant*
 996 *Sciences* 171:370-381.
 997 Epperson BK, Clegg MT (1987) Frequency-dependent variation
 998 for outcrossing rate among flower-colour morphs of *Ipomoea*
 999 *purpurea*. *Evolution* 41: 1302-1311.
 1000 Espindola A, Pellissier L, Alvarez N (2011) Variation in the
 1001 proportion of flower visitors of *Arum maculatum* across its
 1002 distributional range in relation with community-based climatic
 1003 niche analysis. *Oikos* 120:728-734.
 1004 Fehr C, Rausher (MD 2004) Effects of variation at the flower-
 1005 colour A locus on mating system parameters in *Ipomoea*
 1006 *purpurea*. *Molecular Evolution* 13: 1839-1847.
 1007 Freitas L, Sazima M (2003) Floral Biology and pollination
 1008 mechanisms in two *Viola* species – from nectar to pollen flowers?
 1009 *Annals of Botany* 91:311-317.
 1010 Futuyma DJ (2013) *Evolution*. Third edition. Sinauer Associates,
 1011 Inc. Sunderland, Massachusetts.
 1012 Gibson WH, Davie EE (1901) Blossom hosts and insect guests:
 1013 How the heath family, the bluets, the figworts, the orchids and
 1014 similar wild flowers welcome the bee, the fly, the wasp, the moth
 1015 and other faithful insects. Newson and Company, New York.
 1016 Gomez JM (2000) Phenotypic selection and response to selection
 1017 in *Lobularia maritima*: Importance of direct and correlational
 1018 components of natural selection. *Journal of Evolutionary Biology*
 1019 13:689-699.
 1020 Heinrich B (1993) *The hot-blooded insects: Strategies and*
 1021 *mechanisms of thermoregulation*. Harvard University Press.
 1022 Cambridge, Massachusetts.
 1023 Herrera CM (1990) The adaptedness of the floral phenotype in a
 1024 relict endemic, hawkmoth-pollinated violet. I. Reproductive
 1025 correlates of floral variation. *Biological Journal of the Linnean*
 1026 *Society* 40:263-274.
 1027 ---- (1993) Selection on floral morphology and environmental
 1028 determinants of fecundity in a hawk moth-pollinated violet.
 1029 *Ecological Monographs* 63:251-275.

- 1030 Gurevitch J, Scheiner S, Fox, G (2006) *The Ecology of Plants*.
 1031 Second edition. Sinauer Associates, Inc. Sunderland,
 1032 Massachusetts.
- 1033 Hildebrandt U, Hoef-Emden K, Backhausen S, Bothe H, Bozek M,
 1034 Siuta A, Kuta E (2006) The rare, endemic zinc violets of Central
 1035 Europe originate from *Viola lutea* Huds. *Plant Systematics &*
 1036 *Evolution* 257:205-222.
- 1037 Irwin RE, Strauss SY (2005) Flower colour microevolution in wild
 1038 radish: Evolutionary response to pollinator-mediated selection.
 1039 *The American Naturalist* 165:225-237.
- 1040 Johri BM, Ambegaokar JR, Srivastava PS (1992) *Comparative*
 1041 *Embryology of Angiosperms*. Volume I. Springer-Verlag, Berlin.
- 1042 Kenrick J, Kaul V, Williams EG (1986) Self-incompatibility in
 1043 *Acacia retinodes*: Site of pollen-tube arrest is the nucellus. *Planta*
 1044 169:245-50.
- 1045 Kevan P (1972) Heliotropism in some Arctic flowers. *The*
 1046 *Canadian Field Naturalist* 86:41-44.
- 1047 ----- (1975) Sun-tracking solar furnaces in high arctic flowers:
 1048 significance for pollination and insects. *Science* 189:723-726.
- 1049 Lamont BB, Downes KS (2011) Fire-stimulated flowering among
 1050 resprouters and genophytes in Australia and South Africa. *Plant*
 1051 *Ecology* 212:2111-2125.
- 1052 Le Maitre DC, Brown PJ (1992) Life cycles and fire-stimulated
 1053 flowering in geophytes. In: van Wilgen BW, Richardson DM,
 1054 Kruger FJ, van Hensbergen JH (eds) *Fire in South African*
 1055 *Mountain Fynbos*, Springer-Verlag, New York, pp 145-160.
- 1056 Mabberley DJ (1997) *The plant-book*. Second edition. Cambridge
 1057 University Press, Cambridge.
- 1058 Malberla R, Nattero J (2011) Pollinator response to flower colour
 1059 polymorphism and floral display in a plant with a single-locus
 1060 flower colour polymorphism: Consequences for plant re-
 1061 production. *Ecological Research* 27: 377-385.
- 1062 Marcussen T, Borgen L (2011) Species delimitation in the Ponto-
 1063 Caucasian *Viola sie-heana* complex, based on evidence from
 1064 allozymes, morphology, ploidy levels and crossing experiments.
 1065 *Plant Systematics and Evolution* 291:183-196.
- 1066 Martin P, Houf GF (1993) Glade grasslands in southwest
 1067 Missouri. *Rangelands* 15:70-73.
- 1068 McKinney LE (1992) A taxonomic revision of the acaulescent blue
 1069 violets (*Viola*) of North America. Botanical Research Institute of
 1070 Texas, Inc., Fort Worth, Texas.
- 1071 Migdalek G, Wozniak M, Slomka A, Godsik B, Jedrzejczyk-
 1072 Korycinska M, Rostanski A, Bothe H, Kuta E (2013)
 1073 Morphological differences between violets growing at heavy metal
 1074 polluted and non-polluted sites. *Flora* 208:87-96.
- 1075 Mereda P, Hodalova I, Martonfi P, Kucera J, Lihova J (2008)
 1076 Intraspecific variation in *Viola suavis* in Europe: Parallel
 1077 evolution of white-flowered morphotypes. *Annals of Botany*
 1078 102:443-462.
- 1079 Müller H (1883) *The fertilization of flowers*. London.
- 1080 Nieuwland JA, Kaczmarek RM (1914) *Studies in Viola*, I:
 1081 Proposed segregates of *Viola*. *The American Midland Naturalist*
 1082 8:207-217.
- 1083 Pellegrino G, Bellusi F, Musacchio A (2008) Double floral
 1084 mimicry and the magnet species effect in dimorphic co-flowering
 1085 species, the deceptive orchid *Dactylorhiza sam-bucina* and
 1086 rewarding *Viola aethnensis*. *Preslia* 80:411-422.
- 1087 Ramos RR, Venturieri GA, Cuco SM, Castro NM (2005) The site
 1088 of self-incompatibility action in cupassu (*Theobroma*
 1089 *grandiflorum*). *Brazilian Journal of Botany* 28:569-578.
- 1090 Rausher MD (2008) Evolutionary transitions in floral colour.
 1091 *International Journal of Plant Sci-ences*. 169:7-21.
- 1092 Richards AJ (1986) *Plant Breeding Systems*. Allen & Unwin,
 1093 Boston, Massachusetts.
- 1094 Russell AL, Newman SR, Papaj DR (2016) White flowers finish
 1095 last: Pollen foraging bumble bees show biased learning in a floral
 1096 colour polymorphism. *Evolutionary Ecology* (2016).
 1097 Doi:10.1007/s10682-016-9848-1.
- 1098 Rymer PD, Johnson SD, Savolainen V (2010) Pollinator behavior
 1099 and plant speciation: can assortative mating and disruptive
 1100 selection maintain distinct morphs in sympatry. *New Phytologist*
 1101 188:426-436.
- 1102 Sage T, Sampson B (2003) Evidence for Ovarian Self-
 1103 incompatibility as a cause of self-sterility in the relictual woody
 1104 angiosperm, *Pseudowintera axillaris* (Winteraceae). *Annals of*
 1105 *Botany* 91:807-816.
- 1106 Sage T, Strumas F, Cole WW, Barrett SCH (1999) Differential
 1107 ovule development following self- and cross-pollination: the basis
 1108 of self-sterility in *Narcissus triandrus* (Amaryllidaceae). *American*
 1109 *Journal of Botany* 86: 855-870.
- 1110 Schrader MN, LaBerge WE (1978) The nest biology of the bees:
 1111 *Andrena (Melandrena) regularis* Malloch and *Andrena*
 1112 (*Melandrena carlini*) (Hymenoptera: Andrenidae). *Biological*
 1113 *notes*; no. 108. State of Illinois, Dept. of Registration and
 1114 Education, Natural History Survey Division.
- 1115 Sobral M, Losada M, Veiga T, Gruitian J, Guitian J, Guitian P
 1116 (2016) Flower colour preferences of insects and livestock: Effects
 1117 on *Gentiana lutea* reproductive success. *PeerJ* 4:e1685; DOI
 1118 10.7717/peerj.1685.
- 1119 Steyermark J (1963) *Flora of Missouri*. Iowa State Press, Iowa
 1120 City.
- 1121 Wang Y (2008) *Molecular biology of flower development in Viola*
 1122 *pubescens*, a species with the chasmogamous-cleistogamous mixed
 1123 breeding system. PhD Dissertation, Ohio University. UMI
 1124 3302717.
- 1125 Weberling F (1989) *Morphology of Flowers and Inflorescences*.
 1126 Cambridge University Press. Cambridge.
- 1127 Willmer P (2011) *Pollination and Floral Ecology*. Princeton
 1128 University Press. Princeton, New Jersey.
- 1129 Winn AA, Moriuchi KS (2009) The maintenance of mixed mating
 1130 by cleistogamy in the perennial violet *Viola septemloba*
 1131 (Violaceae). *American Journal of Botany* 96:2074-2079.

1132

1133