OPPOSING NATURAL SELECTION FROM HERBIVORES AND PATHOGENS MAY MAINTAIN FLORAL-COLOR VARIATION IN *CLAYTONIA VIRGINICA* (PORTULACACEAE)

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Abstract.—The maintenance of floral-color variation within natural populations is enigmatic because directional selection through pollinator preferences combined with random genetic drift should lead to the rapid loss of such variation. Fluctuating, balancing, and negative frequency-dependent selection mediated through pollinators have been identified as factors that may contribute to the maintenance of floral-color variation, and recently it has been suggested that indirect responses to selection on correlated characters through agents of selection other than pollinators may substantially shape the evolution of floral traits. Here, I provide empirical support for this latter view in *Claytonia virginica* (Portulacaceae) through a multiseason field study, a pollen supplementation study, and an artificial herbivory experiment. These studies show that most individuals fall into one of four discrete color classes, and suggest pollinator-mediated selection for increased floral redness in concurrent years. Floral color is also an indirect target of opposing directional selection via herbivores and pathogens that fluctuates through time. Taken together, these data suggest a novel mechanism by which floral-color variation may be maintained, and illustrate the importance of an inclusive, pluralistic view of selection when investigating the evolution of complex phenotypes.

Key words.—*Claytonia virginica*, floral-color evolution, herbivory, maintenance of polymorphism, phenotypic selection, pollination ecology, pollinator-mediated selection.

Historically, floral-color variation among populations or closely related species has been attributed to either selection generated through spatial variation in pollinator fauna or their preferences for particular color morphs, or the action of random genetic drift (Wright 1943a,b; Grant 1949; Stebbins 1950; Wright 1978; Faegri and van der Pijl 1979; Waser 1983a; Kiester et al. 1984; Proctor et al. 1996). Indeed, several recent studies have shown how floral-color variation can substantially influence the rate and duration of pollinator visitation, and perhaps drive adaptive divergence (Grant 1994; Hodges and Arnold 1994; Campbell et al. 1997; Morgan and Schoen 1997; Wilbert et al. 1997; Schemske and Bradshaw 1999; Jones and Reithel 2001). However, the maintenance of within-population floral-color variation has remained somewhat enigmatic. Given that consistent measures of pollinator-mediated selection for color phenotypes have been reported in several systems (Campbell et al. 1997; Morgan and Schoen 1997; Jones and Reithel 2001), it stands to reason that floral-color variation should be rapidly lost within populations, especially in situations where color has a relatively simple genetic basis.

Because the magnitude of floral-color changes induced through microenvironmental variation in soil pH or the presence of certain metals (Harborne 1967, 1976, 1988, 1991) is relatively minor in comparison to known genetic polymorphisms for floral color (e.g., blue or white flowers in *Linanthus parryae*; Schemske and Bierzychudek 2001), a number of authors have looked for selective mechanisms that might maintain within-population variation for floral color. For example, it is well documented that temporal or spatial variation in the strength and direction of natural selection on floral color (i.e., fluctuating selection) can maintain genetic vari-

ation (Hedrick et al. 1976; Hedrick 1986; Ellner and Sasaki 1996), and recent data suggest that this mode of selection maintains floral-color polymorphism in populations of *Linanthus parryae* (Polemoniaceae) (Schemske and Bierzychudek 2001; Turelli et al. 2001). In addition, a number of studies have shown that both balancing and negative frequency-dependent selection may explain, at least in part, the maintenance of a floral-color polymorphism within populations of *Clarkia gracilis* (Onagraceae) (Jones 1996a,b), *Dactylorhiza sambucina* (Orchidaceae) (Gigord et al. 2001), and *Ipomoea purpurea* (Convovulaceae) (Rausher et al. 1993; Rausher and Fry 1993; Subramaniam and Rausher 2000).

More recently, it has been hypothesized that indirect responses to selection on correlated characters through non-pollinator agents of selection may also influence the evolution of floral traits (Strauss and Armbruster 1997; Armbruster 2002). With respect to floral color, pleiotropic relationships between color and various aspects of plant performance have been identified in *Digitalis purpurea* (Scrophulariaceae), *I. purpurea* (Convolvulaceae), *Lobularia maritima* (Brassicaceae), and *Phlox drummondii* (Polemoniaceae), and a few studies have shown that floral-color variation may be maintained, in part, through fluctuating selection on plant performance (Ernst 1987; Rausher and Fry 1993; Levin and Brack 1995; Gómez 2000). In addition, pleiotropic relationships between pigmentation molecules and vegetative defensive compounds have been discovered in *I. purpurea*, and it appears that selection generated through herbivore pressures may indirectly maintain floral-color variation within *I. purpurea* populations (Simms and Bucher 1996; Fineblum and Rausher 1997).

Floral-color variation in *Claytonia virginica* (Portulacaceae) is associated with the expression of a crimson pigment molecule (cyanidin), and two flavonols that occur throughout the plant body (quercetin and kaempferol; Doyle 1981, 1983).

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These flavonols, combined with cyanidin, have a bluing effect on the overall coloration of the petal. As concentrations of quercetin and kaempferol increase, petal coloration ranges from mauve to light pink or nearly white (Harborne 1967). These two flavonols have a suite of additional biological properties; in particular, they play a significant role in herbivore and fungal defense in a number of different species (Bate-Smith and Swain 1962; Harborne 1991). Given the multiple phenotypic effects of these two compounds, it seems reasonable to predict that selection for increased protection against herbivores or pathogens may indirectly contribute to the evolution of flower color in *C. virginica*.

In this study, I identify the ecological agents of selection on floral color in *C. virginica*, and ask whether floral-color variation might be maintained through variation in direct selection through pollinators, indirect selection generated through herbivores and pathogens, or some interaction among the three. Specifically, I asked the following questions: (1) What is the quantitative pattern of floral-color variation? (2) Is there selection on floral color via pollinators? (3) Is floral color associated with plant damage via herbivores and pathogens? (4) What are the direct fitness consequences of herbivory and infection? These data show that herbivores and pathogens play a major role in the maintenance of floral-color variation by generating opposing directional selection on floral color that fluctuates through time.

MATERIALS AND METHODS

Study System

Claytonia virginica (Portulacaceae) is a perennial, spring-flowering woodland herb distributed throughout northeastern North America. Physiologically independent inflorescences arise from a single corm and are subtended by two opposite, cauline leaves (Jónsdóttir and Watson 1997; Morgan 1998; Whigham and Chapa 1999). Occasionally, plants also produce ''accessory'' leaves, which are separate from inflorescences. Plants in southern Indiana typically emerge in late January to early February, and have fully expanded leaves prior to first flowering (F. M. Frey, pers. obs.). Flowers of *C. virginica* are regular with two bracts (''sepals''), a five-merous petaloid calyx (''petals''), no corolla, five antipetalous stamens, and a three-carpellate gynoecium with basal placentation. Inflorescences bear protandrous flowers that open acropetally, one to two at a time, over a period of about two weeks. The staminate phase lasts for a single day, whereas the pistillate phase may last for up to six days if the flowers remain unpollinated (Doyle 1981). These flowers are self-compatible, but not self-pollinating (Schemske 1977; Motten 1986). In southern Indiana, flowering typically begins in mid-March and continues through mid- to late-April; petals are reflexed from mid-morning to mid-afternoon and become tightly furled in the evening and during rainy weather. The primary pollinator is the solitary bee, *Andrena erigeniae* (Hymenoptera: Andrenidae). The superior ovary typically contains six ovules, however, even with multiple pollinator visits, the longitudinally dehiscent capsules rarely contain six seeds (Davis 1966; Motten et al. 1981). Fruits ripen quickly (within 10 days) and explosively shed their seeds, which are subsequently dispersed by ants (Handel 1978; Doyle 1981).

Soon after first flowering, the leaves of *C. virginica* are attacked at night by slugs (*Agriolimax reticulatum*). Damage is restricted to leaves; inflorescences and flowers remain untouched. Nearly two weeks from first flowering infection by a rust, *Puccinia mariae-wilsonii*, becomes evident. It is thought that plants encounter fungal spores, which have been deposited on the ground from one of several intermediate hosts, upon emergence from the soil (Doyle 1981; Frey 2003). Early stages of infection appear as bright yellow/orange clusters of small pits on the shoots or flower buds, eventually progressing to darker telia stages. Flower bud infections result in complete abortion, shoot infections do not inhibit flowering, and corms remain uninfected (Frey 2003). Previous work has demonstrated wide variation in infection status among individuals, and it has been suggested that variation in flavonoid defense compounds may be correlated with infection status (Doyle 1981).

Study Population and Sampling

There are more than 50 recognized cytotypes in the *C. virginica* aneuploid complex ranging from $2n = 12$ to $2n =$ about 192 (Rothwell 1954, 1959; Rothwell and Klump 1965; Lewis et al. 1967; Lewis and Semple 1977). These cytotypes fall into one of four flavonoid races that differ with respect to geographical distribution, cytotype, leaf width/length ratio, and the accumulation of 12 different kaempferol and quercetin 3-O-glycosides (Doyle 1981, 1983, 1984). This study used a pure population of race III plants ($2n = 16$) in a large woodlot on the Indiana University Bayles Road property in the spring of 2001, 2002, and 2003. There were no other co-flowering spring ephemerals in this woodlot (e.g., *Stellaria pubera*); therefore, variation in reproductive success data due to incompatible pollen types (intra- or interspecific) was not an issue.

In the early spring of 2001, a total of 230 seedlings were identified and marked by placing a four-inch roofing nail with a painted number next to the base of the emerging inflorescence. Ninety of these plants were immediately transplanted to a greenhouse at the Indiana University Botany Experimental Field in four-inch pots containing a 50:50 mix of sterilized soil and Metro Mix (Scotts Co., Marysville, OH) to quantitatively assess floral color. The positions of the remaining 140 plants were mapped onto a coordinate-grid system.

In 2002, 250 seedlings were identified in a different location of the same woodlot, marked with an eight-inch tree spike, and mapped. Because of substantial frost heave, most of the plants used in 2001 and their markers had shifted and only a handful of individuals could be verifiably recovered. As such, nearly all individuals used in 2002 were different from those used in 2001.

Assessing Floral-Color Variation

All plants were assigned a color class by sight using a color chart upon first flowering (Frey 2003; 1, white; 2, light pink; 3, mauve; 4, crimson). In 2001, I took spectral data from plants transplanted to the greenhouse using a S2000 miniature fiber optic spectrometer with a PX-2 pulsed xenon lamp (Ocean Optics, Inc., Dunedin, FL). One first-day flower

on a total of 70 individuals was assessed for percent reflectance at wavelengths between 350 and 750 nm at the approximate beginning, middle, and end of the flowering season (a total of 210 measures). Because the spectral profiles were characteristically M-shaped with peaks at 450 and 650 nm and a single valley at 550 nm, a ''spectral shape'' parameter was calculated to facilitate statistical analyses ($[(\%\text{reflectance}_{450} + \%\text{reflectance}_{650})/2] - \%\text{reflectance}_{550}$). The value of this parameter increases from zero as the depth of the valley at 550 nm becomes more pronounced. Spectral data were not taken from plants in the field in 2001, but were taken from 230 of the 250 plants in the field in 2002. Quantitative measures of floral color among flowers on individual plants were highly repeatable throughout the growing season, and the full range of floral-color variation in the field was recovered in plants transplanted to the greenhouse (F. M. Frey, unpubl. data). To assess the validity of the four floral-color classes, I performed a separate one-way ANOVA on the spectral data from each year (2001: $N = 70$; 2002: $N = 230$), and used a post-hoc Tukey comparison procedure to identify homogeneous subsets.

Estimating Fitness

Flowers of *C. virginica* are self-compatible, but require pollinators to set fruit. Therefore, to assess selection on floral color via pollinator preferences, I estimated the female component of fitness through two reproductive functions in each year: total fruit production and percent fruit set. Total fruit production provides a measure of female reproductive success because it is the result of pollinator visitation, resource allocation and acquisition, and life-history trade-offs. In each year, total fruit production per plant was recorded as the total number of fruits produced on all inflorescences. Percent fruit set was calculated as the ratio of total fruit production to total flower production. Relative fitness through each reproductive performance estimate was calculated by dividing each value by the appropriate population mean value.

Because pollinators are necessary for fertilization, percent fruit set should be tightly associated with pollinator visitation rates or duration. However, Morgan (1998) showed that percent fruit set in *C. virginica* increases with plant size, suggesting that fruit set may reflect differential resource status rather than differential pollination. To test this possibility, I performed a pollen supplementation experiment in 2003, in which I hand-pollinated all flowers on one member of 32 similarly sized paired plants, leaving the other for open pollination. Differences in percent fruit set between the treatments were analyzed using a paired *t*-test. I used a two-way ANOVA with color (four levels) and pollination treatment (two levels) as factors to assess their effects on percent fruit set. To assess the association between flower number and percent fruit set in this experiment, I used a general linear model analysis with percent fruit set as the dependent variable, flower number as the independent variable, and pollination treatment as the covariate. A significant interaction between the independent variable and covariate would indicate that the strength of the relationship between flower number and percent fruit set differed between pollination treatments.

Relationship between Relative Fitness and Floral Color

To quantify the relationship between reproductive success and floral color, I took two different approaches. In the first, I performed a series of separate one-way ANOVAs with color class (four levels) as a factor to assess its effect on each relative fitness measure in each year and used a post-hoc Tukey analysis to identify homogeneous subsets (2001: $N = 98$; 2002: $N = 230$). In the second, I used the quantitative spectral data to estimate the form and strength of selection acting directly on floral color. Because both floral size and total leaf area may affect reproductive success through pollinator preferences and resource availability, respectively, I measured both characters on all plants in the field in 2002 and included them as variables in the statistical analyses. Floral size was estimated as an oval ($0.25 \times \pi \times \text{length} \times \text{width}$) by measuring the length and width of a haphazardly chosen flower from each inflorescence with a set of digital calipers ($N = 230$; 741 flowers measured). In cases where a plant produced multiple inflorescences, floral size estimates were averaged. A separate study in the spring of 2001 showed little within-inflorescence variation for flower size (Frey 2003). I also estimated leaf area as an oval ($0.25 \times \pi \times \text{length} \times \text{width}$) by measuring the length and width of all fully expanded leaves on every plant with a set of digital calipers ($N = 230$; 1482 cauline leaves and 379 accessory leaves measured). Total leaf area per plant was estimated as the sum of these leaf area measures.

Linear, nonlinear, and correlational selection on floral color was quantified by separately regressing each relative fitness measure (total fruit production and percent fruit set) on floral color (spectral shape), floral area, and total leaf area simultaneously (Lande and Arnold 1983). This multivariate technique reveals the strength and direction of selection on a particular trait, or trait combination, independent of other traits used in the analysis (Lande and Arnold 1983; Arnold and Wade 1984). Prior to analysis, each of the three traits was standardized to mean = 0, and variance = 1. Standardized directional selection gradients (β_i) were estimated from the partial regression coefficients of a multiple linear regression of each relative fitness estimate (total fruit production and percent fruit set) on the three traits. Standardized nonlinear (γ_{ii}) and correlational (γ_{ij}) selection gradients were estimated from the partial regression coefficients in a separate multiple linear regression of each relative fitness estimate on each standardized trait, the squared value of each standardized trait, and the cross-product of each standardized trait combination. Separate regressions are required to estimate the linear and quadratic coefficients because each estimate may depend on whether the other is included in the model (Lande and Arnold 1983; Phillips and Arnold 1989; Brodie 1992).

Relationship between Floral Color and Damage through Herbivores and Pathogens

At the end of the growing season, herbivore damage on each individual was recorded as absent (0) or present (1), and digital photographs with a 10 mm scale bar were taken of each damaged leaf (473 total leaves). The area remaining on each damaged leaf was calculated using Scion Image (ver.

beta 4.02, Scion Corp., Frederick, MD). Percent leaf area remaining per plant was estimated as $[1 - (\text{total leaf area following herbivory})/(\text{total estimated leaf area prior to herbivory})]$, and arcsine-square root transformed prior to analyses to improve normality. Relative leaf area remaining was then calculated by dividing each value for percent leaf area remaining per plant by the population mean value. In each year, I estimated infection status as the presence (0) or absence (1) of pustules on the plant body, and performed pustule counts on each individual with a hand lens and fine probe. Relative damage through fungal infection was then calculated by dividing the number of pustules on each individual by the population mean value.

I took three approaches to quantify the relationship between floral color and damage through natural enemies. First, I used a two-way ANOVA with color class (four levels) and year (two levels) as fixed effects and percent leaf removal (transformed) as the response variable to determine whether there was significant variation among color classes with respect to leaf removal via slugs, the intensity of leaf removal varied between years, and different color variants were disproportionately damaged in different years ($N = 332$). Because of low infection rates in 2001 (nine plants), I used a one-way ANOVA to determine whether there was significant variation among the color classes in 2002 with respect to pustule number ($N = 230$). I used separate binary logistic regressions with standardized floral color (spectral shape), standardized floral area, and standardized total leaf area as independent variables to determine whether differences among the color variants in the 2002 field season with respect to herbivore damage or pustule formation were due, in part, to differential attack rates ($N = 230$). To facilitate direct comparisons with the relationship between relative fitness and floral color (selection gradient analysis described above), I calculated linear, nonlinear, and correlational regression coefficients from predicted values obtained from separate logistic regressions (Janzen and Stern 1998). Finally, I calculated partial regression coefficients by regressing relative leaf area remaining following herbivory or pustule number on standardized floral color (spectral shape), standardized floral area, and standardized total leaf area simultaneously ($N = 230$). In addition, I performed separate regression analyses with the squared values of each standardized trait, and the cross-product of each standardized trait to estimate nonlinear and correlational regression coefficients.

Variance inflation factors (VIF) associated with traits in all multiple regressions used to calculate linear and nonlinear regression coefficients were a maximum of 1.3 and 5.0, respectively, indicating that multicollinearity did not skew the least squares estimates (VIF > 10 would indicate otherwise; Neter et al. 1996). With the exception of the logistic standardized selection gradients, which were calculated by hand, all analyses were performed using SPSS 10.2 for Windows (SPSS Inc., Chicago, IL).

Fitness Consequences of Herbivore and Pathogen Damage

To determine the fitness consequences of leaf damage though herbivores and pathogens, two separate lines of study were undertaken. In the early spring of 2002, I created 76 pairs of plants of equal size, subjected one member of each pair to an artificial herbivory treatment upon first flowering (50% leaf removal), and assessed the survivorship of all treatment pairs in the spring of 2003. This damage level was chosen because the mean level of damage in the field in 2001 plus one standard deviation was approximately 50%. Given the life cycle of the rust pathogen, which includes an intermediate tree host, I was unable to perform a similar experiment that varied pathogen levels because I could not reliably artificially inoculate individuals.

To assess future phenotypic effects of past herbivory and fungal infection, I relocated all plants used in 2002 in the spring of 2003. After carefully removing the surrounding leaf litter, I recorded survivorship and measured the inflorescence number, total flower number, and total leaf area of surviving individuals. I then calculated the percent change in each of these traits for each survivor (e.g., [flower number in 2003 − flower number in 2002]/flower number in 2002). To ask whether these phenotypic changes were associated with past herbivore damage or fungal infection, I used a series of one-way ANOVAs with herbivory status (two levels) or fungal infection (two levels) as factors. In addition, I used these data to ask whether floral color (spectral shape) was a good predictor of between-year survivorship in the field with a logistic regression.

RESULTS

### Relationship between Relative Fitness and Floral Color

There was substantial variation for floral color in each year (Fig. 1A), and clear differences among the qualitatively assigned color classes with respect to their spectral profiles (2001: $F_{3,69} = 14.039$, $P < 0.001$; 2002: $F_{3,226} = 28.124$, $P < 0.001$; Fig. 1B). In the 2003 pollen supplementation experiment, the artificial-pollination treatment resulted in substantially higher percent fruit set than the open-pollination treatment (92% ± 1.9 SE vs. 79% ± 3.6 SE, respectively). This difference in percent fruit set was statistically significant (paired t-test: $t = -3.311$, $P < 0.005$; ANOVA: $F_{1,64} = 6.970$, $P < 0.025$), and there was no main effect of color ($F_{1,64} = 0.852$, $P > 0.10$), or interaction between floral color and pollination treatment ($F_{1,64} = 0.587$, $P > 0.10$). These data show that variation in percent fruit set primarily reflects variation in pollinator visitation rates rather than variation in resource status. In addition, the interaction between flower number and percent fruit set in the GLM analysis was not statistically significant ($F_{1,63} = 2.263$, $P > 0.10$), suggesting that the strength of the association between flower number and percent fruit set did not differ between the two treatments.

In both 2001 and 2002, there was a trend suggesting that increased floral redness was associated with higher percent fruit set, and that intermediate floral-color variants set more fruit than either of the extreme floral-color variants (Fig. 2). These trends are supported by the statistical analyses of the 2002 data that treated color as a categorical variable (Table 1). The selection gradient analyses show no evidence of stabilizing selection on floral color through fruit production, but do suggest a positive association between percent fruit set and increased floral redness (Table 2). The marginally significant selection gradient of 0.115 indicates that an increase
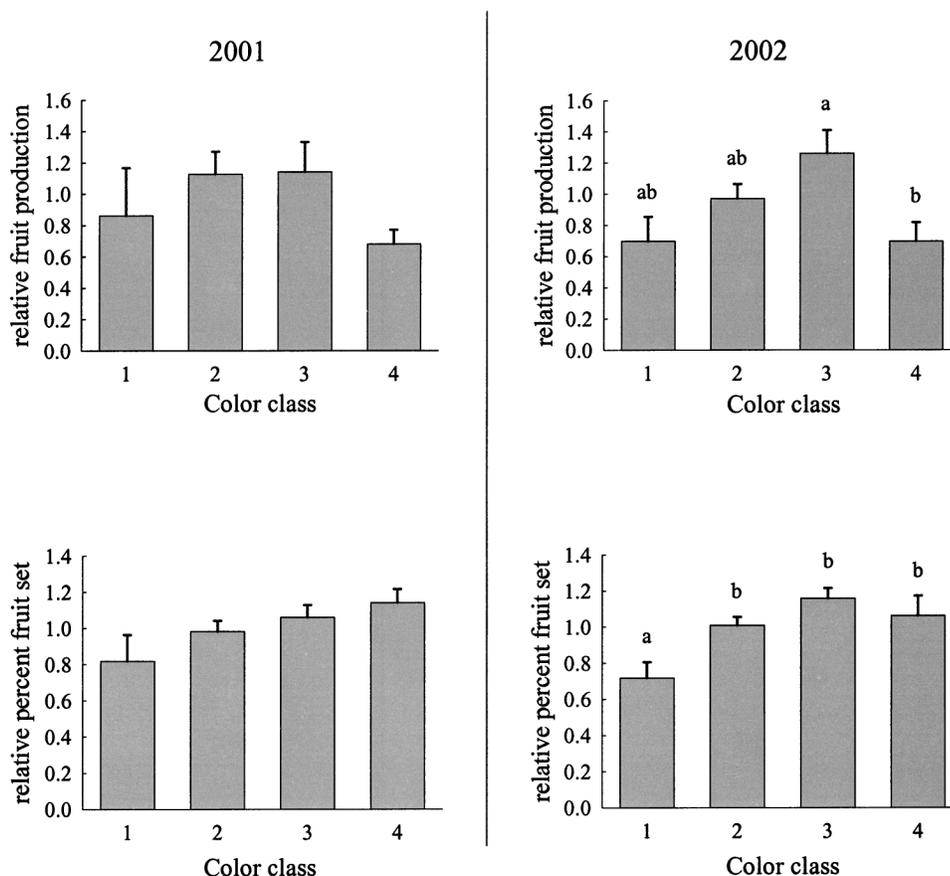
A

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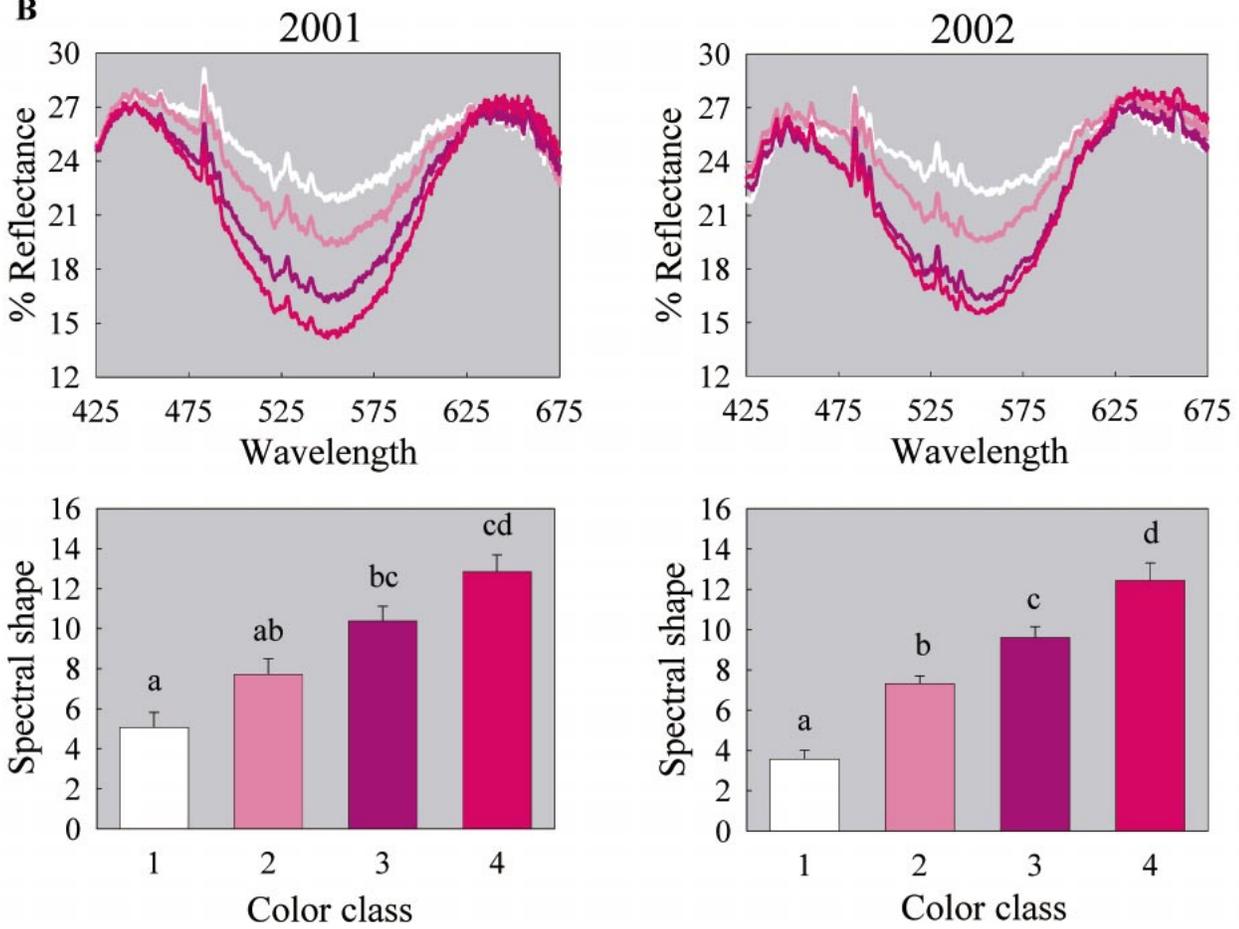
FIG. 2. Female reproductive success estimates for each of the four floral-color classes in consecutive seasons. Average fruit production and percent fruit set values for each color class are shown relative to the appropriate population mean. Error bars denote one SE, and lowercase letters denote homogeneous subsets within each flowering season as identified from a post-hoc Tukey analysis.

in floral redness by one standard deviation is associated with an 11.5% increase in relative percent fruit set. These analyses also suggest that plants with intermediately sized flowers (γ_{ii} = −0.293; Table 2) and large leaves (β_i = 0.636; Table 2) have the greatest total fruit production, and that plants with intermediately sized leaves have the lowest fruit production and percent fruit set (γ_{ii} = 0.371 and 0.325, respectively; Table 2).

Relationship between Floral Color and Damage through Herbivores and Pathogens

The amount of leaf tissue removed by slugs varied across seasons and color classes (color: $F_{3,331}$ = 12.41, $P < 0.001$; year: $F_{1,331}$ = 14.60, $P < 0.001$; color × year: $F_{3,331}$ = 0.655, $P = 0.580$), and in both years redder-flowered plants received substantially more damage than whiter-flowered plants (Fig. 3). The logistic regression analyses did not detect a significant relationship between floral color and likelihood of herbivore attack (Table 3A). Total leaf area, however, was a good predictor of whether or not plants were attacked by slugs; individuals with larger leaves were more likely to be attacked (Table 3A). When considered independent of the effects of floral area and total leaf area, there was a significant, negative relationship between floral redness and the amount of leaf area remaining at the end of the season (Table 3B), supporting the results of the above ANOVA. The regression analysis also showed a positive relationship between the amount of leaf area remaining at the end of the season and the joint value of floral color and floral area (Table 3B). This suggests that plants with small, white flowers received less damage than plants with large, white flowers.

The logistic regression analyses showed that floral redness was inversely related to the probability of fungal infection (Table 3C). When floral color was treated as a categorical variable, there was no detectable association between floral color and pustule number (color: $F_{3,229}$ = 1.664, $P > 0.10$);

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FIG. 1. Floral-color variation in *Claytonia virginica*. The presence of four floral-color classes in the field (A) was supported by spectrographic analysis in two seasons (B). The average spectral profiles and spectral shape parameters (± 1 SE) for each of the four qualitatively assigned color classes in each season are shown in the line and bar graphs, respectively (B). Lowercase letters denote homogeneous subsets within each flowering season as identified from a post-hoc Tukey analysis.

TABLE 1. ANOVA summaries of total fruit production and percent fruit set in 2001 and 2002 with color class as a fixed effect (four levels).

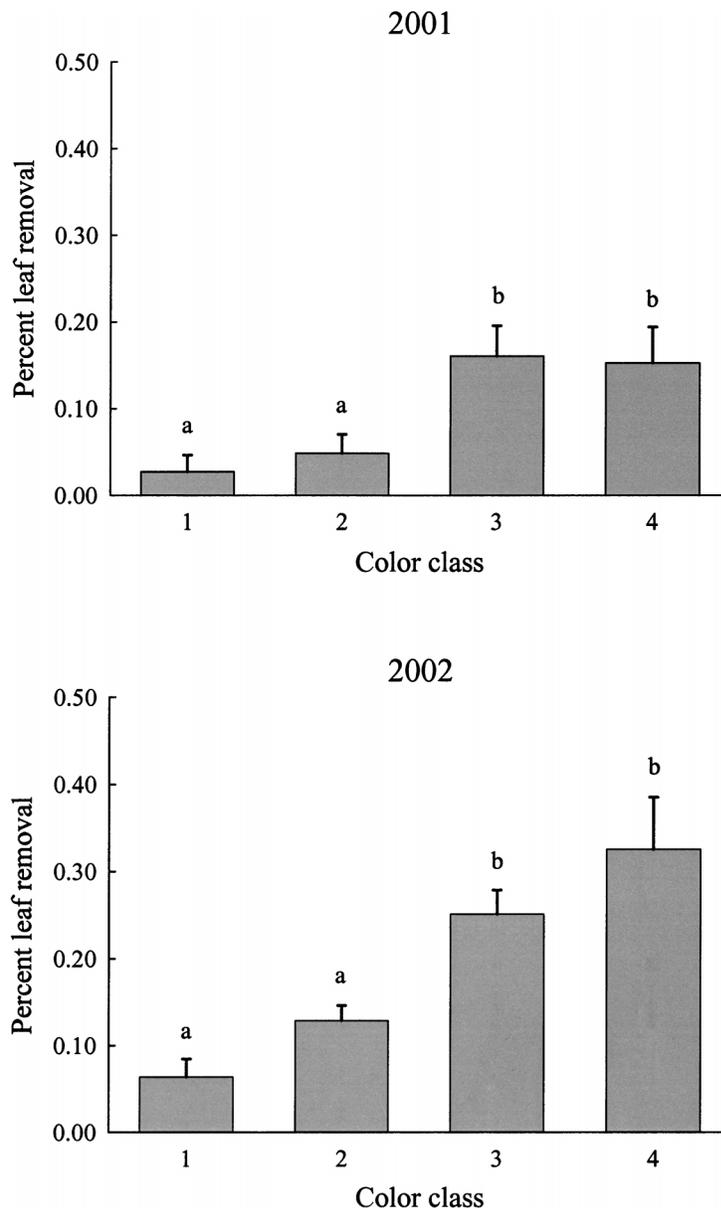|	2001				2002			
	df	N	F	P	df	N	F	P
Total fruit number	3	97	1.491	0.222	3	229	3.364	0.019
Percent fruit set	3	97	2.173	0.096	3	229	6.729	0.001

FIG. 3. In both 2001 and 2002, redder color classes (3 and 4) sustained greater herbivore damage than whiter color classes (1 and 2), and the level of plant damage through herbivory was greater in 2002 than 2001.

TABLE 2. Standardized linear (β'_i), nonlinear (γ'_{ii}), and correlational (γ'_{ij}) regression coefficients for floral color, floral area, and initial leaf area through fitness components associated with reproductive success. P-values are provided in parentheses and significant regression coefficients are bold.

| Fitness estimate | Trait i | β'_i (P) | γ'_{ii} (P) | Trait j | |
				Floral area γ'_{ij} (P)	Leaf area γ'_{ij} (P)
Fruit production	Floral color	0.019 (0.717)	−0.009 (0.870)	0.099 (0.075)	−0.035 (0.504)
	Floral area	0.054 (0.295)	**−0.293 (0.010)**	—	−0.070 (0.214)
	Initial leaf area	**0.636 (<0.001)**	**0.371 (0.002)**	—	—
Fruit set (%)	Floral color	0.115 (0.082)	−0.015 (0.837)	0.093 (0.199)	−0.018 (0.787)
	Floral area	0.122 (0.067)	−0.242 (0.103)	—	−0.115 (0.116)
	Initial leaf area	0.055 (0.412)	**0.325 (0.033)**	—	—

however, when treated as a continuous variable, the multiple regression analysis showed that whiter-flowered plants had more pustules than redder-flowered plants (Table 3D). This discrepancy between the results of the ANOVA and the linear regression analysis is the result of large variation in pustule number within the intermediate color classes. These data also suggested a negative correlation between pustule number per plant and the amount of leaf tissue removed by herbivores (2002: $r = -0.119$, $P = 0.07$).

Fitness Consequences of Herbivore and Pathogen Damage

Both herbivore and pathogen damage had severe fitness consequences. The artificial herbivory experiment showed that a 50% leaf removal treatment significantly reduced the probability of survival to the next growing season (control plant survival: 91% ± 3.3 SE; damaged plant survival: 72% ± 5.16 SE). In addition, plants that experienced natural herbivory in 2002 produced far less leaf area in 2003 than those plants that were not damaged by herbivores ($F_{1,176} = 5.119$, $P < 0.025$; Fig. 4A), but produced similar numbers of inflorescences and flowers (inflorescence number: $F_{1,176} = 0.121$, $P > 0.50$; flower number: $F_{1,176} = 0.008$, $P > 0.50$; Fig. 4A). In contrast, there was no detectable change in leaf area among plants that sustained pathogen infection in 2002 ($F_{1,176} = 1.871$, $P > 0.10$; Fig. 4B), but infected plants produced fewer inflorescences and flowers (inflorescence number: $F_{1,176} = 5.368$, $P < 0.05$; flower number: $F_{1,176} = 4.700$,

$P < 0.05$; Fig. 4B). There was a trend suggesting that whiter-flowered plants were more likely to survive to 2003 than redder-flowered plants (Wald statistic = 3.545, $P > 0.05$); this shows that herbivore damage is not the sole cause of mortality in the field.

Discussion

Quantitative analyses of floral color in this system confirm that individuals can be accurately categorized into four classes, and field studies show that floral color is the target of selection through multiple ecological agents. Herbivore damage and rust infection is strongly associated with floral color, and damage through these natural enemies has severe fitness costs. Taken together, these results show that floral color is the target of opposing directional selection through natural enemies. In addition, there is some evidence for pollinator-mediated selection for increased floral redness. Below, I discuss how these three agents of selection might interact to maintain floral-color variation in this system.

Pollinators

In both seasons, there was a trend suggesting that increased floral redness was associated with higher percent fruit set, and pollen supplementation showed that fruit set was limited by pollen deposition, rather than insufficient resources. Although these results suggest that pollinators are agents of

TABLE 3. Standardized linear (β'_i), nonlinear (γ'_i), and correlational (γ'_{ij}) regression coefficients for floral color, floral area, and initial leaf area through fitness components associated with herbivore and pathogen damage. P-values are provided in parentheses, and significant regression coefficients are bold.

| Fitness estimate | Trait i | β'_i (P) | γ'_{ii} (P) | Trait j | |
				Floral area γ'_{ij} (P)	Leaf area γ'_{ij} (P)
(A) Probability of herbivory	Floral color	0.075 (0.249)	−0.003 (0.965)	−0.068 (0.353)	0.016 (0.818)
	Floral area	0.064 (0.334)	0.141 (0.343)	—	−0.048 (0.512)
	Initial leaf area	**0.183 (0.006)**	−0.081 (0.597)	—	—
(B) Leaf area remaining	Floral color	**−0.186 (0.006)**	−0.022 (0.763)	**0.167 (0.021)**	0.059 (0.393)
	Floral area	−0.015 (0.820)	−0.217 (0.143)	—	0.025 (0.735)
	Initial leaf area	−0.034 (0.605)	0.165 (0.279)	—	—
(C) Probability of infection	Floral color	**−0.150 (0.024)**	0.100 (0.176)	0.129 (0.077)	−0.054 (0.435)
	Floral area	−0.062 (0.356)	−0.160 (0.285)	—	−0.005 (0.947)
	Initial leaf area	0.048 (0.468)	0.140 (0.362)	—	—
(D) Pustule number	Floral color	**−0.140 (0.035)**	0.045 (0.545)	0.081 (0.267)	−0.064 (0.354)
	Floral area	−0.017 (0.798)	−0.188 (0.210)	—	0.040 (0.592)
	Initial leaf area	0.093 (0.163)	0.120 (0.436)	—	—

A

B
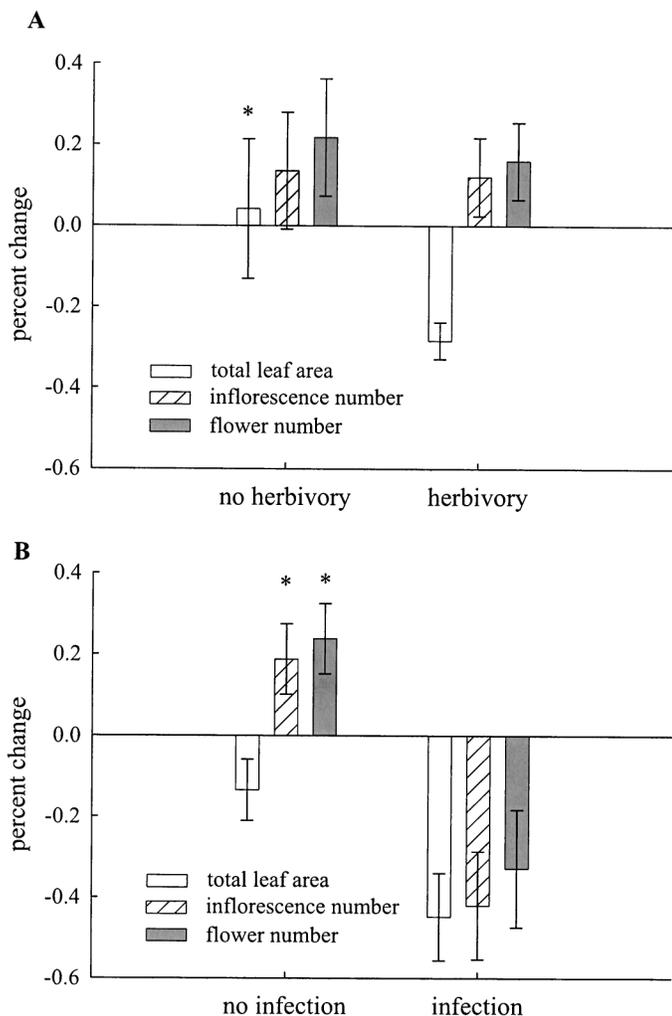
FIG. 4. Fitness consequences of natural herbivory and infection. Individuals that experienced natural herbivory produced significantly less leaf area compared to those individuals that were not damaged, whereas there were no differences with respect to changes in inflorescence number or flower production in 2003 compared to 2002 (A). Individuals that experienced natural infection produced significantly fewer inflorescences and flowers compared with individuals that were not infected, whereas there were no differences with respect to changes in total leaf area in 2003 compared to 2002 (B). Asterisks indicate a significant difference between damaged (either through herbivory or infection) and undamaged plants for each of the three traits.

selection on floral color, it is unlikely that differential pollinator preferences for color alone are sufficient to maintain variation in this population. There was no evidence that pollinator preferences for floral color varied across seasons (i.e., fluctuating selection), or that plants with intermediately colored flowers set more fruit than white- or red-flowered plants (i.e., balancing selection). In addition, the whitest floral-color morph was rare relative to the other floral-color variants in both seasons (Frey 2003) and consistently had the lowest fruit set, suggesting the absence of negative frequency-dependent selection on color. These findings are consistent with those from *Clarkia gracilis*, in which pollinator behavior and postpollination reproductive success was insufficient to ex-

plain the maintenance of floral-color polymorphism (Jones 1996a,b). There was good evidence that plants with intermediately sized flowers had the greatest relative fruit production, but it is not clear how this stabilizing selection on floral size may influence the evolution of floral color without a better understanding of how these traits are associated.

Clearly, further investigation into pollinator foraging behavior is warranted before any general conclusions can be made concerning the role pollinators play as agents of selection in this system. *Claytonia virginica* sometimes coexists with a variety of white-flowered ephemerals (e.g., *Stellaria pubera* [Caryophyllaceae], *Anemonella thalictroides* [Ranunculaceae], *Cardamine concatenata* [Brassicaceae]), and pollinators have been trapped in these populations with mixed pollen loads (F. M. Frey, unpubl. data). The clear fitness costs associated with producing floral phenotypes similar to heterospecifics (e.g., less efficient pollen transfer [Kevan 1978; Waser 1983b], increased pollen interference and stigma clogging [Waser 1978]), should generate selection for producing a ''distinct floral signal'' in mixed floral communities. In addition, there is evidence that pollinators move less frequently between species whose floral signals are distinct to increase reward reliability (Heinrich 1979; Waser 1983a; Menzel and Shmida 1993; Wilson and Stine 1996; Chittka 1997). Therefore, pollinator-mediated selection for increased floral redness in *C. virginica* may be even stronger in communities with an abundance of white-flowered ephemerals, and among-population variation in the strength of this selection may help to maintain floral-color variation. In support of this hypothesis, an among-community survey found a significant, positive relationship between the abundance of *S. pubera* and the relative frequency of red-flowered *C. virginica* (Frey 2003).

Herbivores and Pathogens

Large-leaved plants were attacked more often than small-leaved plants, and color variants did not differ with respect to the probability of leaf damage. This result shows that slugs did not preferentially attack particular color variants; instead, they simply attacked plants that they encountered in the field. Spatial analyses of the patterns of floral-color variation and herbivore attack showed that color variants were not clumped together and that the incidence of slug damage was relatively uniform throughout the population (F. M. Frey, unpubl. data). In both field seasons, there was clear evidence that floral color was strongly associated with leaf damage caused by slug herbivores; red-flowered plants sustained far greater damage than white-flowered plants. These results are in accord with the mechanism by which floral color is manifested in this system and what is known about the biochemistry of the pigment compounds; namely, as concentrations of the two flavonols increase, flowers appear whiter and individuals become better defended against herbivores (Bate-Smith and Swain 1962; Harborne 1967, 1991). These data also suggest that certain combinations of floral color/size traits were better defended against herbivores. Specifically, small, white-flowered individuals received less damage than large, white-flowered individuals, suggesting variation within the color variants with respect to tissue-specific flavonol concentrations.

For example, if all white-flowered plants produce similar quantities of flavonols, then those with larger flowers would necessarily have less to allocate to leaves.

In the 2002 field season, the dispersion pattern of fungal infection was significantly patchy (F. M. Frey, unpubl. data), and white-flowered plants were disproportionately infected within these ''epicenters'' of disease. This result was somewhat unexpected given that redder-flowered plants contain lower concentrations of the two flavonols than whiter-flowered plants (Doyle 1981; Frey 2003), and these compounds deter fungal infection in other species (Bate-Smith and Swain 1962; Harborne 1991). It is possible that herbivore damage caused the up-regulation of general defensive compounds, which in turn provided heavily damaged plants (those with redder flowers) with greater resistance to the rust pathogen than less damaged plants (those with whiter flowers). Consistent with this hypothesis, herbivore damage preceded pustule formation in the field, leaf damage was negatively correlated with pustule number, and plants with both leaf damage and pustules were the whiter morphs (mean spectral shape = 5.4 ± 3.60 SD; see Figure 1B for comparison).

Importantly, both herbivory and infection have direct fitness consequences. Plants that experienced a 50% leaf removal on their cauline leaves upon first flowering were far more likely to die than those plants that remained undamaged. Additionally, individuals with herbivore damage produced substantially smaller leaves than undamaged plants in the next growing season. In contrast, infected plants were equally likely to survive to the next growing season but produced far fewer flowers and inflorescences than uninfected plants. This is consistent with other work that has shown how infection by rust pathogens reduces plant growth and fecundity (Alexander et al. 1984; Alexander and Burdon 1984; Burdon 1987; Melendez and Ackerman 1993, 1994; Kluth et al. 2001).

Maintenance of Floral-Color Variation

Floral-color variants could be accurately categorized into one of four classes, plants transplanted to a common greenhouse recovered the full range of floral-color variation in the field, and a separate reciprocal transplant experiment showed that flower color does not change in response to variation in soil characteristics (Frey 2003). Taken together, these results suggest that a few genes of major effect, and/or several genes of lesser effect, primarily underlie the expression of floral color in *C. virginica*. Although the underlying mechanisms of inheritance and standing levels of allelic variation for floral color remain unresolved in this system, opposing directional selection that is consistent across generations would likely erode floral-color variation rather than maintain it. For example, if color differences are indeed the result of genes of major effect, floral-color variation should rapidly be lost, unless there is a net heterozygote advantage. Likewise, consistent, opposing selection pressures would tend to eliminate quantitative floral-color variation within a population.

In this study, the pattern of selection for increased floral whiteness through herbivore attack was consistent in time and space across two growing seasons; however, the magnitude of herbivore damage was far greater in 2002 compared to 2001. Selection for increased floral redness through pathogen attack varied across field seasons; pathogen attack was extremely low in 2001, but common and patchy in 2002. These results suggest that this population experiences temporal variation in the strength of the covariation between floral color and plant damage through herbivores and pathogens, as well as spatial variation in the strength of the covariation between floral color and infection. Together with selection for increased floral redness through pollinator preferences, which may also vary in space or time depending on the array of co-flowering spring ephemerals, these data suggest that floral-color variation could be maintained in some populations by temporal or spatial fluctuation in the frequency and magnitude of these three patterns of selection. Furthermore, these data suggest that among-population floral-color variation might reflect not only differences in pollinator preferences for particular color variants, perhaps driven through the composition of the spring ephemeral community, but also variation in the distribution and abundance of natural enemies.

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