Effects of a Locus Affecting Floral Pigmentation in *Ipomoea purpurea* on Female Fitness Components

Mark D. Rausher and James D. Fry[1]

Department of Zoology, Duke University, Durham, North Carolina 27706

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ABSTRACT

A locus influencing floral pigment intensity in the morning glory, *Ipomoea purpurea*, is polymorphic throughout the southeastern United States. Previous work has suggested that the white allele at this locus has a transmission advantage during mating because of the effect of flower color on pollinator behavior. The experiment described here was designed to determine whether other effects of the *W* locus may contribute an opposing selective advantage to the dark allele. Dark homozygotes were vegetatively smaller and produced fewer flowers, seed capsules and seeds than either light heterozygotes or white homozygotes. In addition, dark homozygotes produced smaller seeds than heterozygotes, and there is some indication that white homozygotes also produced smaller seeds than heterozygotes. Pleiotropic effects on seed number thus do not seem to contribute to selection opposing the mating advantage associated with the white allele. However, pleiotropic effects on seed size might contribute to overdominance that could stabilize the *W* locus polymorphism.

ACCOUNTING for the maintenance of genetic variation in natural populations has been a focus of evolutionary biology for 50 years. Theoretical analysis has demonstrated that over a dozen processes can explain the maintenance of genetic variation (for summary see HARTL 1980), while experiments with laboratory populations indicate that at least some of these processes can operate under some conditions (*e.g.*, DOBZHANSKY 1948; DOBZHANSKY and PAVLOVSKY 1953; EHRMAN 1967; POWELL and WISTRAND 1978; JONES and PROBERT 1980; CAVENER and CLEGG 1981). Nevertheless, definitive examples of the operation of any of these processes in nature are rare. Moreover, while patterns of genetic variation revealed by several recent investigations have provided strong evidence of the operation of balancing selection on polymorphisms (BARKER and EAST 1980; HEDRICK and THOMSON 1983; HUGHES and NEI 1988; KREITMAN and HUDSON 1991; KARL and AVISE 1992), even these studies have not identified the type of balancing selection involved. Consequently, our understanding of why organisms are so genetically variable (*e.g.*, LEWONTIN 1974; WRIGHT 1977; POWELL and TAYLOR 1979; HEDRICK 1986) is at best rudimentary. Detailed examination of the selective forces acting on genetic variation in a variety of organisms is needed. This report constitutes an initial contribution toward our long-term objective of conducting such an examination on genetic variation influencing floral characteristics in the annual morning glory, *Ipomoea purpurea*.

Previous work (ENNOS 1981; ENNOS and CLEGG 1983; EPPERSON and CLEGG 1988) has identified four major unlinked loci influencing floral pigment hue and intensity. This study concentrates on the *W* locus affecting pigment intensity, which is generally polymorphic in populations throughout the southeastern United States. Plants homozygous for the *w* allele have flowers that are white with pigmented rays (*i.e.*, "whites"); those homozygous for the *W* allele have darkly pigmented flowers that have even more intensely pigmented rays (*i.e.*, "darks"); and heterozygotes have lightly pigmented flowers with dark rays (*i.e.*, "lights"). Frequencies of the white allele are typically 0–0.4 in natural populations that have been surveyed (EPPERSON and CLEGG 1986).

Prior experiments and observations have also revealed several results relevant to understanding the type of selection acting on the *W* locus: (1) when white flowered plants are in the minority, bees undervisit white flowers compared to their frequency in both natural and experimental populations (BROWN and CLEGG 1984; EPPERSON and CLEGG 1987); (2) when in the minority, white flowered plants have lower outcrossing rates (proportion of seeds pollinated by another plant) than do plants with pigmented flowers, probably as a consequence of the reduced visitation (BROWN and CLEGG 1984; EPPERSON and CLEGG 1987); (3) these differences between white and pigmented plants disappear when whites are not in the minority in experimental populations (EPPERSON and CLEGG 1987); and (4) some evidence suggests whites may contribute more pollen *per capita* to the outcross pollen pool (SCHOEN and CLEGG 1985).

[1] Present address: Department of Genetics, North Carolina State University, Raleigh, North Carolina 27695-7614.

These effects would tend to favor an increase in the frequency of the white allele when rare (*e.g.*, FISHER 1941), and hence could explain in part the stability of the *W* locus polymorphism in natural populations. However, they do not explain protection of the dark allele. Therefore, absent any other selective forces acting on this locus, a combination of genetic drift and active protection of the white allele is expected to lead to fixation of the white allele. It would thus be expected that in most populations the dark allele should be the rarer allele. The observation that the mean frequency of the white allele in natural populations is only approximately 0.1 (EPPERSON and CLEGG 1986) thus strongly suggests the operation of some countervailing selection pressure favoring the dark allele.

The purpose of this investigation was to determine whether such countervailing selection operates on survival or female fitness. In particular, we examine the hypothesis that differences among *W* locus genotypes in viability, fecundity, or offspring quality contribute to selection favoring the dark allele. Such differences could arise if the *W* locus has pleiotropic effects on characters affecting viability or seed production. In some plant species, mutations affecting anthocyanin pigmentation are known to influence foliage phenol and lignin content (two potentially defensive characters) and seedling viability (COE, NEUFFER and HOISINGTON 1988). By analogy, similar effects might reasonably be expected at the *W* locus. In addition, EPPERSON and CLEGG (1987) have suggested that fecundity of white genotypes of *I. purpurea* may be reduced because of a combination of reduced pollination visitation (and hence greater reliance on selfing) and inability of some individuals (those with short anthers relative to stigma) to self pollinate (ENNOS 1981).

MATERIALS AND METHODS

Study organism: The morning glory *I. purpurea* grows in disturbed habitats, such as agricultural fields and roadsides, throughout the southeastern United States. Germination occurs between mid-May and August, depending on weather conditions and the timing of soil disturbance. Plants flower continually beginning about 6 weeks after germination until they are killed by the first frost. Flowers are perfect and usually capable of self-pollination, although some individual plants produce flowers with short anthers and can self-pollinate only if pollinators transfer pollen from the anthers to the stigma (ENNOS 1981). Although *I. purpurea* is generally capable of selfing, typical outcrossing rates estimated previously range from 0.5 to 0.9 (BROWN and CLEGG 1984; SCHOEN and CLEGG 1985; EPPERSON and CLEGG 1987). The primary pollinators are bumblebees. At our study site, the bumblebee *Bombus pennsylvanicus* accounted for more than 99.5% of more than 5000 observed visits by pollinators to the plants in our experiments.

Experimental design: Experimental seed of known genotype at the *W* locus and with the remainder of the genetic background randomized (but see below) were generated by three generations of crosses (Figure 1). In the first generation of crosses, plants collected as seedlings from an agricultural field in Orange County, North Carolina, were crossed in pairs ("great-grandparental pairs"), each pair consisting of one dark and one white plant. One heterozygote offspring ("grandparent") from each pair was then allowed to produce a large number of seeds by selfing (Figure 1A). Heterozygote offspring from this selfing were discarded. The homozygous dark and white offspring ("parents") were then crossed with congenerational offspring of the appropriate genotype from another parental pair to produce experimental seed known to be either white, light, or dark (Figure 1B). This third generation of crossing was performed such that all plants descended from a particular great-grandparental pair were crossed with plants from only one other great-grandparental pair, forming a "unit." This type of outcrossing was performed to minimize inbreeding effects in the experimental seeds. A total of seven units, descended from a total of 14 great-grandparental pairs, were used in the experiment.

Within each unit, 12 pairs of third-generation crosses were performed, four yielding each *W* locus genotype ("pair" in the analyses of variance reported). Within each of these parental pairs, plants were crossed reciprocally.

To facilitate estimating success at pollen donation (reported elsewhere), second-generation plants (grandparents) were all heterozygous for an Esterase marker locus. Potential parental plants were scored for Esterase genotype and were chosen so as to generate experimental seeds with a nonrandom association between *W* locus genotype and Esterase genotype. In particular, all dark and white experimental seeds were homozygous for the fast Esterase allele, while light experimental seeds were homozygous for the slow allele. Thus, the genetic background was effectively randomized for seeds within a given unit, except for genetic material closely linked to the the *W* and Esterase loci (chromosome number is $n = 15$). There is thus the potential for confounding the effects of the *W* and Esterase loci.

Two experimental seeds from each third-generation plant were randomly allocated to one of eight blocks in the experimental field. The design may thus be summarized as follows: block, unit, and *W* locus genotype are crossed main effects. Third-generation pairs are nested within unit × genotype combinations, and reciprocals are nested within third-generation pairs. There were 8 blocks, 7 units, 3 *W* locus genotypes, 4 parent pairs per unit × genotype combination, 2 parents per pair, and 2 seeds per parent per block, yielding a total of 2688 experimental plants in the original design. Because a few third-generation crosses did not produce enough seeds, the design was slightly unbalanced at that level, resulting in a total of 2683 plants in the experiment.

Experimental methods: Experimental seeds were germinated individually in wells of microtiter trays and transferred after one day to Root trainers[R] filled with potting mix. In early July 1990, approximately 1 week after germination, seedlings were transplanted into an old agricultural field in Durham County, North Carolina, that we have used previously (RAUSHER and SIMMS 1989; SIMMS and RAUSHER 1987, 1989). Prior to transplanting the field was disked and all native *I. purpurea* plants were removed from the field. The field was otherwise not weeded during the experiment, except for removal of any *I. purpurea* plants that subsequently appeared. Experimental plants were allowed to twine up 1-m high bamboo poles to mimic growth in corn fields and to facilitate plant censuses and seed collection.

Once flowering had commenced (in late August), flow-

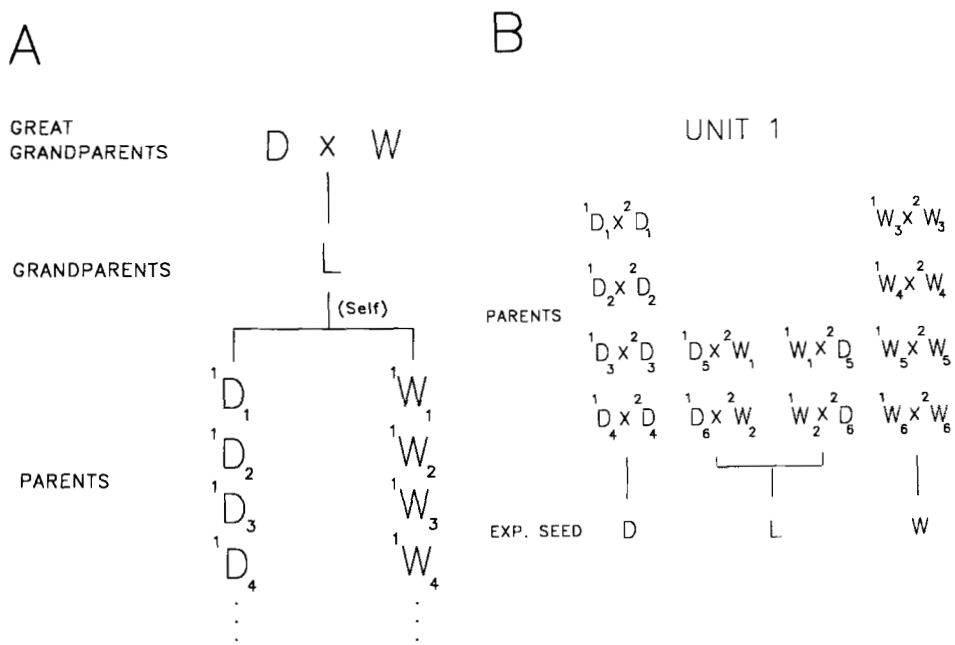

FIGURE 1.—Schematic protrayal of crossing design. (A) The first two generations of crosses performed beginning with a great-grandparental pair. D, L, and W represent dark, light, and white genotypes at the *W* locus. Superscripts indicate the great-grandparental pair from which the parents are derived. Subscripts indicate individual parents. (B) The third generation of crosses used to produce the experimental seed. Symbols as in part A. Note that a "unit" is the set of experimental seeds descended from two great-grandparental pairs. Seven units were used in the experiment.

ering activity was censused biweekly by recording at each census the number of flowers produced by each plant (flowers remain open for 1 day). We also recorded pigment intensity to confirm that each plant was of the expected genotype. Approximately 2 months after transplanting, the leaves on each plant were counted and the length and width of the largest leaf were measured. An index of the area of the largest leaf was calculated from these measurements by multiplying leaf length by leaf width.

Mature capsules remain on the plant for several days before they dehisce. By collecting capsules every 2 or 3 days, we were thus able to collect and count all capsules and seeds produced by each plant in the experiment until frost killed the plants in late November. Seeds that were obviously inviable were not counted. In addition, the total mass of seeds in each capsule was determined. These masses were summed for each plant and divided by the number of seeds produced by that plant to yield mean seed mass.

Statistical analysis: The fitness components of primary interest in this study were the proportion of plants producing at least one seed (viability), the total number of seeds produced (fecundity), and the mean size of seed produced (offspring quality). Differences among *W* locus genotypes in viability were compared using the categorical analysis procedure CATMOD of the Statistical Analysis System (SAS Institute, 1988). This procedure allows a determination of viability differences after the effects of unit or block have been removed.

Fecundity and offspring quality were analyzed using standard analysis of covariance, with leaf number, size of largest leaf, and their squares as covariates (SEARLE 1971). Block and Intensity genotype were treated as a fixed effect, while Unit was considered random. When necessary, response variables were square-root- or log-transformed to render the frequency distribution of residuals approximately normal (SOKAL and ROHLF 1969). Because there was a small but significant effect of *W* locus genotype on these covariates (see below), type I sums of squares (SAS Institute, 1988) with the covariates entered below the genotype main effect, and with the effects of block and unit removed from the genotype main effect, were used instead of the more standard type III sums of squares for analyses of seed number, seed mass, and flower and capsule number (see APPENDIX for justification of this approach). Appropriate denominator mean squares for approximate *F*-tests were constructed based on the type I expected mean square as listed by SAS. When compound denominators were necessary or when an interaction or nested effect mean square was the appropriate denominator, non-significant mean square components were dropped to simplify the *F*-test (NETER, WASSERMAN and KUTNER 1990).

When the effect of intensity genotype was significant, all three pairwise comparisons of the intensity genotypes were performed using appropriate contrasts employing type I sums of squares (see APPENDIX). These contrasts provide information regarding two issues. First, comparison of each homozygote genotype with the heterozygote provides a test of overdominance: only if both contrasts are significant can the existence of overdominance be legitimately inferred. Second, the contrast between homozygotes is instructive in deconfounding the effects of intensity genotype and marker (Esterase) genotype. Because darks and whites had the identical marker genotype, differences between them cannot be attributed to differences in marker genotype.

To facilitate interpretation of the analyses of the primary fitness components, the effects of *W* locus genotype on several additional characters were examined: total number of flowers produced, total capsules produced, and seed number/flower number ratio. In the case of the first two variables, analysis of covariance using leaf number and leaf size as covariates and type I sums of squares were performed as for fecundity. For the ratio, we used analysis of covariance to compare the regression slopes of seed number (square-root transformed) on flower number for different *W* locus genotypes. Type III sums of squares were used in this analysis, since we are interested in comparing adjusted means.

RESULTS

Viability differences: *I. purpurea* plants failed to produce any seed either because they died early in the season or because they did not reach reproductive size before the first killing frost. The proportion of plants

TABLE 1

Proportion of plants producing no seeds, by intensity genotype

	Dark	Light	White
Proportion	0.196	0.195	0.202
Standard error	0.013	0.013	0.013

Chi-square value for null hypothesis of equal proportions is 0.10, $P > 0.95$, d.f. = 2.

failing to reproduce successfully (at least one seed) did not differ among *W* locus genotypes (Table 1). The frequency distribution of seed number is strongly bimodal, with one peak at zero and another at about 15 seeds. Since the proportion producing seeds is virtually identical for the three genotypes, the remaining analyses are restricted to plants producing seeds to avoid complications associated with statistical analyses of bimodal distributions.

Intensity genotype effects: By contrast with viability, fecundity seems to be influenced by *W* locus genotype, but not in a way that would favor the dark allele. On average, light and white genotypes produced approximately the same number of seeds, which was about 4% greater than the number produced by the dark genotype (Figure 2). While there was therefore a trend toward lower seed production by darks, this trend was not quite statistically significant ($P = 0.095$, Table 2).

Because the pattern of differences in seed number is opposite that hypothesized (EPPERSON and CLEGG 1987) to result from an interaction between increased reliance on selfing by whites and inability of some individuals to self (due to relatively short anthers), it is of interest to know whether the pattern is real. We therefore examined the effect of intensity on characters that presumably causally influence seed number. These characters include indicators of plant size (*e.g.*, number of leaves, size of largest leaf), as well as the number of flowers and capsules produced. In our experiment, seed number was highly correlated with each of these variables ($r = 0.72, 0.72, 0.69, 0.98$, respectively, for number of leaves, size of largest leaf, number of flowers, and numbers of capsules, d.f. = 2151, $P < 0.0001$ in each case), suggesting a fairly strong determination of seed number by these variables.

For each of these characters, the effect of intensity genotype is significant (Tables 3 and 4). Evaluation of pairwise contrasts yields the following inferences (Tables 3 and 4, Figure 3): (1) darks produced significantly fewer capsules than lights and nearly significantly fewer than whites; (2) darks produced significantly fewer flowers than either lights or whites; (3) darks had significantly fewer leaves than lights and nearly significantly fewer than whites; (4) the size of the largest leaf differed significantly between lights and each homozygote. All other contrasts were not significant. Thus, except for size of the largest leaf, the pattern exhibited by each of these characters is similar to that seen for seed number: the mean values are smallest for darks, while the means for lights and whites are similar.

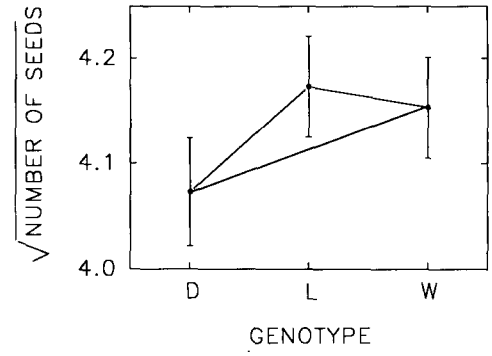

FIGURE 2.—Mean number of seeds produced per plant as a function of genotype at the *W* locus. Values on the *y*-axis are the genotypic means of the square root of the number of seeds produced, as estimated by the appropriate estimable function (see APPENDIX). D, dark (*WW*) genotype; L, light (*Ww*) genotype; W, white (*ww*) genotype. Error bars are one standard error of the mean.

A corollary of EPPERSON and CLEGG's (1987) hypothesis that increased reliance on selfing should lead to reduced fecundity in white-flowered genotypes is that white genotypes exhibit lower seed number/flower number ratio. Such an effect could counteract the expected proportionality between mean flower and mean seed number. We obtained no evidence that the seed/flower ratio differed among intensity genotypes (Figure 4). In particular, there was no evidence of statistical heterogeneity among *W* locus genotypes in the intercept or in either the linear or quadratic coefficients of a regression of seed number on flower number (Table 5). Moreover, elimination of the intensity main effect and both the linear and quadratic interaction coefficients ($I \times \beta$ and $I \times \beta^2$ effects, Table 5) from the model, thus forcing the predicted number of seeds given the number of flowers to be the same for each genotype, did not significantly reduce the explained variance ($F_{4, 1118} = 0.472$, $P > 0.74$).

The second major reproductive fitness component examined, mean seed size, also differed significantly among genotypes at the *W* locus, with light genotypes producing significantly heavier seeds than dark genotypes (Figure 5, Table 2), and nearly significantly heavier seeds than the white genotype, suggesting overdominance for mean seed size.

Unit effects: An effect of Unit on a character indicates that offspring having different great grandparents differ on average for that character. Such an effect is most easily interpreted as an effect of background genotype on the character analyzed. For all

TABLE 2

Analysis of variance for seed number and mean seed mass for plants producing at least one seed

Source of variation	d.f.	Seed number			Mean seed mass		
		SS	*F*	*P*	SS	*F*	*P*
Unit (*U*)	6	57.42	6.02	<0.001	5.04	2.28	<0.05
Block (*B*)	7	1475.42	132.59	<0.001	8.02	4.01	<0.001
Intensity (*I*)	2	7.50	2.36	=0.095	3.81	5.18	<0.01
Dark *vs.* light	1	3.23	2.06	>0.151	2.32	6.28	<0.01
Light *vs.* white	1	0.12	0.08	>0.78	1.33	3.59	<0.07
Dark *vs.* white	1	2.08	1.33	>0.24	0.17	0.46	>0.50
Leaves (LV)	1	3921.59	2466.93	<0.001	14.73	51.64	<0.001
Leaf area (LA)	1	638.66	401.76	<0.001	1.90	6.65	<0.01
LV^2	1	546.02	343.48	<0.001	0.00	0.00	>0.99
LA^2	1	0.06	0.04	>0.83	0.02	0.08	>0.77
$U \times I$	12	10.96	0.57	>0.86	5.69	1.28	>0.25
$I \times B$	14	15.25	0.69	>0.79	3.50	0.67	>0.60
$U \times B$	42	54.90	0.82	>0.78	13.45	0.86	>0.55
Pair within $U \times I$ (*P*)	65	126.13	1.22	>0.11	24.00	1.29	=0.06
$P \times B$	507	737.00	0.91	>0.88	150.08	1.04	>0.29
Reciprocal	82	129.82	1.00	>0.49	25.53	1.09	>0.27
Error	1407	2236.66			657.91		
Total	2148						

Dependent variables were square-root transformed before analysis. Order of effects in table reflects the order of entry of those effects in ANOVA. For mean seed mass, the following effects were tested over the pair (*P*) mean square: *U*, *I*, $U \times I$, and contrasts within *I*. All other effects were tested over error mean square. SS = sum of squares.

TABLE 3

Analysis of variance for flower number (sum of number of flowers observed over all biweekly censuses) and capsule number for plants producing at least one seed

Source of variation	d.f.	Flower number			Capsule number		
		SS	*F*	*P*	SS	*F*	*P*
Unit (*U*)	6	8.88	2.91	<0.001	15.44	7.08	<0.001
Block (*B*)	7	170.11	64.51	<0.001	343.95	135.23	<0.001
Intensity (*I*)	2	9.13	8.98	<0.001	2.92	4.02	<0.02
Dark *vs.* light	1	2.52	4.96	<0.05	1.44	4.08	<0.05
Light *vs.* white	1	0.59	1.16	>0.25	0.02	0.06	>0.81
Dark *vs.* white	1	7.60	14.95	<0.0001	1.11	3.14	<0.08
Leaves (LV)	1	793.60	1720.20	<0.001	813.27	2238.21	<0.001
Leaf area (LA)	1	125.66	272.38	<0.001	146.27	402.56	<0.001
LV^2	1	60.43	130.99	<0.001	113.49	312.36	<0.001
LA^2	1	4.38	9.49	<0.001	0.07	0.19	>0.66
$U \times I$	12	5.72	0.94	>0.50	2.25	0.52	>0.90
$I \times B$	14	6.24	1.18	>0.25	3.86	0.76	>0.71
$U \times B$	42	21.21	1.34	>0.10	13.04	0.85	>0.73
Pair within $U \times I$ (*P*)	65	26.73	0.81	>0.50	29.15	1.23	>0.10
$P \times B$	507	190.97	1.14	<0.02	169.37	0.92	>0.85
Reciprocal	82	37.83	1.40	<0.02	31.42	1.05	>0.35
Error	1407	463.98			511.25		
Total	2148						

Dependent variables were square-root transformed before analysis. For flower number, effects *U*, *I*, $U \times I$, *P* and contrasts within *I* were tested over a compound mean square equal to $MS_{reciprocal} + MS_{P \times B} - MS_{error}$ (d.f. = 85) and effects *B*, $B \times I$, and $U \times B$ were tested over the $P \times B$ mean square. All other effects were tested over the error mean square. SS = sum of squares.

characters examined, including seed number and mean seed size, the Unit effects are strong (Tables 2, 3 and 4), indicating the presence in the base population of variation at loci other than the *W* locus (and the marker locus) affecting these characters. This result is consistent with previous results indicating the existence of considerable additive genetic variation for plant size and seed number (RAUSHER and SIMMS 1989; SIMMS and RAUSHER 1989).

Intensity × unit interaction: An interaction between intensity and unit effects can be caused in two different ways: by differences in the effects of different copies of the *W* locus allele obtained from different sets of great-grandparents (*i.e.*, within-allele class var-

TABLE 4

Analysis of variance for number of leaves and leaf area of largest leaf for plants producing at least one seed

Source of variation	d.f.	Leaf number			Leaf area		
		SS	*F*	*P*	SS	*F*	*P*
Unit (*U*)	6	5.92	4.17	<0.001	17.01	2.81	<0.01
Block (*B*)	7	49.10	29.61	<0.001	203.19	28.79	<0.001
Intensity (*I*)	2	1.38	2.92	=0.054	13.16	6.52	<0.01
Dark *vs.* light	1	1.67	7.19	<0.01	20.82	21.05	<0.001
Light *vs.* white	1	0.16	0.68	>0.40	10.00	10.11	<0.005
Dark *vs.* white	1	0.82	3.55	=0.06	2.27	2.29	>0.13
$U \times I$	12	1.91	0.67	>0.78	5.24	0.46	>0.93
$I \times B$	14	3.15	0.95	>0.50	9.63	0.68	>0.79
$U \times B$	42	11.31	1.14	>0.24	43.50	1.03	>0.42
Pair within $U \times I$ (*P*)	65	11.61	0.75	>0.92	51.70	0.79	>0.88
$P \times B$	507	109.91	0.92	>0.88	472.06	0.92	>0.85
Reciprocal	82	22.83	1.18	>0.14	85.09	1.03	>0.41
Error	1411	334.22			1422.72		
Total	2148						

All effects tested over error mean square. SS = sum of squares.

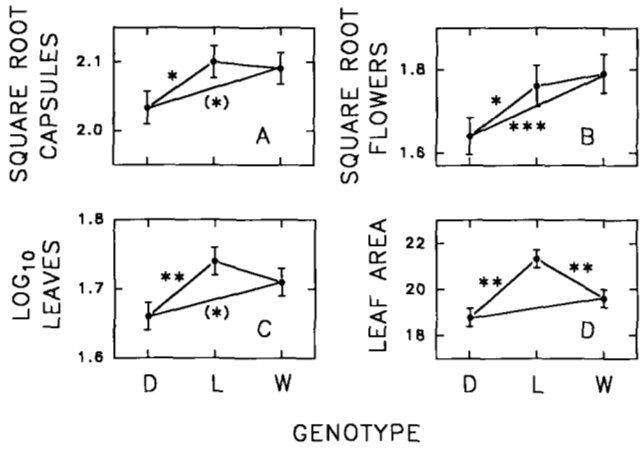

FIGURE 3.—Effect of *W* locus genotype on reproductive and vegetative characteristics. Genotype symbols as in Figure 2. Significance of contrasts between pairs of genotypes indicated by asterisks: (*)$P < 0.08$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (A) Mean of square root of number of capsules per plant; (B) mean of square root of number of flowers per plant; (C) mean of $\log_{10}$ (number of leaves per plant); (D) 10^{-2} × mean of area of largest leaf (mm^2). Error bars are one standard error of the mean.

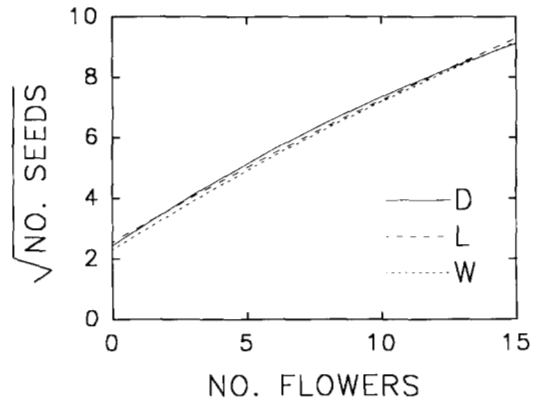

FIGURE 4.—Estimated relationship between number of seeds produced and number of flowers produced for dark, light, and white genotypes. Seed number is square-root transformed. Solid line: darks; dashed line, lights; dotted line, whites. Curves are best-fitting quadratic regressions of seed number on flower number.

iation) or by differences in the effect of particular *W* locus alleles in different genetic backgrounds. For none of the characters examined was there any evidence of either type of difference, as indicated by the lack of unit × intensity interaction (Tables 2, 3 and 4).

Other effects: For seed number and seed size, as well as flower and capsule number, one or more covariates explained significant amounts of variation. Since the covariates are indices of plant size early during vegetative growth, we interpret these effects to mean that early plant size has a positive effect on reproductive output, an effect that is common in plants, including morning glories (RAUSHER and SIMMS 1989). We suspect that much variation in early plant size is caused by heterogeneity in microsite conditions experienced by individual plants (*e.g.*, HARPER 1977).

Reciprocal effects were detected only for flower number (Tables 2, 3 and 4). Thus, maternal effects seem to have little influence on the fitness components examined.

DISCUSSION

Maintenance of the *W* locus polymorphism: Prior work on the *W* locus polymorphism in *I. purpurea* has identified apparent advantages to the white allele during the transmission phase of the life cycle. In particular, when individuals of the white genotype are in the minority, they tend to self more than dark-genotype individuals (BROWN and CLEGG 1984; EPPERSON and CLEGG 1987). In the absence of pollen discounting and inbreeding depression, a greater tendency to

TABLE 5

Analysis of covariance for seed number *vs.* flower number for plants producing at least one seed

Source of variation	d.f.	Seeds *vs.* flowers SS	*F*	*P*
Unit (*U*)	6	12.36	0.92	>0.47
Block (*B*)	7	297.37	19.05	<0.0001
Intensity (*I*)	2	5.42	1.22	>0.29
β	1	511.84	229.54	<0.001
β^2	1	17.58	7.88	<0.005
$I \times \beta$	2	2.99	0.67	>0.51
$I \times \beta^2$	2	2.26	0.51	>0.60
$U \times I$	12	26.98	1.01	>0.43
$I \times B$	14	9.31	0.30	>0.99
$U \times B$	42	81.49	0.87	>0.70
Pair within $U \times I$ (*P*)	65	171.06	1.16	>0.18
$P \times B$	494	1023.21	0.93	>0.82
Reciprocal	82	175.31	0.96	>0.58
Error	1118	2492.95		
Total	1849			

All effects tested over error mean square. Seed number square-root transformed before analysis. β and β^2 are linear and quadratic coefficients of regression of seed number on flower number. Nonsignificant $I \times \beta$ and $I \times \beta^2$ effects indicate homogeneity of coefficients among *W* locus genotypes. Nonsignificant Intensity effect indicates homogeneity of intercepts. SS = sum of squares.

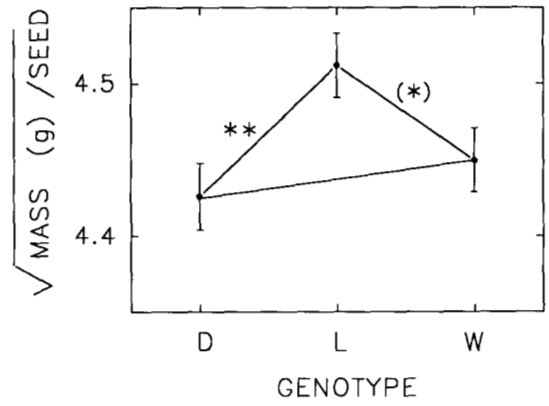

FIGURE 5.—Mean seed mass as a function of genotype at the *W* locus. Symbols as in Figures 2 and 3. Values on *y*-axis are the genotypic means of the square root of mean seed mass, as estimated by the appropriate estimable function (see APPENDIX). Error bars are one standard error of the mean.

self confers a net transmission advantage to an allele that increases selfing (FISHER 1941). It thus seems that in the absence of some countervailing advantage associated with the dark allele, white should become fixed. Since the white allele is in fact in the minority in most populations (EPPERSON and CLEGG 1986), it is probable that such a countervailing advantage exists.

We have not been able to identify such a countervailing advantage in this study. Although we examined the influence of variation at the *W* locus on three components of fitness, *i.e.*, viability (defined broadly as producing at least one seed before dying), fecundity, and offspring quality (seed mass), for none of these fitness components was there any indication that selection favored the dark allele. Moreover, for fecundity, the trend was in the opposite direction, with dark plants producing fewer seeds than lights or whites. It thus remains puzzling why the white allele does not become fixed.

One possible explanation is that overdominance for offspring quality, as reflected in the observed differences among genotypes in seed size, stabilizes the polymorphism. This explanation by itself seems unlikely, however, because in most natural populations the frequency of the white allele is considerably less than 0.3 (EPPERSON and CLEGG 1986). An equilibrium due to overdominance in offspring quality and with gene frequencies of this magnitude would require the offspring of white individuals to be of substantially lower quality than the offspring of dark individuals (ROUGHGARDEN 1979), whereas we observed the opposite trend.

The absence of an advantage to the dark allele in selection on viability, fecundity, or seed size suggests that any dark-allele advantages that exist are likely to be associated with other fitness components, most likely with differential transmission of pollen. One possibility is that white-flowered plants are less successful as pollen donors to other plants than are dark plants. Such an effect could occur in either of two ways. First, the reduced visitation by pollinators to white-flowered plants, which apparently is the cause of their higher selfing rate (BROWN and CLEGG 1984; EPPERSON and CLEGG 1987; RAUSHER, AUGUSTINE and VANDERKOOI 1993), could cause whites to export less pollen to other plants; this would be a form of "pollen discounting" (HOLSINGER, FELDMAN and CHRISTIANSEN 1984). Although an experiment by RAUSHER, AUGUSTINE and VANDERKOOI (1993) gave evidence against pollen discounting, this experiment was performed using small arrays of potted plants without any heterozygote light-flowered plants. It is possible that patterns of pollen transmission in large natural populations containing all three genotypes might be different, especially since some evidence indicates that pollinator behavior can differ among plant arrays of different sizes (STUKY 1984; STANTON *et al.* 1991). Second, white-flowered plants might be less successful as pollen donors for reasons independent of pollinator behavior; for example, they might produce pollen of lower quality, or fewer pollen grains per flower, than dark-flowered plants. The report by SCHOEN and CLEGG (1985) that white plants contributed disproportionately *more* pollen to the outcross pollen pool than darks argues against this possibility, but, as recognized by the authors, this result could have arisen because the authors failed to randomize the genetic background of their experimental plants.

Two other hypotheses about possible compensating

advantages of the dark allele still need to be investigated. The first of these is that there is segregation distortion or gametophytic selection favoring the dark allele. The second possibility is inbreeding depression, which would be expected to effect the progeny of whites more than those of lights and darks. DARWIN (1895) reported substantial inbreeding depression in *I. purpurea*, although the reduction in fitness of selfed progeny was less than the 50% that is usually thought to be necessary to prevent the evolution of selfing (MAYNARD SMITH 1977; LLOYD 1979; CHARLESWORTH 1980; CHARLESWORTH and CHARLESWORTH 1990). However, because DARWIN's seeds may have been a mixture from a variety of sources in South America, it is unclear that DARWIN's results are pertinent to understanding maintenance of variation at the *W* locus in southeastern North America. By contrast, PEAR (1983) found no evidence of inbreeding depression in *I. purpurea* collected from Georgia, but her experiments were performed in a greenhouse under benign conditions, which can ameliorate the expression of deleterious genes causing inbreeding depression (ANTONOVICS 1968; SCHMITT and EHRHARDT 1990).

Pleiotropic effects of the *W* locus: Although our experiment failed to reveal any component of selection favoring the dark allele, it appears that the *W* locus has pleiotropic effects that affect growth and reproduction. By the commencement of flowering, dark-flowered plants were smaller than light- and white-flowered plants. This difference in size presumably accounts in large part for the reduced flower and capsule production by dark-flowered plants. Reduced flower and capsule production would normally be expected to yield reduced seed production. This pattern was observed, but the reduction in fecundity was not quite statistically significant.

Two explanations may be offered for this lack of significance: either (1) the greater flower production by lights and whites was completely counteracted by a reduced seed/flower ratio [EPPERSON and CLEGG (1987) suggest how such a reduction might occur], or (2) darks really do produce fewer seeds than whites and lights, but our analysis was not quite sensitive enough to reveal it unambiguously. Analysis of the relationship between seed number and flower number revealed no evidence of heterogeneity among intensity genotypes in the number of seeds per flower ($P > 0.74$). By contrast, because the intensity effect on seed number approached statistical significance, there is some evidence for the second explanation. We therefore conclude that our results are best interpreted to indicate that the *W* locus has pleiotropic effects on seed production.

Our results also indicate that intensity genotype influences mean seed size, which in many plants influences offspring survival and reproductive success (HARPER, LOVELL and MOORE 1970). It thus seems likely that, in general, pleiotropic effects of the *W* locus are an important determinant of fecundity and offspring quality.

Little is known about the causes of these apparent pleiotropic effects. One possibility is that production of anthocyanins by dark-flowered genotypes diverts resources from functions contributing to growth or defense of plants from enemies. Experiments with other plants have demonstrated that many mutations affecting the anthocyanin biosynthetic pathway result in increased accumulation of potentially defensive substances such as phenolics and lignins (GEE, NELSON and KUC 1968; KIRBY and STYLES 1970; STYLES and CESKA 1981a,b). In addition, several of these mutations have major effects on growth and viability (COE, NEUFFER and HOISINGTON 1988), and it is likely that many others have minor effects. Nevertheless, two observations argue against this hypothesis. First, *W* locus genotypes do not appear to differ in susceptibility to herbivorous insects or a rust fungus (FINEBLUM 1991). Second, there are no visible effects of the *W* locus on pigmentation patterns in the stems or leaves, in contrast to what is seen with other loci affecting floral pigmentation in *I. purpurea* (SCHOEN *et al.* 1984; EPPERSON and CLEGG 1988). Moreover, white-flowered individuals produce normal floral pigments, but these pigments are restricted to a set of rays or "nectar guides" rather than being distributed over the entire corolla. These observations suggest that the *W* locus may regulate the timing or tissue-specificity of pigment synthesis rather than code for an essential enzyme in the anthocyanin biosynthetic pathway, and that its effects on pigmentation may not be expressed outside of floral tissue. If this hypothesis is true, it does not seem that diversion of precursors from the anthocyanin pathway just in floral tissue would be substantial enough to account for the observed effects on growth and fecundity. It is possible, however, that this gene may regulate other biochemical pathways as well, and that this regulation may be expressed in non-floral tissue, producing pleiotropic effects of the type observed.

An altogether different hypothesis for the apparent pleiotropic effects of the W locus is that they are the product of active molding by selection. Plants are presumably under continuous selection for optimal allocation of resources between competing demands of vegetative growth, flower production, and seed production (BAZZAZ *et al.* 1987). The optimal pattern of allocation is likely to be influenced by the frequency at which pollinators visit plants that produce a given number of flowers (*e.g.*, SCHAFFER and SCHAFFER 1979). Because white and pigmented flowers apparently differ in their inherent attractiveness to bumble-

bees (HEINRICH, MUDD and DERINGIS 1977), the sole pollinator of *I. purpurea*, it follows that the optimal allocation of resources between competing functions may differ among *W* locus genotypes. If the *W* locus polymorphism is an ancient one, then differences among genotypes in patterns of growth, flowering and seed production would be expected.

One additional point that needs to be considered is whether pleiotropic fitness effects apparently due to the *W* locus might actually be caused by variation at other loci affecting flower pigmentation, as might be the case, for example, if there were associations between genotypes at the *W* locus and genotypes at the *P*, *I* or *A* loci (for a description of these loci, see ENNOS and CLEGG 1983; EPPERSON and CLEGG 1988). Such associations were unlikely in our experiment for two reasons. First, there was no variation at either the *A* or *I* loci. All individuals were *AA* and *II*. Although there was some variation at the *P* locus, which determines whether flowers are blue or pink, all experimental individuals were phenotypically blue (*PP* or *Pp*) and the majority (an estimated 72%) were *PP*. In addition, the *P* locus (as well as the *A* and *I* loci) is unlinked with the *W* locus. Consequently, selfing of the four *Pp* grandparental plants (the remaining three were *PP*) should have ensured that there was no association between genotypes at the *W* and *P* loci among the 48 parents (16 per *W* locus genotype) that may have been either *PP* or *Pp*. With such randomization, the apparent pleiotropic effects we have detected cannot be attributed to variation at the other pigment loci.

Two caveats must be offered regarding apparent pleiotropic effects of the *W* locus. First, it is possible that these effects may be caused not by the *W* locus itself but by a closely linked locus that is in linkage disequilibrium with the *W* locus. We believe this possibility to be unlikely because of the lack of interaction between the Unit and Intensity effects in any of the analyses, but we cannot rule out this possibility definitively. Second, if the apparent overdominance in mean seed size is real, this pattern could have been caused by effects of the Esterase marker locus (or a closely linked locus) rather than by effects of the *W* locus, since Esterase and *W* locus genotypes were completely correlated in the experimental seeds. Nevertheless, in view of the almost universal existence of pleiotropy (WRIGHT 1977), it would not be surprising that alleles at the *W* locus should also exhibit multiple pleiotropic effects. Evidence for pleiotropy does indicate, however, that a complete understanding of the maintenance of the *W* locus polymorphism can not be attained by viewing the *W* locus exclusively or primarily as a mating-system modifier (*e.g.*, SCHOEN and CLEGG 1985; EPPERSON and CLEGG 1987). Rather, the manifold effects of this locus on all components of fitness must be examined and quantified, and the net advantage or disadvantage accruing to each allele over the entire life cycle must be ascertained.

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APPENDIX

In this appendix we justify the use of type I (sequential) sums of squares in analyses of the effects of genotype on flower, capsule and seed number and on mean seed mass. In this type of analysis, the covariates are entered into the model below the main effects.

The normal type III sum of squares are inappropriate when covariates differ among the treatment of interest (intensity genotype). This may be seen by considering the simplest situation in which there is a response variable y, a single class variable having two levels, t (treatment), and a single covariate, x. The null hypothesis of interest is that the treatment means are equal.

The statistical model for this situation is

$$y_{ij} = \mu + t_i + \beta x_{ij} + \epsilon_{ij},$$

where the subscript i denotes the treatment, the subscript j denotes the replicate of the ith treatment, μ is the grand mean, t_i is the effect of treatment i, and β

is the regression coefficient associated with the covariate. The estimable function for the mean of treatment i is

$$E_i\colon \mu + t_i + \beta x_{i\cdot} = 0,$$

where $x_{i\cdot}$ is the mean of the covariate for treatment i. The difference between treatment means then corresponds to the contrast

$$C_I = t_1 - t_2 + \beta(x_{1\cdot} - x_{2\cdot}).$$

This is the same as the estimable function associated with the type I sum of squares for the treatment effect.

By contrast, the type III sum of squares for the treatment effect is

$$C_{III} = t_1 - t_2,$$

which is obtained from a difference between the estimable functions associated with the *adjusted mean* for each treatment. The general form of these estimable functions is

$$E_{i\cdot}' = \mu + t_i + \beta x_{\cdot\cdot},$$

where $x_{\cdot\cdot}$ is the grand mean of the covariate. The adjusted mean for each treatment is essentially the mean value of y the treatment would have if the mean value of the covariate for that treatment equalled the grand mean of the covariate. The type III sum of squares thus adjusts the mean of each treatment before comparing treatment means. Since in our experiment the null hypothesis of interest is that the unadjusted means are equal, type III sum of squares are not appropriate.

The use of type I sum of squares actually provides conservative conclusions in our study because for all characters examined, the level of significance associated with the intensity genotype effect was smaller (*i.e.*, there is more evidence for rejecting the null hypothesis) with type III sum of squares than with type I sum of squares. It should also be noted that in all analyses, the effect of intensity genotype was entered in the model statement *after* the other main effects. Consequently, the significance of the intensity genotype effect was evaluated after the effects of block and unit had been removed. Although interaction effects are entered after the main effects and covariates, exploratory analyses using type III sum of squares provided essentially the same result as the type I sum of squares for these effects, *i.e.*, that none of them even approached significance (see text). Consequently, the position of entry of these effects has little influence on the overall analysis, and in particular has little influence on significance of the intensity genotype effect.