

A field investigation into potential reproductive and resistance advantages of purple-petalled plants in polymorphic populations of horsenettle (*Solanum carolinense*)

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Abstract

Background and aims – Variation in flower color among individuals within plant populations has long fascinated horticulturalists and evolutionary biologists alike. Nevertheless, the diversity of genetic factors and ecological interactions that act to maintain flower-color polymorphisms over time are understood for very few species. Here, we suggest that *Solanum carolinense* (horsenettle) makes an excellent model system for studying the evolutionary ecology of a flower-color polymorphism, and we begin to shed insight into factors that might affect the stability of the polymorphism.

Material and methods – We transplanted 24 horsenettle ramets of each of 40 field-collected genets (10 genets per each of four source fields) into a common-garden plot (in an oldfield with an existing horsenettle population). For each ramet, we visually characterized petal color and quantified production of flowers, fruits, seeds, and damage by 11 species of herbivores.

Key results – Rather than varying continuously within ramets and among genets, petal color fell into three relatively discrete categories that are common in most horsenettle populations: purple, white, and mauve. In the common-garden experiment, purple-petalled morphs initiated significantly more flower buds, opened more flowers, and produced more fruits and seeds than did the mauve-petalled or white-petalled morphs. Petal-color morphs differed only slightly in resistance to a minority of the herbivore community, with no consistent advantage for color morphs across species of herbivores or types of tissues fed upon.

Conclusion – Horsenettle has numerous characteristics that make it an excellent system for studying the genetics and ecology that drive the maintenance of flower-color polymorphisms. Although pleiotropic effects of flower-color on resistance to herbivory appear to be minimal in horsenettle, potential pleiotropies that differentially affect maternal and paternal reproduction, or sexual and vegetative reproduction, are worthy of further study.

Keywords

andromonoecy, *Anthonomus*, florivory, flower color, horsenettle, multiple herbivores, pleiotropy, polymorphism, resistance to herbivory, Solanaceae

INTRODUCTION

A fundamental goal of evolutionary ecology is to explain the persistence of genetic variation among individuals

within populations of organisms. For botanists, variation in flower color has been a particularly popular subject of study. Flower-color polymorphisms (i.e., when more than one phenotype occurs at a proportion above what can

be explained by mutation alone) are intriguing because they are visually striking, taxonomically widespread, and likely to have consequences on plants' relative fitness (Warren and Mackenzie 2001; Carlson and Holsinger 2010; Arista et al. 2013; Narbona et al. 2018; Tenhumberg et al. 2023). All things being equal, natural selection would be expected to purge less-fit phenotypes and their underlying alleles from a population, leading populations to be monomorphic in flower color. Therefore, the maintenance of polymorphisms in traits that are closely tied to fitness—such as flower color—suggests that interesting ecological and evolutionary interactions are at work (Sapir et al. 2021).

Conventional wisdom holds that the *raison d'être* for colorful flowers is to attract animals to maximize pollination. Although ecological interactions among plants and pollinating agents are a main driver of the evolution of flower color (Eckhart et al. 2006; Muchhala et al. 2014; Caruso et al. 2018; Kellenberger et al. 2019; Narbona et al. 2021; Trunschke et al. 2021), the maintenance of flower-color polymorphisms can rarely be explained solely by plants' interactions with pollinators (Paine et al. 2019; Dafni et al. 2020; Sapir et al. 2021; Sullivan and Koski 2021). Increasingly, researchers are recognizing that plants' interactions with herbivores and other natural enemies may play a major role in enabling the persistence of more than one flower-color morph within plant populations (Irwin et al. 2003; Strauss and Whittall 2006; Rausher 2008; Carlson and Holsinger 2010; Tenhumberg et al. 2023).

Most directly, florivores (herbivores that feed on flowers) may be attracted to the same color cues as pollinators. The costs of florivory could thus counteract the pollinator-derived benefit of a flower color and potentially lead to a fitness advantage of a different color (Giamoustaris and Mithen 1996; Irwin et al. 2003; Strauss and Irwin 2004; de Jager and Ellis 2014). Balancing selection for flower color imposed by pollinators and florivores might thus lead to the maintenance of a polymorphism (Sapir et al. 2021).

In addition to a direct influence of florivory, herbivores of any type of plant tissue may affect flower-color evolution in an indirect manner. On a molecular basis, changes in flower color are often caused by a mutation that prevents the formation of a pigment that is the usual end-product of a branch of a complex biosynthetic pathway (Rausher 2006, 2008; Dick et al. 2011; Del Valle et al. 2019). Many of the chemical precursors of pigments—as well as chemical end-products of other branches of the pigment pathway—serve protective roles in plants (Fineblum and Rausher 1997; Clegg and Durbin 2000). These secondary metabolites include flavones, flavonols, condensed tannins, lignins, and others that are known to confer a degree of resistance against herbivory (Rausher and Fry 1993; Shirley 1996; Davies 2009; Bar-Akiva et al. 2010; Liu et al. 2021; Singh et al. 2021). Depending on where in a pigment's pathway a mutation occurs, a pleiotropic effect could lead either to a reduction or to an increase in the production of chemicals that promote resistance

to herbivory (Simms and Bucher 1996; Fineblum and Rausher 1997). Therefore, evolution of a new flower color could either be constrained by or facilitated by side-effects on resistance to herbivory. In addition, balancing selection due to offsetting effects on pollinator attraction and resistance to herbivores could maintain polymorphisms in flower color in plant populations.

Most flowering plant species are subjected to a community of multiple herbivores of various guilds that collectively feed on all types of plant tissues. The greater the diversity of herbivores, the more likely it is that a mutation in a gene that controls flower color will have pleiotropic effects on the plant's resistance to at least one species of herbivore. Moreover, if damage levels of any pair of herbivore species are influenced in opposite ways by flower-color variants, then the impacts of these two herbivores on plant fitness might also be expected to result in balancing selection on flower color—even if the pollinators are unaffected by the variation in flower color.

While evidence supporting non-pollinator influences on the evolution of flower color exists, the number of plant species examined is still limited. Moreover, very few studies have come close to including all of the herbivores that feed on any species of host plant. More empirical studies on more systems are thus necessary before any firm conclusion can be made regarding the question of how important herbivores are in the maintenance of flower-color polymorphisms in nature. Here, we use data from field surveys and a common-garden experiment on the native herbaceous plant *Solanum carolinense* L. (Solanaceae) to examine the potential influence of herbivores on the maintenance of an apparent polymorphism in flower color.

Solanum carolinense, or horsenettle, is a common weed of old agricultural fields and disturbed areas. Although the color of horsenettle's petals is usually simply reported as varying from white to purple, the range of color variation found within plants, between plants, and among populations has not been well characterized—nor has the genetic control of flower color been studied for this species. Nevertheless, excellent work has been done on the genetics and ecology of flower color in wild populations of other solanaceous plants (Smith and Rausher 2011; Coburn et al. 2015; Berardi et al. 2016; Larter et al. 2018; Li et al. 2019), and research on pigments in well-studied congeners, such as potatoes, eggplants, and tomatoes (*S. tuberosum* L., *S. melongena* L., and *S. lycopersicum* L., respectively), can provide important foundational information (Harborne and Swain 1979; Armbruster 2002). Importantly, horsenettle has a diverse and well-studied community of insect herbivores, including not only florivores but also a variety of leaf, stem, and fruit feeders (Wise 2007b).

This paper has five specific goals: 1) characterize the petal-color variation of horsenettle flowers; 2) quantify the frequencies of the color morphs in field populations; 3) assess the consistency of petal color both within ramets and across ramets of the same genet to help establish

whether petal color is under genetic control rather than (or in addition to) environmental control; 4) quantify fitness-related traits (e.g., flower and seed production) for ramets of the different petal-color morphs to infer whether natural selection is likely to be acting on petal color; and 5) investigate potential relationships between petal color and resistance to a community of eleven species of herbivores that represent a diversity of feeding guilds.

MATERIAL AND METHODS

Natural history

Solanum carolinense L. (horsenettle) is a weedy herbaceous perennial plant that is native to the southeastern United States but that has established populations throughout the U.S., as well as in parts of Europe and Asia (Bassett and Munro 1986; Imura 2003; Follak and Strauss 2010). Within populations, horsenettle genets spread mainly by means of fast-growing lateral roots, while sexual reproduction enables them to spread to newly disturbed sites via animal-dispersed seeds (Ilnicki et al. 1962; Nichols et al. 1991). As is typical for the nightshade family (Solanaceae), horsenettle's flowers are radially symmetric, with five sepals and petals surrounding a columnar ring of five yellow anthers and a single ovary with a single style (Fig. 1). The color of horsenettle's petals has been described as varying from white to purple (or white to blue, mauve, or violet). Although floral pigments have apparently not been studied in horsenettle specifically, the blue, purple, and red coloration of flowers in the nightshade family are conferred by anthocyanins—the most diverse and widespread class of flower pigments across the angiosperms (Grotewold 2006; Davies 2009; Berardi et al. 2016). More specifically, delphinidin-based anthocyanins have been found to be responsible for the purple color in the petals of other species in the genus *Solanum* L. (Jaeger 1985; Eich 2008; Ng et al. 2018).

As is also common for species of *Solanum*, horsenettle is andromonoecious, with individuals producing cosexual (perfect) flowers (Fig. 1B, C) as well as male flowers that have much-reduced styles and infertile ovaries (Fig. 1A) (Whalen and Costich 1986; Anderson and Symon 1989). Horsenettle ramets typically produce more perfect flowers than male flowers, but floral-sex ratio is both phenotypically plastic (Solomon 1985; Steven et al. 1999; Wise and Cummins 2007) and highly heritable in this species (Elle 1998; Wise and Cummins 2006; Vallejo-Marín and Rausher 2007b; Quesada-Aguilar et al. 2008). Horsenettle is obligately outcrossing (Richman et al. 1995; Stone 2004), and it relies primarily on large-bodied bees capable of buzz-pollination to transfer pollen to receptive stigmas of flowers on ramets from a different genet in order for fruit-set and maturation to occur (Connolly and Anderson 2003; Quesada-Aguilar et al. 2008). Ripe fruits are round yellow berries with a typical diameter of ~1.5

cm, but ranging up to ~2.5 cm (Wise and Sacchi 1996; Wise and Cummins 2007).

Native horsenettle populations are attacked by a diverse set of specialist insects that collectively feed on all parts of the plants (Wise 2007b). Previous studies have shown that herbivore damage can negatively affect horsenettle's growth and reproduction (Solomon 1983; Wise and Sacchi 1996; Wise et al. 2008; Underwood and Halpern 2012; Wise and Mudrak 2021; Coverdale and Agrawal 2022). Moreover, many of its herbivores were found to impose natural selection for increased resistance in an experimental field population of horsenettle (Wise and Rausher 2013, 2016).

The current study focuses on damage by eleven species, including ten insects and one cricetid rodent—the eastern meadow vole, *Microtus pennsylvanicus* (Ord, 1815). These voles tend to chew through the pedicels of horsenettle racemes, causing mortality to flowers and fruits, even though they do not appear to actually consume most of them (Wise 2007b).

Five of the ten insect species included in this study specialize on leaves: the eggplant leafminer (*Keiferia inconspicua* (Murtfeldt, 1883), Lepidoptera: Gelechiidae); the eggplant tortoise beetle (*Gratiana pallidula* (Boheman, 1854), Coleoptera: Chrysomelidae); the eggplant flea beetle (*Epitrix fuscata* Crotch, 1873, Coleoptera: Chrysomelidae); the eggplant lace bug (*Gargaphia solani* Heidemann, 1914, Hemiptera: Tingidae); and the citrus gall midge (*Prodioplosis longifila* Gagné, 1986, Diptera: Cecidomyiidae). Two of the insect species specialize on flowers: the potato bud weevil (*Anthonomus nigritus* Boheman, 1843, Coleoptera: Curculionidae); and the second brood of the gelechiid moth *Frumentia nundinella* (Zeller, 1873), which lays eggs on flower buds, while the larvae feed inside parthenocarpic, seedless fruits (Solomon 1980). Adults of the pepper maggot (*Zonosemata electa* (Say, 1830), Diptera: Tephritidae) lay eggs in fruits, and their larvae feed on pulp, causing fruit spoilage. Larvae of the potato stalk borer (*Trichobaris trinitata* (Say, 1832), Coleoptera: Curculionidae) feed and pupate inside horsenettle stems. Finally, adults and larvae of the false potato beetle (*Leptinotarsa juncta* (Germar, 1824), Coleoptera: Chrysomelidae) feed liberally on leaves, flowers, and fruits. With the exception of the citrus gall midge (which is of neotropical origin), all of these insect species are native specialists on horsenettle, though several have also become pests of solanaceous crops (Wise 2007b).

Source and propagation of plants

The horsenettle plants used in this study originated from four field populations in northern Virginia, USA, that represent a variety of land-use and management histories. Two fields were located at Blandy Experimental Farm (BEF) in Clarke County—one an old agricultural field undergoing secondary succession and the other a field managed as a grassland/wildflower meadow. The third

population was located in an old horse pasture at Sky Meadows State Park in Fauquier County (~13 km from BEF), and the fourth population was in a ruderal field between a woodlot and See Lane in rural Frederick County (~7 km from BEF). In the early spring of 1997, perennial roots were excavated from 30 newly emerging horsenettle ramets along transects in each of the four populations. To increase the likelihood that the roots were from different genets, we collected individuals that were a minimum of 6 m apart. Unique genet identity was later tested with pollination trials, wherein progeny of roots originating from adjacent transect locations were confirmed to be separate genets only if their cross-pollinations resulted in successful fruit maturation. If fruit maturation did not occur, then one of the source plants was eliminated and the other was cross-pollinated with progeny of the roots originating from the next position on the collection transect, and so on.

Sections of new root growth of each genet were planted into 18.9-L (5-gal) plastic pots filled with a peat-based commercial growing medium (Wesco Growing Media III, Wetsel Seed Company, Harrisonburg, Virginia, USA) each spring from 1997 to 2001. This procedure served to purge genets of carryover (non-genetic) influences caused by environmental variation in the source fields, as well as to generate multiple genetically identical ramets from each genet for a series of experiments performed between 1998 and 2002.

Experimental design

The analyses for the current paper on flower color in horsenettle involve data collected from a large common-garden experiment performed at BEF in 2001. Twenty-four ramets from each of 40 horsenettle genets (10 from each source population, 960 ramets total) were transplanted in a randomized block design in the midst of an existing oldfield population of horsenettle. The experimental design and data-collection procedures have been described in detail elsewhere (Wise 2007a, 2009; Wise and Hébert 2010; Wise and Rausher 2013). In the spring of 2001, at least thirty equal-sized (2 cm³)

root sections of each genet were planted singly in Wesco Growing Media III in 3.8-L (1-gal) round plastic pots. Between 28 June and 2 July 2001, 320 ramets (8 per genet) were transplanted into the horsenettle field at a spacing of 1.5 m apart within 10 rows that were 2 m apart. This experimental setup was replicated in three spatial blocks within the field. The ratio of transplanted ramets to horsenettle ramets growing naturally in the field was estimated to be ~1-to-30 (Wise 2007b).

Damage to leaves, flowers, fruits, and stems was quantified throughout the summer for eleven species of herbivores. Details on how damage was distinguished among the species and quantified have been published elsewhere (Wise 2007b). Briefly, folivory was quantified for five species as a measure of relative leaf area damaged, and for one species (*viz.* eggplant lace bugs) as the presence or absence of damage caused by a brood of nymphs. Florivory was quantified for each of four species by the proportion of flower buds that were destroyed. Frugivory was quantified for three species as the proportion of maturing fruits that were destroyed (or for pepper maggots, infested and showing appreciable damage that led to rotting). Finally, damage by the one species of stem borer was quantified as either present or absent.

To identify flower sex, characterize petal color, and quantify florivory, each of the 960 ramets was visited every 3–5 d. Three different investigators visited each ramet on a rotating schedule, so the determination of petal color for each ramet was based on a consensus of three observers over a ramet's flowering period.

We quantified five traits related to reproductive performance for each ramet: the number of flower buds matured, the number of flowers successfully opened, the sex ratio of the flowers, the number of ripe undamaged fruits, and the number of seeds produced. Each fruit was collected upon ripening and its size estimated as the mode of at least three measurements of its diameter. The number of seeds per fruit was estimated using the prediction equation ($r^2 = 0.90$) developed by Wise and Cummins (2007) for horsenettle fruits, where d is the fruit diameter in cm:

$$\text{Seeds per fruit} = 70.1 - 23.0d + 2.18d^2 - 0.0415d^3$$

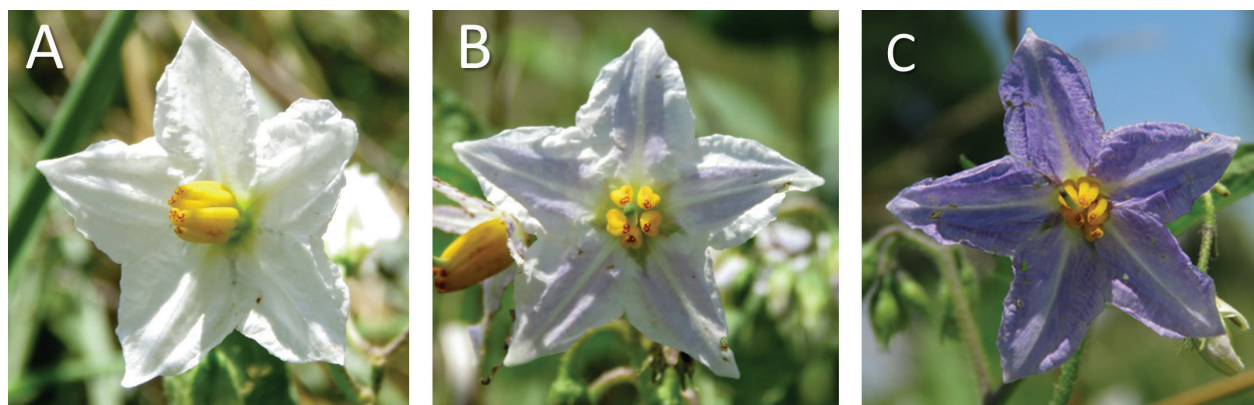


Figure 1. Horsenettle flowers representing white- (A), mauve- (B), and purple- (C) petalled morphs. In these photos, the white flower is male (with no style visible), while the other two are cosexual (with styles protruding beyond the yellow anthers).

The total seed production for a ramet was quantified as the sum of the seed-number estimates for all of the fruits produced by that ramet.

Statistical analyses

To assess differences in sexual-reproductive traits among petal-color morphs, we ran a MANOVA (multivariate ANOVA) with petal color (white, mauve, or purple) as an explanatory variable. The original source population of each plant was also included as an explanatory variable to take into account any fixed differences in the reproductive traits across the four source populations that are independent of petal color. The MANOVA included the following response variables: number of flower buds, number of open flowers, floral-sex ratio (% male flowers), number of fruits, and number of seeds. Following the MANOVA, we ran a separate ANOVA for each of the five reproductive traits, with petal color and source population as the explanatory variables. For the traits that were significantly affected by petal color ($P < 0.05$ in an ANOVA), Tukey's honestly significant difference (HSD) tests were run to determine which of the three petal-color morphs differed significantly from each other. All statistical analyses reported in this paper were performed using JMP IN 4.0.4 (SAS Institute, Cary, North Carolina, USA).

To examine more closely how petal color may have led to differences in seed production, we ran two additional ANCOVAs on seed number—one that included number of flower buds as a covariate (in addition to the petal-color and source-population explanatory variables), and one that included number of open flowers and floral-sex ratio as covariates (in addition to petal color and source population). The first of these ANCOVAs addressed whether petal color had an influence on seed production beyond the seed production's relationship to the number of flower buds initiated (e.g., differences in proportion of buds that opened or that successfully matured their ovaries.) The second ANCOVA addressed whether petal color had an influence on seed production beyond its relationship to the number of flowers opened or the sex ratio of the flowers (e.g., differences in successful pollination and fertilization of ovules). Each of the ANOVAs and ANCOVAs included all 960 ramets, except for the two analyses that involved floral-sex ratio, which included only the 914 ramets that successfully opened flowers. Tukey HSD tests were performed to assess the significance of pairwise differences between petal-color morphs for all response variables.

To assess whether resistance to any of the herbivores differed among petal-color morphs, we ran a MANOVA with petal color as an explanatory variable and damage level by each herbivore (14 total damage metrics across 11 species) as the response variables. The original source population of each plant was also included as an explanatory variable in the MANOVA to take into account any fixed differences in resistance level (independent of

petal color) across the four source populations. Following the MANOVA, we ran a separate ANOVA for each of the 14 types of damage, with petal color and source population as the explanatory variables. For the herbivores in which petal color at least marginally significantly affected damage level ($P < 0.10$ in an ANOVA), Tukey's HSD tests were run to determine which of the three petal-color morphs differed significantly from each other.

RESULTS

Flower-color observations

Across the 960 horsenettle ramets in the field experiment, flower petals exhibited colors ranging from white to deep purple; however, close visual examination showed that the variation in color could be consistently classified into three categories: white petals, purple petals, or petals exhibiting purple streaks on a white background (Fig. 1).

Within each ramet, all flowers fell into the same color category. Similarly, petal color was consistent among all of the ramets within the same genet. Although there was petal-color variation within a ramet, the differences were ontogenetic and predictable. Specifically, in the streaky-flowered individuals, petals darkened from anthesis to senescence as the purple pigment appeared to diffuse gradually into the white sectors of the petals. The shade of purple that these petals attained was noticeably lighter than for the individuals whose petals were completely purple upon anthesis. For simplicity, we refer to the individuals with the streaky or pale purple petals as being "mauve".

Some variation in petal color was observed among ramets of white-petalled genets. Notably, in at least one ramet of five of the white-petalled genets, we observed either small purple spotting on the otherwise all-white petals, purple shading on the edges of the petals, or very light purple streaks on the central veins on the lower surface of the petals. We also observed minor variation in petal color among the purple-petalled genets. Most strikingly, the ramets of one genet were noted for having an especially deep shade of purple in their petals.

Polymorphism in the field

All three petal-color categories were represented by numerous genets in the field populations (Fig. 2). The purple morph was the most common among genets from three of the four source populations; the white morph was the least common overall—represented by only 20% of the 40 genets; and the mauve morph was represented by 25% of the genets. No white-petalled genets were found in the sample of ten genets from the Sky Meadows population, while no mauve-petalled genets were found in the sample from the Blandy Meadow population.

Table 1. Results of the MANOVA of five reproductive traits on the source population of the horsenettle plants and their petal color (white, purple, or mauve) in the common-garden experiment.

Source	Wilks' lambda	Approx. F	Degrees of freedom		P-value
			Num.	Denom.	
Population	0.7593009	21.8883	12	2395	< 0.0001
Petal color	0.9081359	11.1677	8	1810	< 0.0001

Table 2. Summary of ANOVA results for the influence of source population and petal color on traits related to sexual reproduction of horsenettle ramets in the common-garden experiment.

Source	Df	MS	F-ratio	P-value
A. Flower buds				
Population	3	19481.4	87.7	< 0.0001
Petal color	2	6120.1	27.5	< 0.0001
Error	954	222.2		
B. Flowers opened				
Population	3	5251.11	69.85	< 0.0001
Petal color	2	1806.13	24.03	< 0.0001
Error	925	75.17		
C. Fruits				
Population	3	1396.03	52.55	< 0.0001
Petal color	3	539.97	20.33	< 0.0001
Error	954	26.57		
D. Floral-sex ratio				
Population	3	0.419799	11.3013	< 0.0001
Petal color	2	0.029797	0.8022	0.45
Error	908	0.037146		
E. Seeds				
Population	3	482528	13.7621	< 0.0001
Petal color	2	209000	5.9609	0.0027
Error	954	35062		

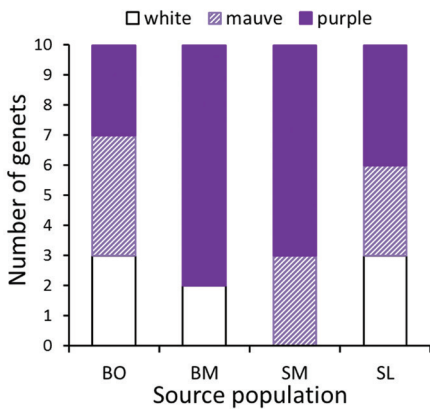


Figure 2. Frequency distribution of petal-color morphs for the 40 horsenettle genets in the common-garden experiment, separated by original source field. BO = Blandy Oldfield; BM = Blandy Meadow; SM = Sky Meadows; SL = See Lane.

Reproductive success

Petal color had a significant influence on the overall pattern of reproductive traits in the field experiment, as determined by the MANOVA that included all five reproductive traits ($P < 0.0001$, Table 1). The subsequent ANOVAs and Tukey HSD tests revealed that, on average, purple-petalled ramets produced 53% more flower buds, opened 66% more flowers, and matured 61% more fruits than the white-petalled ramets did; and purple-petalled ramets produced 19% more flower buds, opened 11% more flowers, and matured 17% more fruits than mauve-petalled ramets did (Table 2, Fig. 3). The floral-sex ratio for white-petalled ramets leaned slightly more heavily toward male flowers (mean = 21%) than did the purple-petalled and mauve-petalled ramets (means = 18%), but this difference was not statistically significant ($P = 0.44$, Table 2D).

The differences among color morphs in the various reproductive traits culminated in purple-petalled ramets producing significantly more seeds than did the ramets with the other petal colors ($P = 0.0027$, Table 2E). Specifically, ramets from purple-petalled genets produced a mean of 22% more seeds than ramets from white-petalled genets and 37% more seeds than ramets from mauve-petalled genets (Fig. 4A). Notably, the mauve-petalled ramets had the lowest mean seed production (Fig. 4A), even though they were the intermediate morph for the other reproductive traits (Fig. 3).

Table 3. Summary of ANCOVA results for the influence of petal color on sexual reproduction of horsenettle ramets in the field experiment.

Source	Df	MS	F-ratio	P-value
A. Seeds				
Population	3	222512	7.9455	< 0.0001
Flower buds	1	6760658	241.4111	< 0.0001
Petal color	2	108870	3.8876	0.021
Error	953	28005		
B. Seeds				
Population	3	203414	7.8619	< 0.0001
Flowers opened	1	8625509	333.3744	< 0.0001
Floral-sex ratio	1	1053503	40.7177	< 0.0001
Petal color	2	230241	8.8988	0.0001
Error	908	25873		

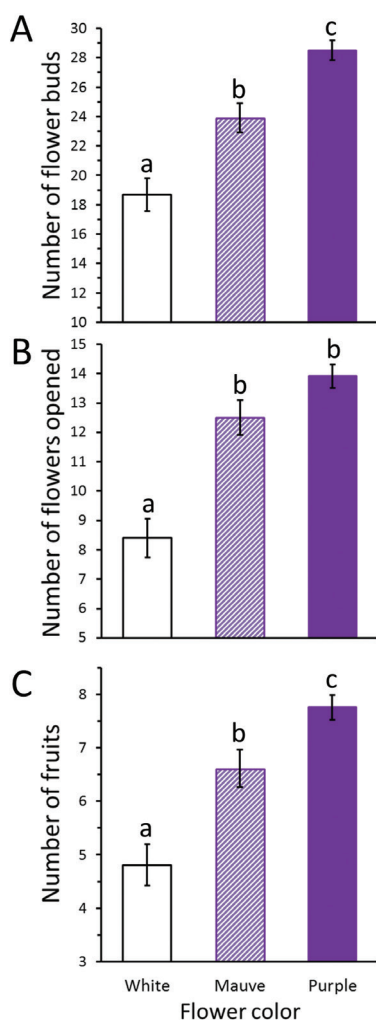


Figure 3. Comparison of reproductive traits by white-, mauve-, and purple-petalled ramets. **A.** Number of flower buds. **B.** Number of flowers successfully opened. **C.** Number of fruits matured. The bars represent least-squares means $\pm$ 1 SEM from the ANOVAs (Tables 1, 2). Bars that do not share a lower-case letter are significantly different from each other at $P < 0.05$, as determined by Tukey HSD tests.

When the influence of the number of flower buds or number of open flowers and floral-sex ratio was extracted by the ANCOVAs on seed production, petal color had a much smaller, but still statistically significant, effect on seed production ($P = 0.021$ and $P = 0.0001$, respectively, Table 3). On average, purple-petalled ramets produced more seeds than mauve-petalled ramets—beyond what would be predicted by the number of flower buds they matured (Fig. 4B) or by the number of flowers that successfully opened and the floral-sex ratio (Fig. 4C). In contrast, the difference in seed production between purple- and white-petalled ramets disappeared completely when the number of flower buds or the number of flowers opened and floral-sex ratio were taken into account (Fig. 4).

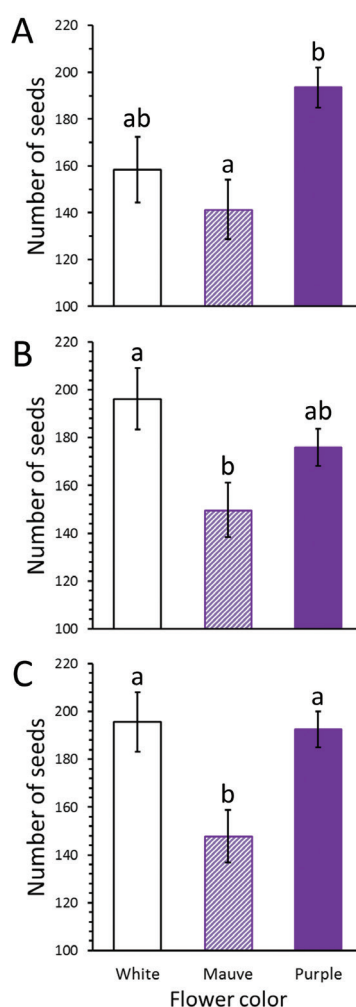


Figure 4. Comparison of seed production by white-, mauve-, and purple-petalled ramets in the common-garden experiment. The bars represent least-squares means $\pm$ 1 SEM of seed production calculated from the following analyses: **A.** the ANOVA that includes petal color as the only explanatory variable (Table 2E), **B.** an ANCOVA with the influence of the number of flower buds removed (Table 3A), and **C.** an ANCOVA with the influence of the number of opened flowers and floral-sex ratio removed (Table 2B). Bars that do not share a lower-case letter are significantly different from each other at $P < 0.05$, as determined by Tukey HSD tests.

Resistance to herbivory

The horsenettle plants in the common-garden experiment suffered a substantial, but widely variable, amount of damage by each of the eleven species of herbivores recorded in this study (Fig. 5). Every single ramet displayed evidence of leaf-feeding by both the eggplant flea beetles and false potato beetles. Damage by the other four folivores was less widespread, with 46%, 42%, 5%, and 5% displaying evidence of feeding by the eggplant leafminers, eggplant tortoise beetles, eggplant lace bugs, and citrus gall midges, respectively. Overall, the ramets

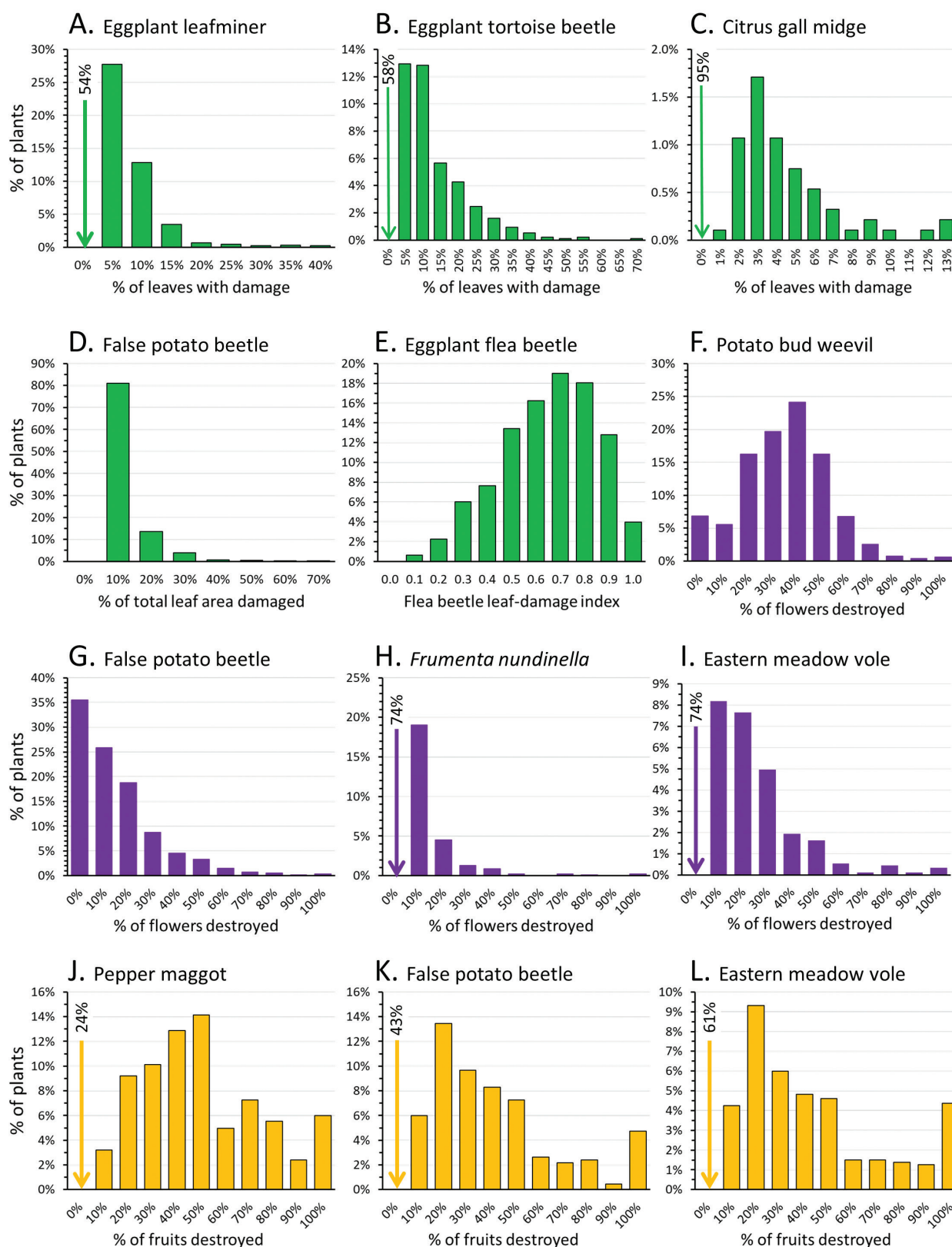


Figure 5. Histograms of damage levels for 12 of the 14 types of damage measured in the common-garden experiment. The percentages of ramets that suffered no measurable damage by an herbivore are shown either by columns or by numbers accompanied by arrows pointing to the 0% position on the x-axis. The 10% column represents the percentage of ramets that displayed greater than 0% up to 10% damage, the 20% column represents the percentage of ramets that displayed greater than 10% up to 20%, etc.

Table 4. Results of the MANOVA of 14 types of herbivory on the source population of the horsenettle plants and their petal color (white, purple, or mauve) in the common-garden experiment.

Source	Wilks' lambda	Approx. F	Degrees of freedom		P-value
			Num.	Denom.	
Population	0.861085	3.2067	39	2424	< 0.0001
Petal color	0.938498	2.0216	26	1630	0.0017

lost an average of ~50% of their flowers to herbivory, with potato bud weevils, false potato beetles, *Frumentella nundinella*, and meadow voles respectively responsible for destroying a mean of 31%, 12%, 3%, and 5% of the flower buds that were initiated by each ramet. Pepper maggots, false potato beetles, and meadow voles were respectively responsible for destroying a per-ramet mean of 35%, 21%, and 16% of the fruits that could have otherwise matured seeds. Finally, potato stalk borers successfully infested and damaged 73% of the horsenettle ramets.

Petal color had a significant influence on the overall pattern of herbivory by horsenettle's community of herbivores in the field experiment, as determined by the MANOVA that included all 14 damage measurements ($P = 0.0017$, Table 4). Subsequent ANOVAs for the 14 damage types provided insight into which species of herbivores may have been influenced by the petal colors of its hosts (Table 5). For none of the 14 ANOVAs were the P -values for petal color significant at the Dunn-Šidák experiment-wise alpha value of 0.0037. However, for three of the herbivores, petal color was a statistically significant explanatory factor at a test-wise alpha level of 0.05, and for two additional herbivores, the effect of petal color was at least marginally significant at a test-wise alpha of 0.1 (Table 5). With 14 separate ANOVAs, we would not expect more than one test to yield a significant result at an alpha of 0.05 due to chance alone—that is, a positive inference due to a Type I error. Likewise, we would expect 1.4 tests to yield marginally significant results due to chance alone (i.e., at an alpha of 0.1).

The fact that five of the ANOVAs yielded at least a marginally significant indication of a petal-color effect suggests that there may be some signal, even if the effect of petal color on resistance to any one herbivore is not particularly strong. Specifically, of the three petal morphs, mauve-petalled ramets suffered the least damage by eggplant tortoise beetles (Fig. 6A) but the most damage by eggplant flea beetles (Fig. 6B). Similarly, mauve-petalled ramets suffered the least flower damage by potato bud weevils (Fig. 6C) but the most flower damage by false potato beetles (Fig. 6D)—though the difference for the latter species was only marginally significant (Table 5I). Finally, mauve-petalled ramets experienced a marginally significant resistance advantage against potato stalk borers (Fig. 6E, Table 5G).

DISCUSSION

Flower-color characterization

Although a cursory observation of a typical horsenettle population gives the general impression of a diversity of flower colors ranging continuously from white to purple, closer scrutiny reveals that there is a degree of discreteness and order to the apparently continuous variation. Specifically, individual ramets produce only one floral-color morph: all-white petals, all-purple petals, or white petals with purple streaks. This discreteness is blurred by the streaky petals turning more uniformly light purple (mauve) as a flower ages. Nevertheless, the presence of discrete categories suggests that flower-color may be polymorphic in horsenettle—if the variation is under genetic control and is maintained over time by ecological factors.

The results of our common-garden experiment provide anecdotal evidence that petal color in horsenettle is likely under genetic control. All of the ramets that were derived from the same genet consistently produced flowers in the same color category. Because of the propagation regime and experimental design, it is highly unlikely that environmental conditions had much influence on this consistency of petal color among ramets. Specifically, to minimize potential carryover (non-genetic) influences of the source environment on the phenotypes of the plants, the genets had undergone four years of growth in standard commercial growing medium under as identical conditions as possible prior to the common-garden experiment. Each spring, the genets were grown from cuttings of new roots that had been initiated the previous summer—rather than from older roots. Moreover, in the common-garden experiment, the 24 ramets of each genet were dispersed in a randomized block design. Regardless of where a single genet's ramets were located, the petal color among ramets within a genet was consistent.

A conclusion that petal color is under genetic control in horsenettle would not be particularly surprising, given the fact that the genetics of flower color has been the focus of numerous studies of plants in the same family (Solanaceae) and even the same genus (*Solanum*) as horsenettle (Eich 2008; Smith and Rausher 2011; Ho and Smith 2016; Li et al. 2019). The patterns of petal-color phenotypes found in the current study are consistent with

Table 5. Summary of ANOVA results for the influence of source population and petal color on damage levels by the various herbivores in the common-garden experiment. The herbivores are identified by genus name, and the type of tissue damaged is identified in parentheses.

Source	Df	MS	F-ratio	P-value
<i>A. Keiferia</i> (leaves)				
Population	3	0.001851	0.9055	0.44
Petal Color	2	0.001985	0.9711	0.40
Error	929	0.002044		
<i>B. Gratiana</i> (leaves)				
Population	3	0.020883	3.1063	0.026
Petal Color	2	0.035919	5.3431	0.0049
Error	929	0.006723		
<i>C. Prodiplosis</i> (leaves)				
Population	3	0.000125	0.8601	0.46
Petal Color	2	0.000075	0.5161	0.60
Error	929	0.000145		
<i>D. Leptinotarsa</i> (leaves)				
Population	3	24.7126	0.4604	0.71
Petal Color	2	45.3098	0.8441	0.43
Error	924	53.6808		
<i>E. Epitrix</i> (leaves)				
Population	3	0.501049	13.500	< 0.0001
Petal Color	2	0.148471	4.0005	0.019
Error	925	0.037113		
<i>F. Gargaphia</i> (leaves)				
Population	3	0.146162	2.9472	0.032
Petal Color	2	0.110265	2.2234	0.11
Error	945	0.049593		
<i>G. Trichobaris</i> (stems)				
Population	3	0.865732	4.4755	0.0040
Petal Color	2	0.515742	2.6662	0.070
Error	945	0.193438		
<i>H. Anthonomus</i> (flowers)				
Population	3	0.173928	5.5527	0.0009
Petal Color	2	0.134534	4.2950	0.014
Error	923	0.031323		
<i>I. Leptinotarsa</i> (flowers)				
Population	3	0.063260	2.5832	0.052
Petal Color	2	0.065773	2.6858	0.069
Error	923	0.024489		
<i>J. Frumentia</i> (flowers)				
Population	3	0.086684	11.8326	< 0.0001
Petal Color	2	0.010321	1.5524	0.21
Error	923	0.006648		
<i>K. Microtus</i> (flowers)				
Population	3	0.058181	3.6022	0.013
Petal Color	2	0.015233	0.9431	0.39
Error	923	0.016151		
<i>L. Leptinotarsa</i> (fruits)				
Population	3	0.109247	1.4803	0.22
Petal Color	2	0.033466	0.4535	0.64
Error	863	0.073802		
<i>M. Zonosemata</i> (fruits)				
Population	3	0.258596	2.2916	0.033
Petal Color	2	0.082887	0.9347	0.39
Error	863	0.088681		
<i>N. Microtus</i> (fruits)				
Population	3	0.650855	9.4050	< 0.0001
Petal Color	2	0.068297	0.9889	0.37
Error	863	0.069203		

a trait controlled by a major gene with two incompletely dominant alleles. However, further studies would be necessary to investigate that speculation. Moreover, we observed a few anomalies that suggest that there may be additional petal-color alleles or that other loci act as modifiers or regulators of pigment expression in horsenettle's petals. For instance, we observed small purple spots in otherwise all-white petals of a few genets, as well as noticeably different shades of purple among some of the purple-petalled genets.

Evidence for a stable polymorphism

Regardless of the details of the genetic control of petal color in horsenettle (which may be resolved in future studies), the field component of this study provided evidence that

horsenettle populations tend to be polymorphic for petal color. Even in the relatively small sample of ten genets from each of the four fields, all samples included more than one petal-color phenotype, and samples of two fields included all three phenotypes. Of course, the absence of a phenotype from the sample of only ten genets per field does not preclude the possibility that genets displaying the missing phenotype were present but did not make it into the sample. The results of this study, combined with the common characterization of the variability of the color of flowers in horsenettle populations, suggest that the flower-color polymorphism within horsenettle populations is stable.

Both within and among the four field populations in this study, purple-petalled genets tended to more abundant than mauve- or white-petalled genets. This

pattern is consistent with the substantial advantages that purple-petalled ramets had in flower, fruit, and seed production in the common-garden experiment. Such a reproductive advantage—generally interpreted in terms of pollinator preferences—has been observed for individuals with darker-pigmented flowers than with white flowers in several other plant species (Waser and Price 1981; Frey et al. 2011; Joseph and Siril 2013; Buide et al. 2021), but this pattern is not universal (Carlson and Holsinger 2013). Like these other species, the results for horsenettle raise two main questions: 1) why do purple-petalled plants produce more flowers and seeds? and 2) with purple-petalled plants having such a large reproductive advantage, why has natural selection not caused the white allele(s) to be eliminated from the populations?

Flower color and resistance to herbivory

If a mutation that disrupts the biochemical pathway leading to anthocyanin is indeed responsible for the lack of purple pigment in the flowers of some horsenettle genets, then any pleiotropic reduction in defensive chemistry in petals (and other tissues) would be expected to lead to greater damage by herbivores in white-petalled and perhaps mauve-petalled individuals. Evidence that differences in flower color can lead to differences in levels of herbivory has been accumulating across a number of species (Irwin et al. 2003; Johnson et al. 2008; Caruso et al. 2010; de Jager and Ellis 2014; Sobral et al. 2016; Buide et al. 2021). However, studies that show that these differences in turn lead to fitness differences

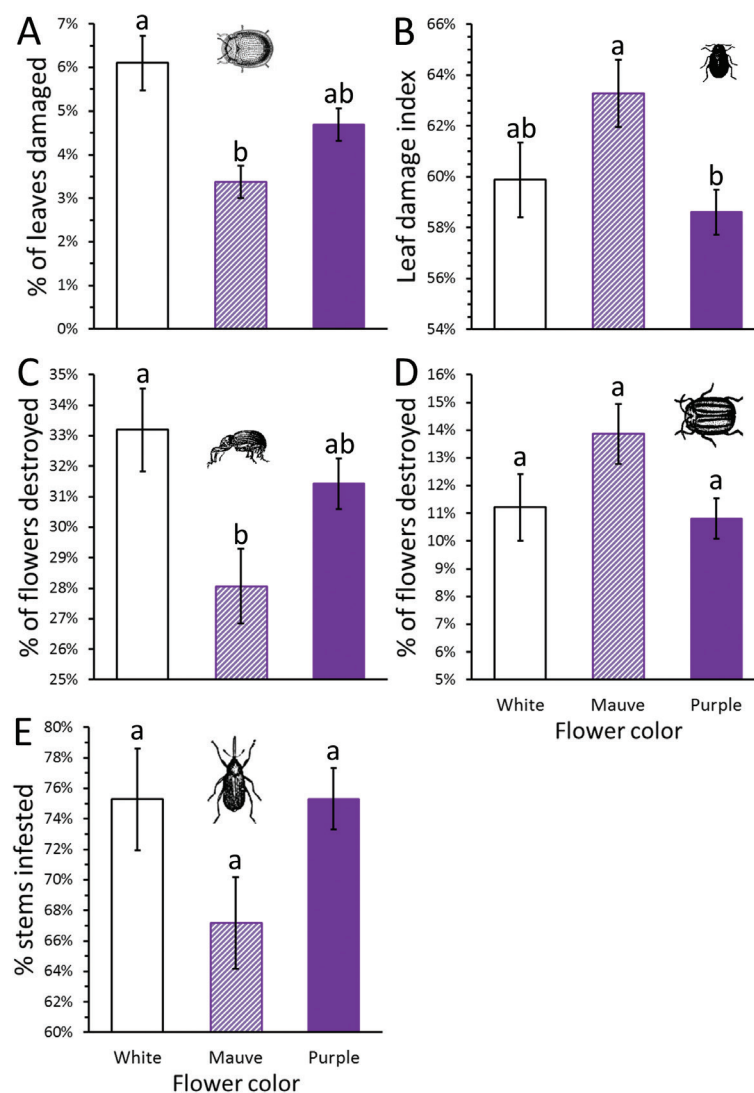


Figure 6. Comparison of resistance to herbivory in white-, mauve-, and purple-petalled ramets to herbivores for which the ANOVAs suggested a significant ($P < 0.05$) or marginally significant ($0.05 < P < 0.10$) influence of petal color on damage levels (Table 5). **A.** Leaf damage by eggplant tortoise beetles ($P = 0.0049$). **B.** Leaf damage by eggplant flea beetles ($P = 0.0049$). **C.** Flower damage by potato bud weevils ($P = 0.014$). **D.** Flower damage by false potato beetles ($P = 0.069$). **E.** Stem infestation by potato stalk borers ($P = 0.070$). The bars represent least-squares means $\pm$ 1 SEM calculated from ANOVAs (Table 5). Bars that do not share a lower-case letter are significantly different from each other at $P < 0.05$, as determined by Tukey HSD tests.

among plant phenotypes (or genotypes) remain scant for the few systems for which this phenomenon has been studied (Strauss and Whittall 2006). If this phenomenon is common in nature, then this comprehensive study of the impact of horsetnettle's herbivores would be likely to provide such evidence. However, despite the large number of herbivores considered, this study revealed no evidence that herbivory played any more than a minor role in the reproductive advantage of purple-petalled ramets.

If the white-petalled ramets had been less defended, then we might expect to see the largest effect on the florivores because it is the flowers where pigment expression is most obvious. Indeed, across several species in a variety of plant families, florivorous insects have been found to prefer white flowers over colored flowers (Irwin et al. 2003; Carlson and Holsinger 2010; McCall et al. 2013; Tsuchimatsu et al. 2014; Vaidya et al. 2018; Buide et al. 2021). In the current study, horsetnettle ramets lost an average of about half of their flowers (including buds) to combined damage by four species of florivores. However, that percentage differed only very slightly among the three petal-color morphs. For only one species of florivore—the potato bud weevil—was there a statistically significant disadvantage for white-petalled morphs, with these ramets suffering a 5% greater flower loss than mauve-petalled plants and only a 2% greater loss than purple-petalled plants to the weevils. This slight resistance advantage enjoyed by mauve-petalled morphs was counteracted by the mauve-petalled plants suffering a 4% greater loss of flower buds to false potato beetles than the white or purple morphs suffered.

Because of the intimacy of their feeding relationship, it is the potato bud weevil for which we expected the greatest potential of a resistance difference among petal-color morphs. Specifically, female weevils have a chance to sample flowers as they chew through petal tissue prior to laying an egg inside a flower bud, and larvae complete their entire development inside a severed flower bud. However, countervailing forces may temper this expectation. For instance, these weevils oviposit in flower buds when the petals are immature and have not yet expressed pigmentation fully. Moreover, the colors of the petals may not be important to the weevils because their larvae feed primarily on anthers, and not at all on the petals (Wise 2007b). The bright yellow color of the anthers is not likely to be related to the anthocyanins that imbue the purple hue, and thus the health of the weevil larvae may not be expected to be affected by the color of the petals.

In several plant species, researchers have found that differences in chemistry of pigments expressed in the petals have been accompanied by differences in levels of defensive chemicals expressed in leaf tissues or by differences in performance of folivores (Ritchey 1999; Irwin et al. 2003; Frey 2004; Strauss et al. 2004; Berardi et al. 2013; Sobral et al. 2016; Vaidya et al. 2018). Although concentrations of neither pigments nor defensive chemicals were measured in horsetnettle tissues in this

study, the leaf-damage patterns by the six main folivores offer at least a small amount of evidence that defense of leaf tissues may be related to petal color in horsetnettle. Specifically, leaves of mauve-petalled ramets suffered the least damage by eggplant tortoise beetles and the most damage by eggplant flea beetles. Because the resistance advantage of a given petal-color morph was weak and differed in direction among leaf-feeding species, the net selective effect of the community of folivores on petal-color is likely very low in populations of horsetnettle.

Several studies of other plant species have found that flower color can influence resistance to fruit-feeding herbivores and seed predators—particularly when oviposition occurs inside flower tissue while petals are still present (Caruso et al. 2010; Tsuchimatsu et al. 2014; Veiga et al. 2015). Despite the high rate of frugivory in the common-garden experiment, and the highly significant impact of frugivory on horsetnettle's seed production (Wise and Rausher 2013), flower color appeared to play no role in frugivores' preferences in this study. One likely reason for this lack of effect is the feeding behavior of the frugivores. Female pepper maggot flies oviposit into horsetnettle fruits after the fruits have begun to enlarge—before they are ripe, but long after the petals are gone. Similarly, fruit damage by false potato beetles and meadow voles generally occurs after the petals have senesced and fallen.

Nevertheless, resistance to frugivory might still be related to petal color if genes controlling petal pigmentation have pleiotropic effects expressed in the fruit tissue. The fruits of many species of *Solanum* (e.g., eggplants) are purple or black due at least in part to the presence of anthocyanins (Harborne and Swain 1979; Cipollini et al. 2002). In contrast, the yellow fruits of horsetnettle are protected from natural enemies by a variety of defensive chemicals (e.g., alkaloids) that are unrelated to the anthocyanins that confer the purple hues to flowers and fruits (Harborne 1986; Cipollini and Levey 1997a, 1997b). If any of the defensive chemicals in horsetnettle fruits are related to petal color, they did not appear to affect frugivores' choices in this study.

Maintenance of white petals

Because the purple-petalled ramets in this study had a definitive fitness advantage in terms of seed production, one might expect that natural selection would lead to fixation of the purple allele, thus preventing the establishment of flower-color polymorphisms in field populations of horsetnettle. However, seed production is not the only component of a plant's fitness. Moreover, because ovules of a horsetnettle genet are not normally capable of fertilization by pollen of the same genet (i.e., horsetnettle is obligately outcrossing), seed production represents only the maternal component horsetnettle's genetic contribution to the next generation. If purple-petalled flowers produce less pollen, are visited less often by pollinators, or produce less-competitive pollen

than white-petalled flowers, then the maternal-fitness advantage enjoyed by purple-petalled individuals may be countered by a disadvantage in components of paternal fitness (Sapir et al. 2021).

As an andromonoecious plant, horsenettle produces some female-sterile flowers, but all of its flowers produce pollen and can potentially sire offspring on individuals of different genets. Therefore, the total number of flowers a ramet opens is a rough indicator of the potential for a ramet to pass on its genes through pollen. In this study, purple-petalled ramets opened significantly more flowers than did the white- and the mauve-petalled ramets. Therefore, the reproductive advantage of purple morphs in terms of maternal fitness appears to have been echoed by a similar advantage in potential paternal fitness. However, genetic analyses of the parental and offspring generations would be necessary to determine how closely the number of open flowers predicts the fitness of the ramets in terms of genetic contribution through successful pollination and fertilization (Elle and Meagher 2000; Vallejo-Marín and Rausher 2007a). Observational studies of the pollinators as they visit ramets of the different petal-color morphs would also be valuable in interpreting potential effects of petal color on components of paternal fitness in horsenettle.

In the absence of direct observations of pollinators in the current study, the analyses in which flower number was used as a covariate can provide some insight into patterns of pollination as they relate to petal color. For instance, when the influence of flower number was statistically accounted for in an ANCOVA, the white-petalled ramets produced just as many seeds as did the purple-petalled ramets. This result suggests that the difference in seed production between these two petal-color morphs can be explained by the number of flowers opened alone. As such, white-petalled and purple-petalled flowers were equally successful on average at receiving pollen to fertilize their ovules. That result is at least consistent with pollinators being equally attracted to white and to purple flowers. Of course, the result cannot address the evolutionary significant question of how much of the flowers' pollen was collected on the visits and successfully transferred to flowers of other genets.

The results of the same ANCOVA on seed production suggest that pollinators found mauve-petalled flowers significantly less attractive than purple- or white-petalled flowers. Specifically, on a per-flower basis, the mauve morphs produced an average of ~20% fewer seeds than did the white or purple morphs. Such a result suggests that mauve-petalled flowers either produced fewer ovules (and smaller fruits) or that they received less pollen—which could result from being visited less by pollinators. If mauve flowers were indeed less preferred by pollinators, then it is reasonable to expect that mauve-petalled ramets contributed fewer genes to the offspring generation through their own pollen. Such disadvantages of an intermediate phenotype are consistent with assortative mating caused by preferences of bees for flowers with

all-white or all-purple petals over flowers with mauve petals (Sapir et al. 2021). Observational studies and genetic analyses would be needed to test this hypothesis for pollinator-driven maintenance of the flower-color polymorphism in horsenettle populations.

Sexual reproduction is obviously a central aspect of fitness in most plants. Indeed, horsenettle must produce fruits and seeds so that its progeny can disperse into new locations as successional changes make a current location unsuitable for horsenettle. However, in order to survive and occupy territory within a population, horsenettle relies on the growth of deep taproots and rapidly spreading lateral roots, from which new ramets emerge and receive nutrients (Ilnicki et al. 1962; Nichols et al. 1991). In clonal plants like horsenettle, resources committed to sexual reproduction are not available to allocate toward perennial tissues that enable future vegetative growth and reproduction. Therefore, natural selection for a strategy of increased sexual reproduction may be counteracted by an accompanying cost of a reduction in growth of perennial tissues, which likely result in a reduction of future clonal reproduction (Silvertown 2008; Narbona et al. 2018; Tenhumberg et al. 2023).

Prior studies of horsenettle have shown that both the number of flowers and the biomass of perennial roots that a ramet produces in a growing season are highly heritable traits that display a wide range of variation among genets (Wise and Cummins 2006; Wise 2018; Wise and Mudrak 2021). In addition, studies have shown that horsenettle ramets that produce a relatively large number of fruits tend to allocate less biomass to the production of perennial roots than those that produce fewer fruits (Wise et al. 2008; Wise and Mudrak 2021). What we do not know is whether allocation to root growth is related to petal color—or why one might even suspect that it would be. A clue to this intriguing question was provided by Twyford et al.'s (2018) study on the common yellow monkeyflower, *Mimulus guttatus* DC. (Phrymaceae). A mutation that prevented the production of anthocyanin in the plant and thus caused the loss of pigmentation in the flowers (and other tissues) was also associated with the production of significantly more stolons, by which monkeyflower plants can reproduce clonally. Twyford et al.'s (2018) study thus provided a rare example of the potential for tradeoffs between sexual and clonal reproduction to lead to the maintenance of a flower-color polymorphism in nature. Research on the biomass-allocation strategies of white- and purple-petalled horsenettle morphs is thus a logical next step in determining whether a tradeoff between sexual and vegetative reproduction plays a role in the maintenance of the flower-color polymorphism in horsenettle populations.

Conclusions

In this study, we found that petal color in horsenettle—rather than being a continuously varying trait—comes in three relatively discrete categories: white, purple,

and mauve. As all three color categories tended to be represented within horsenettle populations, ecological factors likely act to maintain flower color as a stable polymorphism. White-flowered ramets were more susceptible to florivory by the potato bud weevil and to folivory by the eggplant tortoise beetle. However, the slightly greater resistance to herbivory of purple-petalled ramets to these two herbivores goes only a very short way in explaining why these ramets produced substantially more seeds than did the white- or mauve-petalled ramets. Thus, the reason that white-petalled morphs are maintained in horsenettle populations remains unidentified. We suggest that tradeoffs in fitness components—perhaps brought about by pollinator behaviors and differential allocation to sexual and clonal reproduction—may maintain flower-color polymorphism in field populations of horsenettle and are thus logical topics of future studies. Parallel studies addressing the identity of the pigments, the mutations that result in variation in petal colors, and the genetic mechanism responsible for the phenotypic variation in horsenettle's flowers (and potentially correlated traits) would also be quite valuable in completing the story of the polymorphism in flower color for this important native species.

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