

THE ROLE OF HERBIVORES IN THE MAINTENANCE OF A FLOWER COLOR POLYMORPHISM IN WILD RADISH

REBECCA E. IRWIN,¹ SHARON Y. STRAUSS, SHONNA STORZ, AIMEE EMERSON, AND GENEVIEVE GUIBERT

Center for Population Biology, University of California, Davis, California 95616 USA

Abstract. Plant species exhibiting polymorphisms with respect to flower color are widespread. Our understanding of the selection pressures that may maintain these color polymorphisms has primarily been confined to one set of organisms—pollinators. Yet, selection on flower color may also be driven by other agents, such as herbivores, especially in cases where pollinators and herbivores are using the same or correlated traits to select plants. A wealth of studies have documented pollinator preference for anthocyanin-recessive color morphs (A⁻; yellow and white flowers) of wild radish, *Raphanus sativus*, over anthocyanin-dominant morphs (A⁺; pink and bronze flowers); yet, differences in pollination alone do not explain the maintenance of the flower color polymorphism. Here, we ask whether variation in flower color in *R. sativus* influences the preference and performance of herbivores for A⁻ flower color morphs vs. A⁺ flower color morphs in four types of herbivores (generalist and specialist Lepidoptera, slugs, aphids, and thrips). We found that all herbivores except for aphids and thrips preferred flowering A⁻ color morphs compared to A⁺ morphs of *R. sativus*. Furthermore, all herbivores except larvae of specialist and generalist Lepidoptera performed better on A⁻ color morphs. Differences in plant secondary chemistry may play a role in differential herbivore preference and performance on the anthocyanin flower color morphs. Chemical analyses of leaf secondary compounds (indole glucosinolates) revealed that A⁺ color morphs produced higher concentrations of indole glucosinolates than A⁻ morphs in the presence of herbivore damage. Therefore, herbivores may exhibit lower preference for A⁺ color morphs, and these morphs may support lower herbivore performance because they are heavily defended once damaged. This is the first study, to our knowledge, to document differential preference and performance of herbivores for different flower color morphs. Previous studies have shown that increased herbivore damage can have profound negative direct and indirect effects on the reproduction of *R. sativus*. The data presented here suggest that differential preference and performance of herbivores for *R. sativus* color morphs may counter selection on flower color exerted by pollinators.

Key words: *anthocyanins; California, USA; flower color polymorphism; herbivory; plant–insect interactions; Raphanus; wild radish.*

INTRODUCTION

Plants in natural environments experience myriad direct and indirect interactions with mutualistic as well as antagonistic species, and the traits expressed by plants are likely shaped by the simultaneous integration of all of these selection pressures (Strauss and Armbruster 1997). Despite the complexity of interactions occurring in natural systems, studies in plant–animal interactions have traditionally focused on interactions between a plant and one type of visitor, for example, interactions between plants and pollinators, herbivores, seed predators, or nectar robbers (e.g., Inouye 1983, Proctor et al. 1996, Karban and Baldwin 1997, Chittka and Thomson 2001). Traits that are involved in one set of interactions may, however, be co-opted into other, very different interactions. For

example, work by Armbruster et al. (1997) suggests that resins, now serving as rewards for highly specialized pollinators, may have initially functioned as antiherbivore and antipathogen defenses. Similarly, showy bracts that attract pollinators also have antiherbivore properties when they close at night and protect stamens and pistils from florivores (Armbruster and Mziray 1987, Armbruster 1997). Thus, the identities of important selective agents on particular traits are not always obvious. In this paper, we examine whether variation in flower color, traditionally viewed as a pollinator-selected trait, might also influence the preference and performance of herbivores.

Plant species exhibiting polymorphisms with respect to flower color are well documented (Kay 1978). Flower color has traditionally been viewed as a trait that is essential in attracting certain suites of pollinators (Grant 1950, Faegri and van der Pijl 1979, Proctor et al. 1996). Although many studies have found selective foraging by pollinators for specific color morphs (e.g., Levin 1972, Mogford 1974a, Kay 1976, Hannan 1981, Waser and Price 1981, Brown and Clegg 1984, Jones

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¹ Present address: Institute of Ecology, Ecology Building, University of Georgia, Athens, Georgia 30602 USA.
E-mail: rirwin@arches.uga.edu

et al. 1986, Stanton 1987a, Stanton et al. 1989, Odell et al. 1999, Comba et al. 2000, Gigord et al. 2001, Jones and Reithel 2001), other studies have found pollinators indifferent to flower color variation within a species (e.g., Ernst 1987, Wolfe 1993, Schemske and Bierzychudek 2001). Many pollinator-centric hypotheses have been proposed as factors maintaining flower color polymorphisms, including color preferences and the constancy of specific suites of pollinators to certain color morphs (Stanton 1987a, Jones and Reithel 2001). Yet, in some natural systems, pollination alone does not explain the maintenance of flower color variation within populations (Hannan 1981, Stanton 1987a, Stanton et al. 1989).

For example, previous studies have shown strong pollinator preferences for anthocyanin-recessive color morphs (yellow and white flowers) of *Raphanus sativus* compared to anthocyanin-dominant color morphs (pink and bronze flowers), suggesting that anthocyanin-recessive morphs should increase in relative frequency in natural populations, especially via increased male reproduction (Stanton 1987a). Although the relative frequencies of the anthocyanin-color morphs vary widely among *R. sativus* populations in California, USA (Panetsos 1964; S. Y. Strauss and R. E. Irwin, unpublished data), within a population, the frequencies of the morphs remain relatively constant over short (2 year) and long (30 year) time periods (S. Y. Strauss and R. E. Irwin, unpublished data). Stanton et al. (1989), after exploring several aspects of pollinator preference and foraging, called for the incorporation of broader population-level processes in order to understand factors affecting the maintenance of flower color variation in *Raphanus* spp. Given that herbivores reduce plant fitness in *Raphanus* spp. through both direct and indirect pathways (e.g., Snow and Stanton 1988, Mauricio et al. 1993, Strauss et al. 1996, Lehtilä and Strauss 1997, 1999, Agrawal 1999), we propose that herbivores could provide countering selection pressures to those exerted by pollinators if herbivores prefer anthocyanin-recessive morphs.

That genes controlling flower color might influence plant resistance to herbivory is not implausible. Three lines of evidence suggest the validity of this hypothesis. First, some floral and pollen herbivores discriminate among flower color morphs, including thrips (Vernon and Gillespie 1990, Gaum et al. 1994, Chyzik et al. 1995) and pollen-feeding beetles (Giamoustaris and Mithen 1996), actions that result in higher attack and damage levels on some color morphs over others. Second, pleiotropic effects may exist between the synthesis of floral pigments and defensive plant compounds (Simms and Bucher 1996, Fineblum and Rausher 1997), and thus may influence herbivore preference and performance. Third, resource allocation trade-offs (Mole 1994) could exist between floral pigment and defensive compound synthesis, especially if synthesis is costly. In this case, vegetative tissue may differ nu-

tritionally or defensively with respect to herbivores, depending on floral-color expression.

Using a combination of laboratory, greenhouse, and field experiments, we evaluated the preference and performance of herbivores in relation to anthocyanin-color morphs of *Raphanus sativus*. We hypothesized that herbivores may act as agents of selection on anthocyanin flower color expression in wild radish populations via differential herbivore preference and performance. *Raphanus sativus* is attacked by a wide range of generalist and specialist herbivores, many of which have different physiologies and searching and feeding strategies. To gain a more general understanding of the role of herbivores, we examined the relationships between floral pigments and herbivore preference and performance for a variety of different herbivores. These species included adults and larvae of *Pieris rapae*, adults of *Spodoptera exigua*, *Agriolimax reticulatus* slugs, *Brevicoryne brassicae* aphids, and *Frankliniella occidentalis* flower thrips. We chose these herbivores because they were the most abundant, and all were observed to eat *R. sativus* in herbivore damage censuses across a wide range of California sites, excluding *S. exigua* (S. Y. Strauss and R. E. Irwin, unpublished data; R. Karban, personal communication). Specifically, we asked: (1) Do herbivores exhibit differential preference for certain anthocyanin flower color morphs? And (2) does herbivore performance vary with anthocyanin flower color morph? Finding differential preference and performance by some of the herbivores, we then examined whether there were differences in *R. sativus* secondary compounds (concentrations of indole glucosinolates) between anthocyanin flower color morphs in the presence and absence of herbivore damage, which might help explain the herbivore preference and performance patterns observed.

METHODS

Study system

Wild radish, *Raphanus sativus* (Brassicaceae), is a naturalized, herbaceous, annual common along roadsides and disturbed areas in valley and coastal areas of California, USA. Seeds germinate early in the rainy season (October to December) with plants blooming near the end of the rainy season (early March) for ~3–4 months. In California, *R. sativus* individuals possess one of four different petal colors: yellow, white, pink, or bronze. Petal color is determined by two independently assorting loci controlling the expression of carotenoids (*W*) and anthocyanins (*P*) (Panetsos 1964). Carotenoid pigments produce yellow petals with yellow (presence of carotenoid) recessive to white (absence of carotenoid). Anthocyanin pigments produce pink petals with white (absence of anthocyanin) recessive to pink (presence of anthocyanin). Therefore, plants with yellow petals are recessive at both loci (genotype: *ppww*), pink dominant at both loci (genotype:

P-W-), white dominant at the carotenoid locus (genotype: *ppW-*), and bronze dominant at the anthocyanin locus (genotype: *P-ww*). Floral display size does not differ significantly among the color morphs (Stanton 1987b). In addition, silique size, which is controlled by a different set of loci, does not differ among color morphs (S. Y. Strauss, unpublished data). All data below were analyzed with respect to the anthocyanin locus, as work with *Ipomoea purpurea* suggested that anthocyanin production may influence herbivore performance (Simms and Bucher 1996). Therefore, all references to the "color morphs" indicate plants that produce anthocyanins (pink and bronze flower color; hereafter referred to as A+) or plants that are recessive for anthocyanin production (yellow and white flower color; hereafter A-).

Raphanus sativus is damaged by herbivores throughout its lifetime, from the cotyledon to the flowering adult stage (S. Y. Strauss and R. E. Irwin, personal observation). Common herbivores of *R. sativus* include naturalized foliage-feeding cabbage white butterfly larvae (*Pieris rapae*), gray garden slugs (*Agriolimax reticulatus*), and cabbage aphids (*Brevicoryne brassicae*). Larvae of the generalist moth *Spodoptera exigua* also feed on *R. sativus*. In addition, native western flower thrips (*Frankliniella occidentalis*) feed on pollen and floral parts in sunny locations.

Plants used in the experiments below came from a variety of sources, all of which originated from naturalized populations in California. In some experiments, we used seed from lines that were created to breed true to flower color. Another set of experiments used plants that were the result of two generations of backcrossing plants into the yellow background (double recessive). These crosses were done to homogenize the genetic background in which color genes were expressed. Backcrossing should homogenize the background with respect to all genes except those strongly linked to flower color. Finally, we also used seed collected directly from wild populations. All experimental plants were grown in the greenhouse in individual 10 cm square pots using University of California greenhouse soil mix. Plants were watered using a subirrigation system ad libitum and fertilized at the two-leaf stage with 2 g of Osmocote Plus 15-11-13 slow release fertilizer (Scott's, Marysville, Ohio).

Specialist and generalist Lepidoptera

Preference.—We presented each of 72 flying specialist female *P. rapae* with the simultaneous choice of A- and A+ color morphs grown from seed in a circular array in screened outdoor arenas. Because the outdoor arenas were relatively small (2 × 2 × 2 m), each array contained only three plants and, thus, unequal sample sizes of A- and A+ color morphs. Seeds for this experiment were collected from the UCD Bodega Bay Marine Reserve, Bodega Bay, California. We used both wild-caught adult female *P. rapae* and lab-

oratory-reared females, which were the offspring of several individuals from Davis, California and Sacramento, California. Individuals were cooled for one hour prior to central release in the arenas in the early morning. Gradual warming of the insects diminishes behavioral disturbances resulting from transport into the arena, and the use of single insects allowed individuals to oviposit independently from each other. Trials for each individual female ran from sunup to sundown. We recorded the total number of eggs laid by each female on each morph at the end of the trial. Each female was presented with a new set of plants; plants were never reused among females. In addition, plants within the same trial were of similar height, leaf area, and phenology. We conducted trials that included flowering plants (19 trials) and nonflowering rosettes (53 trials) to discern whether flower color itself was a cue for herbivores or whether plant-foliage quality played a role in *P. rapae* preference. Nonflowering rosettes were grown to flowering adults to determine flower color once the trials were over. We used the number of eggs per plant to assign ranks (from 1 as most preferred to 3 as least preferred) in oviposition preference by the females for plants in the flight arena (Appendix). We evaluated the preference of *P. rapae* for anthocyanin morphs by calculating the difference between the rank in oviposition for different anthocyanin morphs minus their rank availability within the flight arena (Johnson 1980, Appendix). We then analyzed this difference using the multivariate Hotelling's T^2 to test the null hypothesis that A- and A+ morphs were equally preferred by *P. rapae* for flowering and nonflowering plants (Roa 1992, Appendix). We used a multivariate approach to control for nonindependence of plants in the flight arenas (Appendix).

Performance.—To measure the performance of specialist *P. rapae* and generalist *S. exigua* on anthocyanin flower color morphs of *R. sativus*, we used seeds of *R. sativus* from lines of known flower color created by M. Stanton at the University of California at Davis (UCD). These seeds were created by selfing color morphs using bud pollinations. Progeny of selfed plants were grown to flowering to determine homozygosity of the parent. Homozygous parents were then outcrossed with pollen from homozygous plants of the same color.

One seed from each of 47 maternal families (30 A- and 17 A+ color morphs) was assigned to *P. rapae* and *S. exigua* in the rosette stage prior to flowering. *Pieris rapae* were grown from laboratory-reared colonies made of multiple maternal and paternal parents collected from Davis, California and Sacramento, California. The colonies were infused with wild-caught adults every few generations to reduce inbreeding depression and loss of genetic variability. *S. exigua* were obtained from USDA (Stoneville, Mississippi). First instar neonates were placed on rosette leaves in clip cages made from two petri plates ~5 cm in diameter held together by hair clips. These cages were placed

next to the leaf midvein and confined larvae to one-half of the leaf (as in Strauss et al. 1996). For each leaf that was fed upon by individual larvae, leaf length and phenological number were recorded, and the means of these variables were used as covariates to control for potential chemical and nutritional changes associated with plant phenology. After ten days, the larvae were removed and weighed. We repeated this experiment 20 days later for *P. rapae* using the same plant families and new neonate instars. To test whether *P. rapae* and/or *S. exigua* varied in final mass (ln-transformed) on A- vs. A+ color morphs, we used an ANCOVA (type III ss) with anthocyanin morph (A- vs. A+), insect species, plant family, and trial as fixed effects, and leaf length and number as covariates. Plant family had no significant effect on larval mass ($F_{46,52} = 0.97$, $P = 0.54$) and was removed from the final analysis to increase statistical power.

Slugs

Tests of *A. reticulatus* preference and performance for A- and A+ color morphs were conducted using plants produced from two generations of backcrossing into the yellow background, as described previously. For both the preference and performance experiments, we used wild-caught *A. reticulatus* from Davis, California.

Preference.—In the field, *A. reticulatus* feed on cotyledons as well as true leaves (S. Y. Strauss and R. E. Irwin, *personal observation*); therefore, we examined slug preference using both leaf types. To test whether slugs exhibited preference for anthocyanin flower color morphs at the cotyledon stage, we planted nine backcrossed radish seeds in a circle in a circular pot in the greenhouse. Each seed was 2.8 cm from its nearest neighbor. In total, we planted 80 replicate pots. Three days after cotyledons emerged, we placed one slug in the center of each pot. Slugs were preweighed to ensure that similar-sized slugs were used (between 3.0 and 5.0 mg). Copper tape was wrapped around the pots to prevent slugs from escaping. Twice daily for three days, we estimated the percentage of cotyledon area consumed on each seedling. Nonflowering rosettes were grown to flowering adults to determine flower color once the trials were over. Future flower color of nonflowering rosettes was unknown at the time of trials, though efforts were made to select seeds from mothers of different colors; thus, the availability of each morph to slugs differed among pots. Therefore, we calculated slug preference for anthocyanin morphs by taking the difference of the rank consumption of A- and A+ color morphs minus their rank availability within a pot, as described in the Lepidoptera preference experiments previously (Johnson 1980, Appendix). We evaluated this difference using Hotelling's T^2 to test the null hypothesis that A- and A+ color morphs were equally preferred (Roa 1992, Appendix).

To test whether slugs differed in preference for true leaves, 10 plants from each of 50 backcrossed families

were grown to flowering. As plants initiated flowering, we haphazardly chose three plants that were A- and A+ from each family and removed a leaf disk from each that was 1.8 cm in diameter, taking care to avoid the midrib. Leaf disks from the anthocyanin flower color morphs in each family were placed together in a petri dish. One preweighed *A. reticulatus* (between 3.0 and 5.0 mg) was placed into each dish and the percentage of leaf area consumed on each disk was recorded after 17 hours. Therefore, in each petri dish, slugs had the simultaneous choice of foraging on A- and A+ plants within the same family, thus controlling for plant-family effect. These data were analyzed as those in the cotyledon experiment previously.

Performance.—We tested the performance of *A. reticulatus* on the anthocyanin morphs by presenting the slugs with disks of leaf tissue in a no-choice test. Upon flowering, a leaf disk ~5 cm in diameter was removed from a fully expanded leaf from each of 45 plants and placed individually into a moistened petri dish. We used leaf disks from 29 A- morphs and 16 A+ morphs. When collecting the leaf disk, care was taken to avoid the midvein. Individual *A. reticulatus* were weighed and then added to each petri dish for three days. The proportion of leaf disk consumed was recorded on day 2 and 3, and on day 2, the leaf disk was replaced. After three days, we reweighed each *A. reticulatus* and calculated the absolute change in mass per 24 h. To test whether slugs varied in mass gain on the anthocyanin morphs, we used an ANCOVA (type III ss) with anthocyanin morph (A- vs. A+) as the main effect and mean proportion of leaf area consumed as the covariate (to control for *A. reticulatus* preference for the anthocyanin morphs).

Aphids

Preference.—To estimate *B. brassicae* preference for anthocyanin flower color morphs, we used field surveys of *B. brassicae* abundance in 17 natural populations of *R. sativus* throughout northern California and two experimental arrays of *R. sativus* with equal numbers of each of the anthocyanin morphs. In the 17 natural populations, we ran a transect line in a randomly chosen direction and censused up to 200 *R. sativus* that fell along that line. For each plant, we recorded petal color (A- vs. A+) and whether or not it had aphids. We then used a replicated G test to compare the observed frequency of anthocyanin morphs with *B. brassicae* to the expected frequency (the frequency of the morphs in the population). Across all populations, we found that the observed frequency of aphids on the morphs differed from the expected frequency ($G_T = 47.38$, $P < 0.001$); therefore, we then portioned the total G into contributions due to each population to determine in which populations and on which anthocyanin morphs aphid colonization was deviating from expectation (significance values were adjusted using sequential Bonferroni corrections).

In the two experimental arrays, we used plant progeny produced from controlled crosses into the yellow background. Plants were grown in the greenhouse, and upon flowering, we randomly chose 20 plants for each array, each from different families. The arrays had equal representation of anthocyanin morphs. The arrays were haphazardly placed in two populations of *R. sativus* growing along an orchard off of Hutchinson Road in Davis, California. We censused the arrays two times per week for three weeks and counted the number of *B. brassicae* on each plant. Because the experimental arrays were embedded within a larger matrix of *R. sativus* plants, we considered the experimental plants as independent units in the analysis. We used a MANOVA to examine the effects of anthocyanin flower color (A- vs. A+), array, and their interaction on the number of days to aphid colonization (square-root transformed) and the number of aphids per plant on the final census date (square-root transformed).

Performance.—Progeny produced from two generations of crossing into the yellow background were used to test *B. brassicae* performance on the color morphs. Upon flowering, 66 plants growing in individual pots were inoculated with five *B. brassicae*. The aphids were collected from wild-growing plants in Davis, California. We used plants from 25 different families and approximately equal numbers of anthocyanin morphs. Pots were placed in moats of water so that aphids could not escape by walking down the plant; in addition, during the study period, we detected no winged aphids, indicating no aphid emigration or immigration. Every other day for two weeks, we counted the number of *B. brassicae* on each plant. By the end of the two-week period, many plants had >400 *B. brassicae*. To compare aphid-colony growth on the morphs, we used the total colony size on the last census day and the per capita growth rate (natural-log-transformed) in a MANOVA with anthocyanin morph (A- vs. A+) as the main effect. A significant MANOVA was followed by individual ANOVAs (type III ss).

Thrips

Preference.—We examined *F. occidentalis* preference for anthocyanin flower color morphs in two natural populations of *R. sativus* in California, one along Highway 116 in 1996 and the second along the Putah Creek in 2000. Both populations had dense numbers of both anthocyanin morphs growing intermixed. We collected single flowers from plants in closed vials and later counted the number of thrips in each flower in the laboratory. In the Highway 116 population, we collected one flower from each of 91 plants: 64 A- morphs and 27 A+ morphs. In the Putah Creek population, we collected one flower from each of 90 plants: 45 each from A- and A+ morphs. Because we only counted thrips in flowers at one point in time and we did not measure population growth rates of thrips in the flowers, thrips densities reflect preference, performance, or a combination of both traits. For

simplicity, we refer to the counts as measuring preference. To compare the number of *F. occidentalis* in flowers of each of the anthocyanin morphs, we used a nonparametric Kruskal-Wallis test. We analyzed the two populations separately.

Phytochemical measurements

To understand why some of the herbivores exhibited differential preference and performance on the anthocyanin flower color morphs (see *Results*), we examined whether the anthocyanin morphs varied in their production of indole glucosinolates in the presence and absence of herbivore damage. Indole glucosinolates appear to be the dominant class of inducible glucosinolates with known biological effects (McDanell et al. 1988, Koritsas et al. 1991, Bodnaryk 1992, Gross 1993, Doughty et al. 1995). We planted 65 seeds from 12 plant families that were produced from one generation of backcrossing into the yellow background. In total, we planted 39 A- color morphs and 26 A+ color morphs. At the four-leaf stage, the A- and A+ color morphs were randomly assigned to one of two treatments: (1) 50% of all leaves consumed by caged *P. rapae* larvae (except the fifth and eighth true leaves), or (2) unmanipulated control. In the 50% leaf-removal treatment, we caged third to fifth instar larvae in clip cages, as described in the Lepidoptera performance experiments previously. Cages were placed along the midvein of a leaf, and caterpillars fed on the leaf tissue in the cages. We moved the cages along the midvein until one-half of each leaf was consumed (as in Strauss et al. 1996). On the leaves of unmanipulated control plants, we placed clip cages with no larvae to control for clip-cage effects. As plants initiated flowering, the fifth undamaged true leaf was removed with a razor blade, weighed, and immediately microwaved for ~30–45 seconds to denature endogenous myrosinases. Samples were then dried for 48 h at 60°C and stored at 0°C until further chemical analysis.

We followed the basic sephadex/sulfatase glucosinolate extraction and purification protocols described in Hogge et al. (1988). Samples were placed into deep-well microtiter tubes. We added four 2.3-mm ball bearings, and the samples were ground into a fine powder in a paint shaker by high-speed agitation. To extract glucosinolates, we added 400 μ L of methanol, 10 μ L of 0.3 mol/L lead acetate, and 120 μ L of water. The samples were mixed for 1 min and then allowed to incubate for 60 min at 180 rpm on a rotary shaker. The tissue and protein were pelleted by centrifugation, and the supernatant was used for anion-exchange chromatography.

We loaded 96-well filter plates from Millipore (model MAHVN4550, Millipore, Billerica, Massachusetts) with 45 μ L of DEAE Sephadex A-25. We then added 300 μ L of water to each column and allowed the mixture to equilibrate for 2–4 hours. We removed the water with 2–4 seconds of vacuum and then added 150 μ L

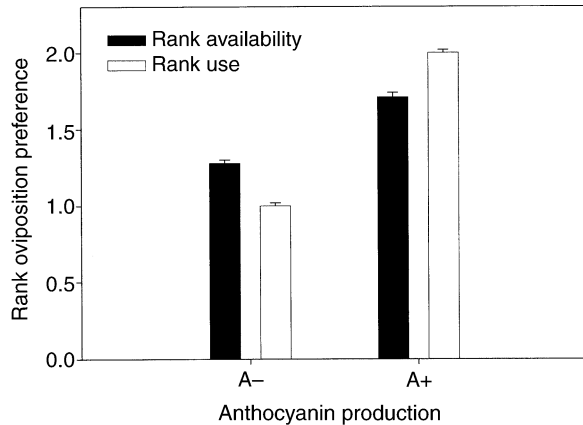


FIG. 1. At the flowering stage, *Pieris rapae* adults laid significantly more eggs on A- color morphs of *Raphanus sativus* than was expected based on their availability in the flight arenas, and *P. rapae* laid significantly fewer eggs on A+ color morphs than was expected. Bars are mean rank use (based on oviposition preference) and mean rank availability of anthocyanin flower color morphs in the flight arenas (error bars show 1 SE). Smaller values indicate higher rank use and rank availability.

of the supernatant to the 96-well columns. The liquid was removed by 2–4 seconds of vacuum, and this step was repeated once to bring the total volume of plant extract to 300 μ L. The columns were washed four times with 150 μ L of 67% methanol, three times with 150 μ L of water, and three times with 150 μ L of 1 mol/L sodium acetate. To desulfate the glucosinolates on the columns, we added 10 μ L of water and 10 μ L of sulfatase solution to each column, and the plates were incubated overnight at room temperature (Hogge et al. 1988). To elute the desulfoglucosinolates, the DEAE Sephadex was washed twice with 100 μ L of 60% methanol and twice with 100 μ L of water. We ran 40 μ L of the glucosinolate extract on a Hewlett-Packard 1100 series high-pressure liquid chromatograph with a Hewlett-Packard Lichrocart 250-4 RP18e 5- μ m column (Hewlett-Packard, Palo Alto, California). Glucosinolates were detected at 229 nm and separated and identified using the following programs with aqueous acetonitrile: (1) a 6-min gradient from 1.5% to 5.0% acetonitrile, (2) a 2-min gradient from 5% to 7% acetonitrile, (3) a 7-min gradient from 7% to 25% acetonitrile, (4) a 2-min gradient from 25% to 92% acetonitrile, (5) 6 min at 92% acetonitrile, (6) a 1-min gradient from 92% to 1.5% acetonitrile, and (7) a final 5 min at 1.5% acetonitrile. We provide results for one particular glucosinolate, indolyl-3-methyl (hereafter referred to as an indole glucosinolate for simplicity). We calculated the concentration of the indole glucosinolate indolyl-3-methyl in μ mol/mg. To understand the effects of anthocyanin floral pigment and induction via herbivore feeding on indole glucosinolates, we used an ANOVA (type III ss) with anthocyanin morph (A-

vs. A+), damage treatment (50% leaf removal vs. control), their interaction, and plant family as main effects.

RESULTS

Specialist and generalist Lepidoptera

Preference.—*P. rapae* adults did not distinguish between rosettes of A- and A+ plants ($T_{2,104}^2 = 0.72$, $P = 0.70$). Females oviposited on the rosettes based on their availability in the flight arena (mean rank availability vs. rank use \pm 1 SE; A-, 1.39 ± 0.08 vs. 1.32 ± 0.06 ; A+, 1.60 ± 0.07 vs. 1.68 ± 0.07). However, at the flowering stage, *P. rapae* laid significantly more eggs on A- plants (yellow and white) than was expected based on their availability in the flight arena and significantly fewer eggs on A+ plants (pink and bronze) than was expected ($T_{2,36}^2 = 7.83$, $P = 0.03$), suggesting that adult *P. rapae* prefer to oviposit on A- morphs at the flowering stage (Fig. 1).

Performance.—Floral anthocyanin morph had a significant effect on larval performance ($F_{1,100} = 7.27$, $P = 0.008$). Both the specialist *P. rapae* and the generalist *S. exigua* performed better on A+ color morphs (Fig. 2), which was opposite to the preference pattern for flowering anthocyanin morphs observed previously. We found a significant anthocyanin pigment \times caterpillar species interaction ($F_{1,100} = 4.79$, $P = 0.03$) because the difference in mass gain on the anthocyanin morphs was larger for *S. exigua* than for *P. rapae* (Fig. 2). We found no significant effect of trial on larval performance of *P. rapae* ($F_{1,100} = 1.50$, $P = 0.22$). Moreover, the covariates, leaf length and leaf number, had no significant effect on larval growth (leaf length, $F_{1,100} = 3.11$, $P = 0.08$; leaf number, $F_{1,100} = 1.00$, $P = 0.32$), suggesting that chemical and nutritional changes associated with changing plant phenology did not influence larval growth in these experiments.

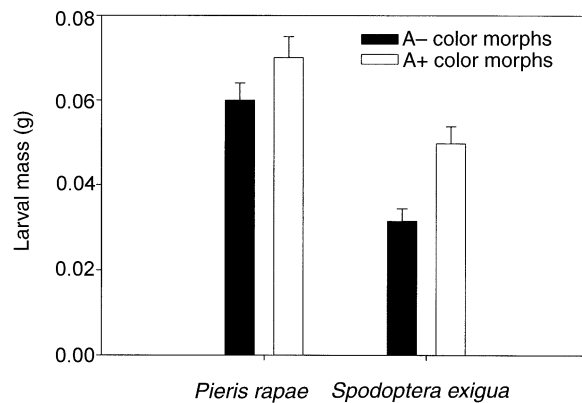


FIG. 2. The specialist *Pieris rapae* and the generalist *Spodoptera exigua* gained more mass feeding on A+ color morphs than on A- color morphs of *Raphanus sativus*. Bars are mean larval mass in g (error bars show 1 SE).

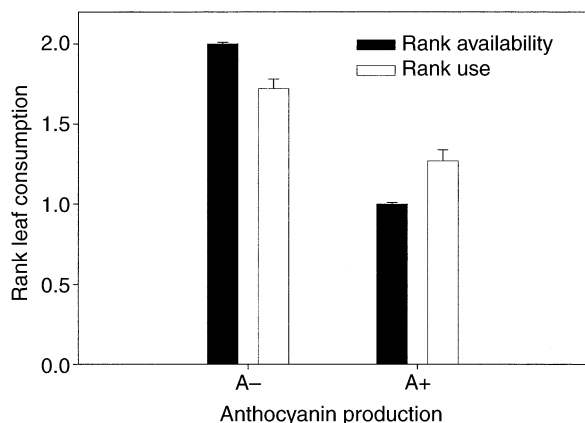


FIG. 3. At the flowering stage, *Agriolimax reticulatus* ate significantly more leaf tissue from A- color morphs of *Raphanus sativus* than was expected based on their availability in the experimental arenas, and the slugs ate significantly less leaf tissue from A+ color morphs than was expected. Bars are mean rank use (based on leaf consumption) and mean rank availability of anthocyanin flower color morphs in the arenas (+1 SE). Smaller values indicate higher rank consumption and rank availability.

Slugs

Preference.—*Agriolimax reticulatus* exhibited no preference for particular anthocyanin-color morphs at the cotyledon stage ($T_{2,98}^2 = 1.04$, $P = 0.50$). In both trials, slugs fed on seedlings in proportion to their availability (mean rank availability vs. rank use ± 1 SE; A-, 1.94 ± 0.13 vs. 2.00 ± 0.12 ; A+, 3.07 ± 0.08 vs. 3.01 ± 0.11). However, at the true-leaf stage, slugs consumed significantly more leaf tissue from A- color morphs (yellow and white) than was expected based on their availability in the experimental arenas ($T_{2,98}^2 = 33.29$, $P < 0.01$; Fig. 3).

Performance.—We found significant variation in *A. reticulatus* growth on the anthocyanin morphs ($F_{1,39} = 8.29$, $P = 0.006$). Over three days of feeding, slugs gained mass feeding on foliage from A- color morphs (yellow and white) while slugs exhibited relatively little change in mass feeding on A+ color morphs (pink and bronze) (Fig. 4). We also found a significant effect of the covariate, proportion of leaf area consumed, on *A. reticulatus* growth ($F_{1,39} = 9.62$, $P = 0.004$) but no interaction between anthocyanin morph and proportion of leaf area consumed ($F_{1,39} = 3.14$, $P = 0.08$). For both A- and A+ color morphs, slugs that consumed more leaf area gained more mass.

Aphids

Preference.—In 5 of the 17 populations of *R. sativus* in northern California, we found that the frequency of *B. brassicae* colonization of the anthocyanin morphs differed significantly from the expected frequency ($P < 0.05$ in all five cases). Yet, *B. brassicae* colonization showed no consistent trend on specific anthocyanin-color morphs. In three populations, A- morphs had

more aphids than expected, and in two populations, A+ morphs had more aphids than expected.

Moreover, in the experimental arrays with equal frequencies of the anthocyanin morphs, we found no differential colonization of the morphs by *B. brassicae* (MANOVA: $F_{2,34} = 2.06$, $P = 0.14$), measured as the number of aphids on the morphs after three weeks (A- vs. A+ mean ± 1 SE: 21.20 ± 7.43 aphids vs. 27.90 ± 7.93 aphids) and as the number of days until individual plants were colonized by aphids (A- vs. A+ [mean ± 1 SE]: 8.60 ± 2.14 d vs. 12.61 ± 2.91 d). Array had a significant effect on aphid colonization ($F_{2,35} = 5.28$, $P = 0.009$); however, we found no interaction between array and anthocyanin-color morph ($F_{2,35} = 1.46$, $P = 0.24$).

Performance.—*B. brassicae* colony growth differed on the anthocyanin morphs (MANOVA: $F_{2,63} = 4.64$, $P = 0.013$). Aphids on A- morphs (yellow and white) had larger colony sizes on the last census date ($F_{1,64} = 9.43$, $P = 0.003$; Fig. 5) and had higher per capita growth rates ($F_{1,64} = 7.76$, $P = 0.007$; Fig. 5) than those on A+ morphs (pink and bronze).

Thrips

Preference.—In both the Highway 116 and the Putah Creek populations, the A- color morphs had more thrips per flower than A+ color morphs; however, this difference was not statistically significant (Highway 116, $\chi^2 = 2.62$, $df = 1$, $P = 0.11$; Putah Creek, $\chi^2 = 1.34$, $df = 1$, $P = 0.25$). *F. occidentalis* densities ranged from 0 to 17 thrips per flower, with a median of 5.5 thrips per flower in A- morphs and 4.0 thrips per flower in A+ morphs.

Phytochemical measurements

In the analysis of leaf chemistry, we found that 50% leaf removal by *P. rapae* increased concentrations of

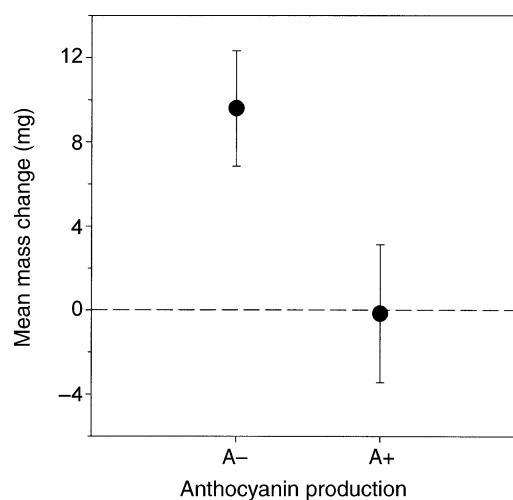


FIG. 4. *Agriolimax reticulatus* gained significantly more mass feeding on A- color morphs than on A+ color morphs of *Raphanus sativus*. Symbols are mean change in mass in mg (± 1 SE) after three days of feeding.

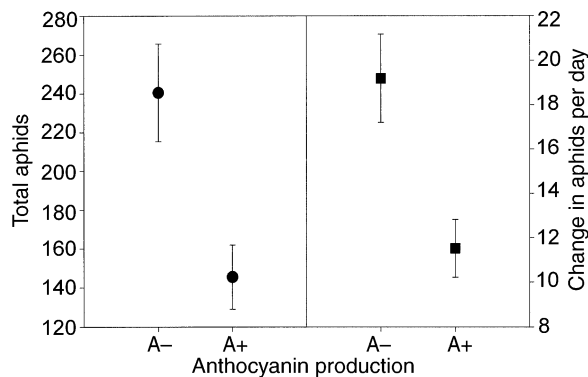


FIG. 5. A- color morphs of *Raphanus sativus* supported higher colony growth rates of *Brevicoryne brassicae* compared to A+ color morphs, measured as the number of aphids per plant after 14 days (circle) and the per capita growth rate (square). Symbols are means \pm 1 SE.

indole glucosinolates in the undamaged fifth true leaf by 62% ($F_{1,50} = 21.60$, $P < 0.0001$; Fig. 6). We found no effect of plant family nor of anthocyanin morph on indole glucosinolate concentrations (family, $F_{1,50} = 1.34$, $P = 0.23$; anthocyanin morph, $F_{1,50} = 1.49$, $P = 0.23$). However, we found a significant damage \times anthocyanin morph interaction ($F_{1,50} = 7.61$, $P = 0.008$; Fig. 6). In damaged plants, A+ morphs induced higher concentrations of indole glucosinolates than A- morphs (Fig. 6). Induction of indole glucosinolate concentrations in damaged *R. sativus* was 38% greater in A+ morphs than A- morphs.

DISCUSSION

Taken together, our work provides evidence that anthocyanin pigment in floral tissue influences herbivore preference and performance. However, results from the different herbivores were not always congruent (Table 1). Herbivores varied in their preference of the anthocyanin flower color morphs, especially at nonflowering vs. flowering stages (Table 1). Both Lepidoptera and slugs exhibited no preference among nonflowering color morphs. However, upon flowering, A- morphs (yellow and white flowers) experienced higher lepidopteran oviposition and slug consumption than A+ morphs (pink and bronze flowers). Neither aphids nor thrips differed in their preference for anthocyanin-color morphs. For herbivore performance, A- color morphs (yellow and white flowers) supported higher herbivore growth and/or reproduction than A+ color morphs (pink and bronze flowers). The exception to this pattern was that A+ morphs supported higher growth of larval *P. rapae* and *S. exigua* (Table 1). We did not expect herbivore performance on the anthocyanin-color morphs to be correlated among all of the different herbivores due to differences in feeding modes and sensory abilities, and herbivore preference and performance are often uncorrelated, both among and within herbivores (see Thompson [1988] for a review).

Pleiotropic effects may exist between the synthesis of floral pigments and secondary compounds in *R. sativus*, as has been suggested with *Ipomoea purpurea* (Fineblum and Rausher 1997), potentially influencing herbivore preference and performance. Chemical analyses of leaf glucosinolates revealed that A+ color morphs produced higher concentrations of indole glucosinolates than A- color morphs in the presence of herbivore damage. A wealth of studies have shown that glucosinolates can negatively affect the preference and performance of herbivores, including many of the herbivores studied here (e.g., Blau et al. 1978, Louda and Rodman 1983, Chew 1988a, b, Louda and Mole 1992) and that damage to wild radish by *P. rapae* larvae induces increased concentrations of indole glucosinolates (e.g., Agrawal et al. 1999). In this study, most herbivores exhibited lower preference for A+ color morphs and these morphs supported lower herbivore performance because, potentially, A+ morphs were heavily defended once damaged. That specialist *P. rapae* performed better on A+ morphs was not surprising as previous studies have shown that increased concentrations of glucosinolates do not adversely affect the relative growth of *P. rapae* per se (e.g., Blau et al. 1978). However, that generalist *S. exigua* exhibited similar performance patterns to *P. rapae* was unexpected, especially given the difference in indole glucosinolates between damaged A+ and A- morphs, and deserves additional consideration. We are currently exploring the biochemical links between anthocyanin floral pigments and glucosinolate production and their combined effects on herbivores.

Clearly, plant defensive chemistry is just one mechanism that might explain the preference and performance patterns observed, and it is very unlikely that this one mechanism could be used to explain the patterns exhibited by all of the herbivores. Differences in

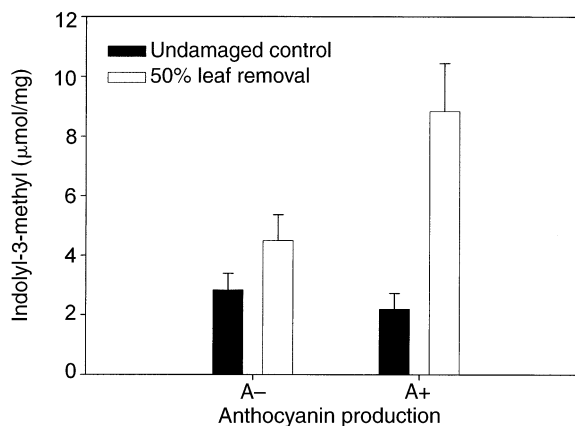


FIG. 6. In the presence of 50% leaf removal by *Pieris rapae* larvae, A+ color morphs produced higher concentrations of the indole glucosinolate indolyl-3-methyl than A- color morphs of *Raphanus sativus*. Bars are means (\pm 1 SE) measured in $\mu\text{mol/mg}$.

TABLE 1. Patterns of preference and performance of four different types of herbivores for anthocyanin flower color morphs of *Raphanus sativus* in laboratory, greenhouse, and field experiments.

Herbivore	Trial	A- (yellow and white)	A+ (pink and bronze)
Lepidoptera			
Preference	nonflr	=	=
	flr	+	-
Performance	nonflr	-	+
Slugs			
Preference	nonflr	=	=
	flr	+	-
Performance	flr	+	-
Aphids			
Preference	flr	=	=
Performance	flr	+	-
Thrips			
Preference	flr	=	=

Notes: A- color morphs produce yellow and white flowers; A+ morphs produce pink and bronze flowers. "Nonflr" refers to nonflowering plants, and "flr" refers to flowering plants. Symbol definitions: + indicates higher preference or performance; -, lower; =, equal. For Lepidoptera performance, both specialist *P. rapae* and generalist *S. exigua* exhibited similar performance patterns.

herbivore behavior, physiology, sensory ability, and mode of feeding will likely influence their growth on host plants and the cues they use to select plants. Flower color itself is an important cue for many insects, facilitating long- and short-range detection of host plants (Kevan 1983). Our results for *P. rapae* suggest that flower color, along with plant defensive chemistry, could be an important cue, as females showed no difference in oviposition among nonflowering morphs, but did show differential oviposition on flowering morphs. Moreover, a previous study found that *P. rapae* prefer to nectar feed on A- color morphs over A+ morphs (Stanton et al. 1989), and female *P. rapae* may nectar feed and oviposit on the same individual plants. However, phenological changes in the color morphs associated with flowering, including possible changes in plant chemistry (A. L. Shelton, *personal communication*), cannot be ruled out, especially because slugs did not discriminate among morphs in nonflowering plants, while there were clear preferences for A- morphs post-flowering. And although not statistically significant, more western flower thrips were found in A- color morphs, and floral pigments may serve as cues (Vernon and Gillespie 1990, Gaum et al. 1994), as thrips feed primarily on pollen and floral parts. In addition, A- color morphs produce significantly more pollen than A+ morphs (S. Y. Strauss and R. E. Irwin, *unpublished data*), making them more profitable for thrips.

Given that variation in flower color influenced herbivore preference and performance, could herbivory contribute to maintaining the flower color polymorphism in natural *R. sativus* populations, especially in

the face of strong pollinator selection for A- color morphs? The results suggest that herbivores may provide selection pressures that counter the pressures generated by pollinators. Both lepidoptera and slugs preferred A- color morphs, and slugs also performed better on these morphs. Increased damage to *Raphanus* spp. has strong negative direct and indirect effects on male and female relative fitness (e.g., Mauricio et al. 1993, Strauss et al. 1996, Lehtilä and Strauss 1997, 1999, Agrawal 1999; but see Strauss et al. 2001). For example, 50% leaf removal by herbivores reduced flower production and reproductive biomass by 25% (Mauricio et al. 1993) and indirectly influenced pollinator visitation (Lehtilä and Strauss 1997). In the case of flower thrips, large thrips infestations in A- color morphs deform flowers and reduce pollen availability (Gaum et al. 1994). Changes in the attractiveness of flowers can reduce male reproductive success in *Raphanus* spp. by 60% (Stanton et al. 1986), results suggesting that there may be selection against A- color morphs via male function. While we did not find differences in aphid preference of color morphs, colony growth rates of cabbage aphids were almost two times larger on A- color morphs than on A+ morphs. In this case, selection could be imposed via herbivore performance rather than preference. For example, Snow and Stanton (1988) showed that large aphid infestations reduced fruit production by 50% in *R. sativus* at similar sites. As a whole, these results suggest strong negative direct and indirect effects on the survival and male and female reproductive success of A- color morphs via changes in herbivore preference and performance. Each of these herbivores may provide counter selective pressures on floral anthocyanin morph compared to those exerted by pollinators.

Petal color polymorphisms in flowering angiosperms are widespread (Kay 1978), and recent empirical studies have begun to investigate a variety of factors that might help maintain these polymorphisms. For example, Schemske and Bierzychudek (2001) found that in *Linanthus parryae*, precipitation was negatively correlated with reproductive success in blue-flowered plants but was positively correlated with reproduction in white-flowered plants. Here we provide evidence of differential herbivore preference and performance between anthocyanin flower color morphs of *Raphanus sativus*, suggesting that herbivores may provide counter-selection pressures to those exerted by pollinators. We realize that herbivory provides just one alternative selection pressure, and other factors, such as competition, water and nutrient resources, and differential demographic characters (i.e., differential germination or seedling survival) may influence the maintenance of flower color polymorphisms (e.g., Mogford 1974b, Horovitz 1976, Abbott 1981, Ernst 1987, Schemske and Bierzychudek 2001; S. Y. Strauss and R. E. Irwin, *unpublished data*), or variation in these floral traits may be neutral. Clearly, complex studies that incorporate mul-

multiple sources of selection can provide us with a better understanding of the evolution of plant and floral traits.

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APPENDIX

In the analyses of all experimental preference studies in which the anthocyanin morphs were offered simultaneously to the study animals, we used multivariate analyses because the anthocyanin morphs in these experiments were nonindependent (i.e., the consumption of one morph is likely dependent on the presence of the other) and the error terms within animals are likely correlated (Johnson 1980, Roa 1992). The response variable in these analyses was a vector of consumption (i.e., rank consumption of the proportion of leaf area consumed). We ranked the consumption data (rather than comparing proportional-usage data) because the experimental studies were only meant to test the relative use of the anthocyanin morphs and not absolute preference, per se (Johnson 1980). We tested the null hypothesis that there was no preference among anthocyanin morphs by the consumer

by testing whether the mean vector of consumption was equal to a constant, k , calculated from the data as:

$$k = \left(\frac{1}{pn} \right) \sum_i \sum_j x_{ij}$$

where p is the number of anthocyanin morphs (recessive or dominant), n is the number of replicates, and x_{ij} are the measured rank consumptions (Roa 1992). To center the data around zero, k was subtracted from each point so that the constant of no preference was zero and the null hypothesis of no preference was:

$$H_0: [\mu_{\text{anthocyanin recessive}}, \mu_{\text{anthocyanin dominant}}] = [0, 0].$$

To test this hypothesis, we used the multivariate Hotelling's T^2 which is distributed as T^2_{p, n_1+n_2-2} (Roa 1992).