

Analysis of a chalcone synthase mutant in *Ipomoea purpurea* reveals a novel function for flavonoids: amelioration of heat stress

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Abstract

Flavonoids are thought to function in the plant stress response and male fertility in some, but not all, species. We examined the effects of a self-fertile chalcone synthase null allele, *a*, for the effects of heat and light stress on fertilization success and flower production in *Ipomoea purpurea*. Pollen recipients and pollen donors of both homozygous genotypes exhibit reduced fertilization success at high temperatures, indicating that high temperature acts as a stress-lowering fertilization success. Homozygous *aa* individuals exhibit reduced male and female fertilization success, compared to *AA* individuals, at high temperatures but not at low temperatures. In addition, *aa* individuals produce fewer flowers than *AA* individuals at low temperatures, but not at high temperatures. These results suggest that flavonoids alleviate heat stress on fertilization success. They also suggest that pleiotropic effects at the *A* locus may explain the low frequency of the *a* allele in natural populations.

Keywords: chalcone synthase, evolution, fertilization, flavonoids, heat stress, *Ipomoea purpurea*

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Introduction

Historically, floral colour variation has served as a useful tool for examining evolutionary mechanisms in plants (e.g. Grant 1950; Levin & Kerster 1970; Waser & Price 1983; Brown & Clegg 1984; Oberath & Bohning-Gaese 1999). Often, flower colour variants are controlled by a single Mendelian locus, rendering genetic analysis straightforward (e.g. Mendel 1865 in Bateson 1909; Sangwan 1998). In many cases, alleles at floral-colour loci exhibit incomplete dominance, allowing inference of a plant's genotype from simple observations of flower-colour phenotype (e.g. Ennos & Clegg 1983). Finally, because floral-colour variation often influences pollinator attraction, it can have direct effects on fitness that are large enough to be measured in field experiments (Brown & Clegg 1984; Rausher *et al.* 1993; Levin & Brack 1995). While these direct effects of flower colour on reproductive success clearly play a major role in guiding the course of floral colour evolution, it is becoming increasingly evident that genes affecting floral pigmentation have pleiotropic effects on fitness. For example, in *Phlox drummondii*, white-flowered plants appear to have reduced

survivorship and reduced flower production relative to pigmented plants (Levin & Brack 1995); in *Linanthus parryae*, the relative fitness of the two colour morphs is correlated with rainfall and not with pollinator visitation (Schemske & Bierzychudek 2001); in several species with pink/purple:white polymorphism, pigmented plants have a higher resistance to drought than the white-flowered plants (Warren & Mackenzie 2001); and, in *Ipomoea purpurea*, flower-colour genotype at the *W* locus influences vegetative size, flower production and total seed production (Rausher & Fry 1993).

These pleiotropic effects are not surprising, given the known physiological functions of plant pigments and biochemically related compounds (Gleiss *et al.* 2001). For example, white-flowered genotypes in many plant species result from the inactivation of genes in the anthocyanin pigment pathway (e.g. Gerats *et al.* 1982; Bonas *et al.* 1984). Because the enzymes encoded by these genes are also involved in the production of other flavonoids, plants containing these knockouts could have reduced ultra-violet light (UV) protection, increased susceptibility to natural enemies, disrupted interactions with fungal symbionts, and reduced self-fertility (Koes *et al.* 1994). Consequently, a complete understanding of the processes that guide the evolution of flower colour will require characterization

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of the magnitude of all the fitness effects, both direct and indirect, associated with variation at floral-colour loci. In this report, we describe a novel pleiotropic effect of a floral pigment locus and estimate the magnitude of its influence on fitness.

The common morning glory, *Ipomoea purpurea* (L.) Roth has at least two polymorphic floral colour loci, the *W* and the *A* loci, across the eastern United States. Flowers of *WWAA* individuals are pigmented throughout the corolla limb. By contrast, *WWaa* and *wwAA* individuals are white flowered. The white-flowered genotypes are phenotypically similar except that the latter genotype has pigmented floral rays and pigmented stems. Both loci are co-dominant so that heterozygotes are lightly pigmented (Ennos & Clegg 1983). Despite the similarity of the associated phenotypes, the *a* and *w* alleles exhibit very different frequencies in wild populations. While the *w* allele is relatively common, with frequencies reaching 0.5 in some populations (Epperson & Clegg 1986; R. Miller and M. D. Rausher, unpublished data), the *a* allele rarely occurs at frequencies higher than 0.005 (Epperson & Clegg 1987; C. Coberly, unpublished data), despite being present since at least the early 1800s (Sims 1814).

Previous work suggests that the direct effects of floral colour on visitation by pollinators are similar for the two loci. Compared to pigmented flowers, white flowers of both genotypes, when rare, are visited less often by bumblebees, their primary pollinator, and as a result have higher selfing rates. This reduced visitation, however, does not result in reduced outcross pollination, i.e. there is no detectable pollen discounting for either white-flowered genotype (Brown & Clegg 1984; Rausher *et al.* 1993; Fry & Rausher 1997; C. Fehr, unpublished data). Consequently, the direct fitness effects of flower colour are expected to increase the frequency of the white-flower allele when it is rare (Fry & Rausher 1997). The relatively high frequency of the *w* allele in natural populations is consistent with this expectation, as is the experimental demonstration of an increase in *w*-allele frequency when rare (Subramaniam & Rausher 2000). This is also consistent with experimental observations showing an apparent absence of deleterious pleiotropic effects of the *w* allele (Rausher & Fry 1993; Mojonnier & Rausher 1997). It thus appears that the evolutionary dynamics of the *w* allele are dominated by direct effects of flower-colour variation on pollinator attraction, at least when the allele is rare.

By contrast, the uniformly low frequency of the *a* allele in natural populations suggests that there are relatively strong deleterious pleiotropic effects associated with this allele, and that these effects more than offset the direct benefits associated with increased selfing, and thus prevent the allele from increasing when rare. This reasoning led us to examine the magnitude of one particular pleiotropic effect that was suggested by our observations. While per-

forming greenhouse crosses during the summer months, one of us (L.C.C.) observed that white-flowered (*aa*) plants failed to set as many seeds as plants with pigmented flowers (*AA*). Increases in both temperature and light might reasonably be expected during the summer. However, while flavonoids have been shown to provide some protection from UV light (Li *et al.* 1993), much of the UV is filtered by greenhouse glass. We therefore reasoned that an increase in the UV light could not explain the increased damage to the chalcone synthase mutant plants. Instead, we hypothesized that flavonoids present in *AA* plants, but not present in *aa* plants, may ameliorate the effects of high temperature (characteristic of our greenhouses in the summer) on fertilization or abortion of ovules. The experiments reported here were designed to test this hypothesis, and to estimate any differential effect of high temperature and high light on the fitness of pigmented and white-flowered plants.

Materials and methods

System

Ipomoea purpurea is an annual, self-compatible vine common throughout the southeastern USA. It grows commonly in disturbed areas, particularly agricultural fields. The *A* locus is one of several duplicated loci (Durbin *et al.* 1995) that encode the enzyme chalcone synthase (CHS), the first enzyme in the biosynthetic pathway that produces the flavonoids, including the anthocyanin pigments (Fig. 1). The *a* (white-flowered) allele differs from the *A* (pigmented) allele in having an Ac/Ds transposon inserted into the CHS-D, preventing transcription of a functional copy of the gene (Johzuka-Hisatomi *et al.* 1999). The *a* allele reported here is indistinguishable from the *a** and *a^{flaked}* alleles reported elsewhere (Epperson & Clegg 1987; Habu *et al.* 1998; Coberly, unpublished data). Because CHS-D is the primary chalcone synthase locus expressed in most plant tissues (Durbin *et al.* 2000), *aa* plants lack pigmentation not only in flowers, but also in most vegetative tissues (Epperson & Clegg 1987; Ishikawa, personal communication).

Plant breeding

Two separate inbred *I. purpurea* lines were used in this experiment (line 1 and line 2). Each line was nearly homozygous (homozygosity 87.5%), but segregating for the CHS-D mutation. The inbred lines were created in the following manner (see Fig. 2): seeds were collected from separate maternal plants from wild populations in Durham County, NC in the autumn of 1983. Individual seeds were germinated and grown to fruit in the Duke University greenhouses. Plants were self-pollinated and

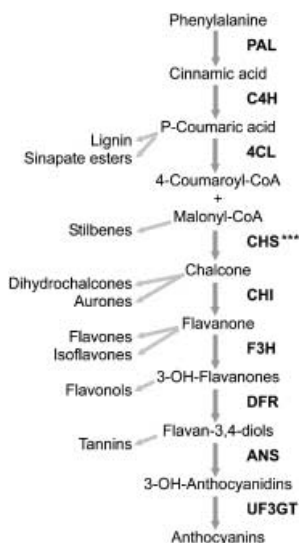


Fig. 1 Simplified diagram of the flavonoid metabolic pathway (see Harborne 1967 for additional information). The main substrates and products of the anthocyanin pathway are presented in the centre, enzymes catalysing the reactions are given in bold on the right (see below for abbreviations), and alternate products are given on the left side of the diagram. Abbreviations are as follows: PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, coumarate; CHS***, chalcone synthase (***) null mutant of the A locus described in this paper; CHI, chalcone isomerase; F3H, flavone-3-hydroxylase; DFR, dihydroflavone reductase; ANS, anthocyanidin synthase; UF3GT, UDP-glucosyltransferase.

propagated via single seed descent for 13 generations to create homozygous inbred lines. Two separate pairs of *AA* and *aa* inbred lines were crossed to create heterozygous offspring, which were then self-pollinated and propagated via single-seed descent for a total of four generations (Fig. 2), yielding two partially inbred experimental lines. The F_4 generation of each experimental line was planted out in bulk, and the homozygous progeny were used as experimental plants. This crossing design randomized the genetic background of the two flower-colour genotypes (except for loci closely linked to the *A* locus) and ensured that plants of each line and treatment were, on average, equally inbred.

Rearing of experimental plants

Seeds were scarified and planted in seedling flats filled with MetroMix 2000®. At 21 days plants were transferred to 4" (10-cm) standard pots in research mix soil (contact authors for recipe). Each plant was fertilized with 1.5 oz (c. 40 g) Osmocote™ time-release fertilizer at the time of transplanting. All plants were grown from seed to flowering in a common greenhouse environment from

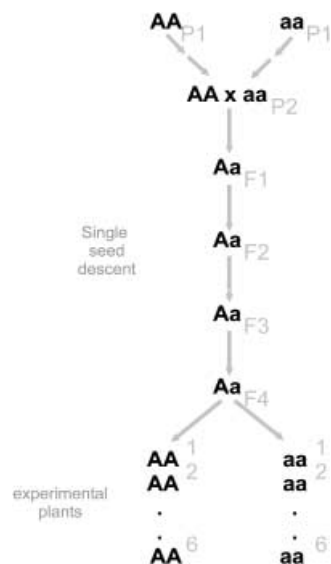


Fig. 2 The breeding design for the experimental plants. P1 plants were collected from wild populations and inbred for 13 generations via single seed descent. The resulting inbred homozygous lines were used as the P2 parental plants for the current experiment. Two independent crossing units were used to create lines 1 and 2. For each crossing unit (diagrammed above) a single wild-type and mutant P2 plant were crossed to create F_1 heterozygous offspring. An F_1 plant from each cross was self-fertilized, and the heterozygous offspring were propagated via single seed descent for another three generations. The F_4 heterozygous plants were allowed to self fertilize and their homozygous offspring were reared and placed in the experimental treatments.

approximately 1 February 2001 until 15 March 2001, when most plants had started flowering. The common greenhouse environment consisted of a single greenhouse chamber with ambient temperature ranging between 18° to 28 °C night/day, ambient humidity (70–80%), and daily watering with double-distilled water. Upon flowering, plants were randomly assigned to treatment and position and transferred to treatment chambers. Plants were acclimatized to treatment conditions for 1 week prior to initiating pollinations. All experiments were performed at the Duke University phytotron.

Treatment conditions

The purpose of our experiment was to determine whether fertilization success of *AA* and *aa* plants differed under different heat and light stresses. To address this question, we employed a full-factorial design with the following factors: temperature; light; pollen-parent (male) genotype; seed-parent (female) genotype; cross parent (male or female); and experimental line. This design was implemented by setting up two treatment chambers (one for each temperature treatment) and dividing each of these

into two subchambers (for the low and high light treatment within each temperature treatment). Each subchamber was set for one of four temperature/light combinations: (i) high temperature/high light; (ii) high temperature/low light; (iii) low temperature/high light; and (iv) low temperature/low light. Each subchamber contained six plants of each line \times genotype combination for a total of 24 plants in each treatment combination and 96 plants overall.

Low-temperature chambers were maintained at 27 °C/18 °C (day/night, respectively) for the entire experiment. High-temperature chambers were maintained at 36 °C/24 °C for the first 2 weeks following acclimatization; the temperature was then reduced for the remaining 16 days of the experiment to 32 °C/20 °C to ensure adequate flowering. The temperatures were chosen by monitoring greenhouse temperatures for 2 months, and then comparing these temperatures to outside temperatures National Climate Data Center (NCDC), database to verify that they occurred in nature. The water-vapour pressure was maintained at 75/90% relative humidity (day/night, respectively; high temperature) and 59/86% relative humidity (low temperature), to maintain equal physiological water stress across treatments (D. Tremmel, personal communication). Humidity values were chosen to represent realistic but slightly high humidity, as might be experienced during the flowering months in nature. We chose slightly high values of humidity, and watered the plants to saturation twice daily, to eliminate any confounding effects of drought stress from the experiment.

In all chambers, lighting was provided by 80% halogen and 20% tungsten light (by wattage) to provide a broad-spectrum, sunlight-mimicking, light source from 06.00 h to 20.00 h daily. In the high-light subchambers incident light at median plant height was 1180 $\mu\text{mol}/\text{m}^2/\text{s}$ (light saturation in *Arabidopsis*, another weedy species, occurs around 1000 $\mu\text{mol}/\text{m}^2/\text{s}$; Jansen *et al.* 1996), approximately equivalent to a moderately sunny day in the northern hemisphere. In the low-light subchambers, two layers of greenhouse shade screen were mounted just below the lights to provide broad spectrum shading. Incident light in these subchambers was 800 $\mu\text{mol}/\text{m}^2/\text{s}$, corresponding to a mildly overcast day (D. Tremmel, personal communication).

Pollinations and fertilization success

Pollinations were performed within and between all combinations of experimental line, male and female genotype (AA vs. aa), and treatment type (temperature/light). In general, each plant was used only once, or rarely twice, per treatment combination. Each pollination pair (see below) was scored as a single event (proportion of fruits matured/pollinations). No self-pollinations (within plant) were

performed; however, we did perform crosses between individuals within the same treatment combination (temperature/light/genotype/line). Two individual flowers per plant were emasculated between 17.00 and 20.00 h each evening to prevent self-pollinations. These flowers, which functioned as pollen recipients, were hand pollinated between 06.00 and 08.00 h the following morning using one or two anthers per stigma from a predetermined treatment combination (light, temperature, genotype, line). Occasionally, only one maternal flower was available, in which case we pollinated the available flower. Additionally, not all plants produced flowers on all days, resulting in unequal sample sizes (Table 1). Excess flowers were removed between 06.00 and 08.00 h every morning to control for the latent effects of seed set on future flowering and capsule abortion. These flowers were used immediately as pollen donors in the appropriate treatments. Pollinated flowers were tagged and fruits were collected at maturity, although nearly all ovaries that remained on the plant for more than 2 days following pollination matured fully (personal observation). Successful pollination was scored as number of fruits matured/number of flowers pollinated for each maternal treatment/paternal treatment combination. A total of 1342 pollination pairs were scored overall.

Flower number

As in many plant species, in *I. purpurea* flower number is directly proportional to male outcross success (Devlin *et al.* 1992, R. Miller and M. Rausher, unpublished data). We therefore also examined whether temperature and light stress had adverse effects on flower production and whether a plant's genotype at the *A* locus influenced the magnitude of these effects. The number of flowers on each plant was counted daily, and summed for each maternal plant over the course of the experiment.

Statistics

We analysed the data using the General Linear Models in SAS version 6.12 (SAS Institute Inc. 1996). In analysing fertilization success, we treated all factors as fixed effects and included main effects plus all two- and three-way interactions of temperature, light, genotype, experimental line, and cross parent (Table 2). Genotype was treated as a fixed effect because we were interested in the effect of the specific genotypes, and not in genotype as representative of any of several genotypes. In analysing flower number, we analysed the data in the same manner as above (excluding cross-parent, since flower number is a function of the individual plant; Table 2 and Table 3, respectively). For brevity, only significant effects are reported. For the dependent variable fertilization success, we analysed the untransformed data as well as the arcsine, natural

Table 1 Mean proportion of capsules set, by treatment. Each observation corresponds to the pooled data from a pair of flowers pollinated on each plant on a given day

Genotype		Light		P H T				P L T			
				M H T		M L T		M H T		M L T	
M	P	M	P	<i>n</i>	Mean (SE)	<i>n</i>	Mean (SE)	<i>n</i>	Mean (SE)	<i>n</i>	Mean (SE)
AA	AA	HL	HL	19	0.237 (0.087)	24	0.271 (0.077)	18	0.306 (0.089)	24	0.625 (0.077)
			LL	20	0.350 (0.085)	24	0.396 (0.077)	18	0.250 (0.089)	20	0.550 (0.085)
		LL	HL	15	0.367 (0.098)	16	0.375 (0.095)	14	0.179 (0.101)	15	0.633 (0.098)
			LL	15	0.333 (0.098)	16	0.219 (0.095)	15	0.433 (0.098)	16	0.594 (0.095)
	aa	HL	HL	19	0.211 (0.087)	24	0.229 (0.077)	20	0.225 (0.085)	24	0.708 (0.077)
			LL	23	0.261 (0.079)	24	0.229 (0.077)	18	0.250 (0.089)	23	0.652 (0.079)
		LL	HL	12	0.250 (0.109)	32	0.422 (0.067)	15	0.200 (0.098)	31	0.677 (0.068)
			LL	14	0.321 (0.101)	32	0.422 (0.067)	16	0.281 (0.095)	32	0.719 (0.067)
aa	AA	HL	HL	20	0.225 (0.085)	24	0.333 (0.077)	21	0.238 (0.083)	24	0.604 (0.077)
			LL	18	0.333 (0.089)	24	0.354 (0.077)	18	0.250 (0.089)	22	0.727 (0.081)
		LL	HL	24	0.229 (0.077)	31	0.452 (0.068)	19	0.079 (0.087)	29	0.724 (0.070)
			LL	20	0.350 (0.085)	32	0.234 (0.067)	21	0.214 (0.083)	32	0.766 (0.067)
	aa	HL	HL	20	0.075 (0.085)	24	0.292 (0.077)	20	0.175 (0.085)	24	0.688 (0.077)
			LL	18	0.083 (0.089)	24	0.417 (0.077)	21	0.143 (0.083)	21	0.690 (0.083)
		LL	HL	20	0.275 (0.085)	16	0.375 (0.095)	18	0.083 (0.089)	14	0.786 (0.101)
			LL	23	0.217 (0.079)	16	0.375 (0.095)	20	0.175 (0.085)	16	0.563 (0.095)

In general, each plant was used once, or rarely twice, per treatment combination. Lines are not shown separately in the table, but are pooled to keep the table size manageable. Gtype = genotype at the *A* locus (*AA* = wild-type, *aa* = mutant), HT = high temperature, LT = low temperature, HL = high light, LL = low light, M = maternal plant (pollen recipient), P = paternal plant (pollen donor), *n* = sample size.

Variable	Df	Type III SS	<i>F</i> -value	<i>P</i> > <i>F</i>
M temp.	1	21.965747	155.21	< 0.0001
P temp.	1	6.735097	47.63	< 0.0001
M line	1	0.588327	4.16	0.0416
M genotype × M temp.	1	1.035237	7.32	0.0069
P genotype × M temp.	1	0.685788	4.85	0.0278
M genotype × M line	1	0.667649	4.72	0.0300
M temp. × P temp.	1	11.131646	78.73	< 0.0001
M line × P line	1	0.775271	5.48	0.0194
P temp. × P light × M light	1	0.638049	4.51	0.0338
P temp. × P line × M line	1	0.586762	4.15	0.0418
M light × P light × P line	1	0.741045	5.24	0.0222

Table 2 Effects of temperature and light on fertilization success

Data were analysed using the general linear models in sas version 6.1. All variables were treated as fixed effects. All major effects, plus two- and three-way interactions were included in the analysis. For brevity, only significant results are reported here. *n* = 1342. M = maternal plant (pollen recipient), P = paternal plant (pollen donor).

logarithm, and LOGIT-transformed data. All analyses gave approximately the same results, and, since the residuals were most normally distributed in the untransformed data, we only report those results. For the analysis of flower number, we analysed only the untransformed data since the residuals were normally distributed. Individual

contrasts were performed on biologically relevant comparisons and are shown in the relevant figures (Figs 3, 4 and 5). We used the Dunn–Sidak correction for multiple comparisons on all two-way interactions and the Tukey's *post hoc* correction for comparisons within the three-way interactions (Sokal & Rohlf 1995).

Table 3 Effects of heat and light on total number of flowers

Variable	Df	Type III SS	F-value	P > F
Genotype	1	2762.76	4.33	0.0474
Temperature	1	84431.00	132.35	< 0.0001
Light	1	11463.00	17.97	0.0003
Line	1	5056.51	7.93	0.0091
Genotype × temp.	1	5596.76	8.77	0.0065
Genotype × light	1	4200.26	6.58	0.0164
Genotype × temp. × line	1	8797.51	13.79	0.0010

Data were analysed using the general linear model in SAS version 6.1. All variables were treated as fixed effects. All major effects, plus two- and three-way interactions were included in the analysis. For brevity, only significant results are reported here; $n = 96$.

Results

Effects of temperature on fertilization success

High temperature adversely affected fertilization success in both pollen donors and pollen recipients, as reflected in the highly significant main effects of male and female temperature in Table 2. When acting as pollen recipients, both flower-colour genotypes exhibited marked reduction in fertilization success at high temperatures (Fig. 3a). When acting as pollen donors, by contrast, the pattern was more complex. While pollen from plants grown at high temperatures had significantly lower fertilization success than pollen from plants grown at low temperatures, this effect depended on the temperature at which the recipient plant was grown (Fig. 3c). In particular, there was an adverse effect of high temperature on pollen when the pollen donor was grown at high temperature and the pollen recipient was grown at low temperature. However, pollen from plants grown in high and low temperatures did equally poorly when the recipient plant was grown at high temperatures [maternal (M)-temperature × paternal (P)-temperature effect, Table 2], suggesting a post-pollination as well as pre-pollination effect of heat stress on pollen. These effects of male temperature were similar for both flower-colour genotypes, as suggested by the lack of significant three-way interaction involving male temperature and either male or female genotype (Table 2). These patterns indicate that, in general, high temperature acts as a stress that reduces fertilization success for both pollen donors and pollen recipients.

Genotype at the *A* locus influenced the magnitude of this stress effect. For pollen recipients, both genotypes had similar fertilization success when grown at low temperature, but the white-flowered genotype had an approximately 26% lower fertilization success than the pigmented genotype at high temperature (Fig. 3a; M-genotype × M-temperature effect in Table 2). Similarly, when pollen recipients were

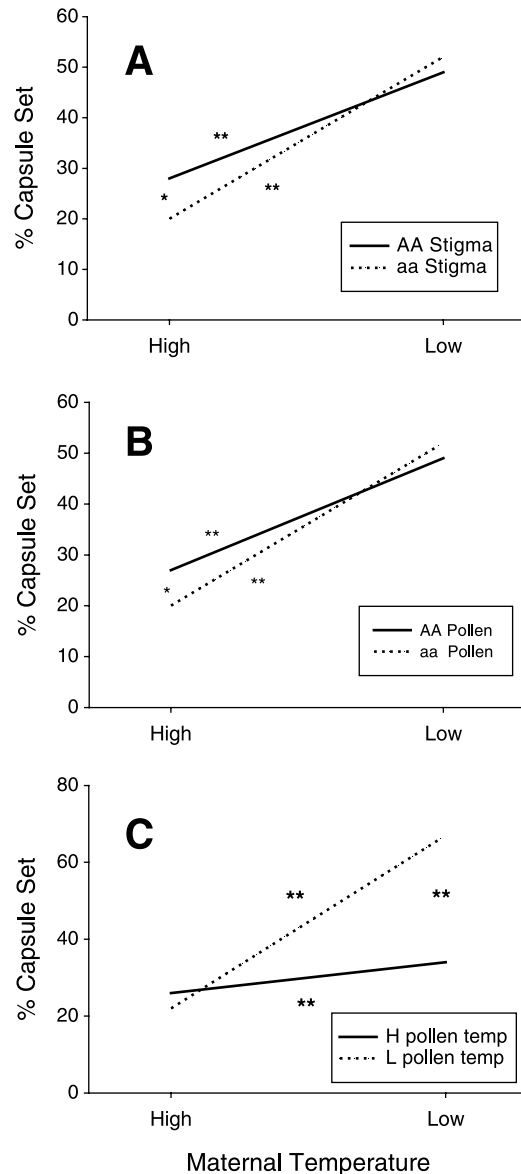


Fig. 3 The effects of genotype and temperature on the proportion of capsules successfully fertilized. Asterisks beside line indicate significant effect of treatment (e.g. maternal temperature). Asterisks between lines indicate significant effect of genotype. * $P < 0.05$, ** $P < 0.01$. (A) Both maternal genotypes set fewer seeds in the high-temperature than in the low-temperature environment; and, *aa* maternal plants set fewer capsules than *AA* maternal plants in the high-temperature environments. (B) Both pollen genotypes perform worse on stigmas in high temperatures than on stigmas in the low temperatures; and, *aa* pollen fertilizes fewer capsules than *AA* pollen when placed on stigmas in high temperatures. (C) Pollen from plants raised at either temperature has reduced fertilization success when placed on stigmas in the high-temperature environment. At low maternal (stigma) temperature, pollen from plants grown at high temperature has reduced fertilization success compared to pollen from plants grown at low temperature.

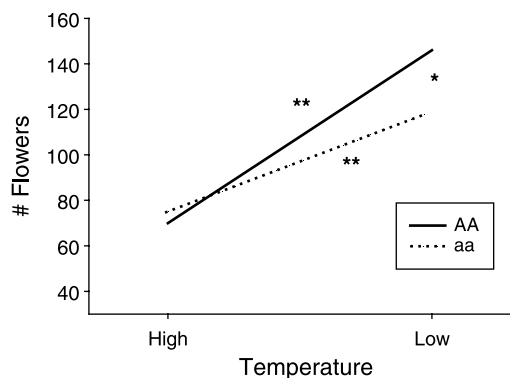


Fig. 4 The effects of temperature on flower number. Both genotypes have fewer flowers in high-temperature treatments than in low-temperature treatments. Mutant (*aa*) plants have fewer flowers than wild-type (*AA*) plants in low-temperature treatments. Asterisks are as in Fig. 3.

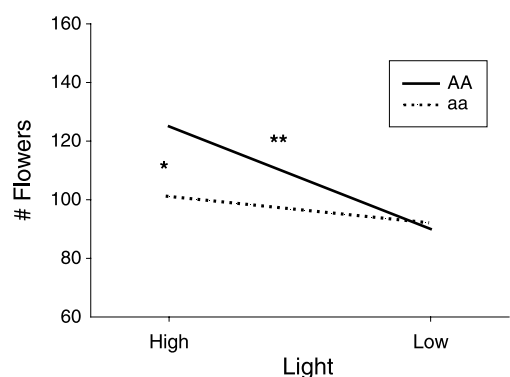


Fig. 5 The effects of light on flower number. Mutant (*aa*) plants have fewer flowers than wild-type (*AA*) plants in high light treatments. Wild-type (*AA*) plants have more flowers in the high-light treatments than they do in the low-light treatments. Asterisks are as in Fig. 3.

grown at high temperature, pollen from white-flowered plants had an average 24% reduction in fertilization success compared to pollen from pigmented plants (Fig. 3b; P-genotype \times M-temperature effect in Table 2). By contrast, when recipients were grown at low temperatures, the flower-colour genotype of the pollen donor had no effect on fertilization success. The lack of a significant three-way interaction involving male genotype and male temperature indicates that these patterns are similar for pollen donors grown at both low and high temperatures. Additionally, the effects of male genotype are only evident when the pollen recipient is in the high-temperature environment, suggesting that flavonoids are important in post-pollination, but not necessarily pre-pollination, aspects of pollen development. Overall, these results indicate that for both pollen donors and pollen recipients, heat stress had a greater adverse effect on fertilization success in the white-flowered (*aa*) than in the pigmented (*AA*) genotype.

Effects of temperature on flower number

Flower production was substantially reduced at high temperatures (Fig. 4, Table 3). The two flower-colour genotypes differed in the magnitude of this response, though in an unexpected way: at high temperatures, the two genotypes produced similar numbers of flowers, while at low temperatures, the white-flowered plants produced an average of 15% fewer flowers than the pigmented plants (Fig. 4; Genotype \times Temperature effect in Table 3).

Effects of light

Light intensity had little discernible overall effect on fertilization success (absence of significant P-light and M-light main effects, Table 2), with no effect on either pollen donor or pollen recipient. Moreover, flower-colour genotypes were similar in their response to light intensity (absence of genotype main effects and interactions involving light and genotype, Table 2), despite clear induction of anthocyanins in leaves of the wild-type plants in high light, but not low light, treatments (personal observation).

By contrast, light treatment did affect flower number. Under low-light conditions, the two flower-colour genotypes produced similar numbers of flowers. Under high-light conditions, however, white-flowered plants produced 20% fewer flowers than pigmented plants (Fig. 5a; Genotype \times Light effect, Table 3).

Discussion

A new function for flavonoids

Flavonoids are a diverse group of compounds that are produced by a branching biochemical pathway that begins with the enzyme chalcone synthase (Fig. 1). They have been demonstrated to perform a variety of functions in plants: attracting pollinators, conferring resistance to natural enemies, facilitating interaction with symbionts, protection from ultraviolet light, regulating hormones, mediation of pollen-stigma interactions (Shirley 1996), and ameliorating drought stress (Warren & MacKenzie 2001). Our results point to an additional function of flavonoids that has not previously been reported: amelioration of adverse effects of heat stress on fertilization and early seed maturation.

In general, there are few data available on the effects of flavonoids in the response to heat stress, although flavonoids are known to be induced by high light, cold stress, and heavy metals (Koes *et al.* 1994; Marrs & Walbot 1997). Flavonoid gene expression, on the other hand, may actually be inhibited in response to high temperatures (e.g. Oren-Shamir & Levi-Nissim 1997). Atanassova *et al.* (2001) show that flavonoid mutants have a reduced time to

germination under normal- and high-temperature environments, although the effective difference in response times between wild-type and mutants is not clear, nor is it clear how this response might affect plant fitness.

Although the most striking effect of the *a* allele in *Ipomoea purpurea* homozygotes is to produce flowers that lack pigmentation, it is likely that many tissues of *aa* plants produce no flavonoids. The enzyme chalcone synthase is represented by a multigene family in *I. purpurea* with as many as five different loci (Durbin *et al.* 1995). Nevertheless, CHS-D, which corresponds to the *A* locus, is the predominant copy expressed in foliage, the floral corolla, pistils and anthers (Durbin *et al.* 2000). Individuals that are *aa* contain nonfunctional copies of CHS-D and produce few flavonoids in the floral corolla (Coberly, Morita, and Saito, unpublished data). Neither are flavonoids expressed in the rest of the plant, based upon comparisons of pigment patterns between wild-type and mutant plants.

Two general possibilities exist to explain our results: (i) heat stress decreases overall plant function and flavonoids ameliorate this stress (for example, by scavenging free radicals, Torel *et al.* 1986), or (ii) heat stress inhibits one or more processes directly involved in fertilization and/or ovule maturation, and flavonoids ameliorate this stress. Our observations show a reduction in both female and male fertilization success. A reduction in female fertilization success is consistent with either of the above hypotheses (for example, stressed plants may abort ovules, or ovules lacking flavonoids may fail to develop properly). However, it is less easy to explain the reduction in male success through generalized plant stress, particularly since the reduction in male success occurs at high temperatures even when the pollen was obtained from plants grown at low temperatures (Fig. 3C). Thus, the reduced fertilization success of *aa* plants at high temperature revealed by our study seems likely to be the result of an absence of some sort of flavonoid in one or more floral tissues, although it remains possible (even probable) that flavonoids ameliorate stress in other plant tissues.

Since we only used one chamber per treatment, we cannot rule out the possibility that our results simply reflect chamber differences. We consider this to be unlikely for the following reasons. First, about 90% of *I. purpurea* flowers grown under greenhouse conditions set seed, unless severely stressed (as planned in our experimental design). It seems unlikely that the types of unintentional environmental variation that occur between chambers or greenhouse rooms could have produced the magnitude of effects seen in our experiment. Second, stresses such as disease, infestation, or drought could potentially have caused such dramatic differences between chambers. However, we saw no evidence of infestation or disease throughout the course of the experiment. In addition, we controlled for water availability, air flow and carbon dioxide concentra-

tion across the chambers. Indeed, plants within the experiment were generally healthy (large glossy green leaves, continued growth, and little to no leaf drop). Third, as mentioned above regarding general plant stress, pollen transferred between chambers often exhibited different effects from pollen transferred within chamber (e.g. *aa* pollen from plants grown in the low temperature and transferred to plants in the high-temperature environment, Fig. 4). Thus, any chamber effects observed in these cases must be effective post-pollination, which seems unlikely if the hypothesized chamber effects are the result of generalized plant stress such as disease or infestation. Finally, the effects that we observe here are consistent with our original observation and hypotheses. It seems unlikely that any unintended chamber effect would differentially affect mutant and wild-type plants in a way so similar to our original hypotheses. In sum, it seems unlikely to us that any unintended (and unobserved) chamber differences would affect our plants to the degree observed, and so consistently coincidental with the treatment effects.

In maternal tissues, auxins are thought to function in early embryogenesis (Muday & DeLong 2001) and flavonoids have been shown to inhibit auxin transport (Jacobs & Rubery 1988; Brown *et al.* 2001). Flavonoids are thought to interact with auxin via low-affinity binding proteins (Muday & DeLong 2001), which are interactions likely to be disrupted by thermal energy. The flavonoids could conceivably function either by stabilizing a weak interaction with these proteins, or by functioning in a heat-sensitive signalling reaction important in early embryogenesis.

While flavonoid regulation of auxin may be a promising mechanism to explain its involvement in maternal fertilization success, it does not explain the effect of flavonoids on pollen success, since auxins have not been found to act in pollen germination or tube growth (Taylor 1995; Cheung 1996). However, the processes of pollen germination and tube growth are similar to the basic functions that auxin regulates — that of directed cell growth. It seems possible that one or more flavonoids may be involved in the general mechanisms of cell growth and/or cell polarity, and may help to stabilize these functions in high-temperature environments. Flavonoids may act in vesicle transport as well as regulating membrane transport proteins (Murphy 2002) — functions that are likely to be important in both auxin-mediated cell growth and pollen tube germination and growth. In any case, further examination of the mechanism of flavonoid action in fertilization seems warranted.

In maize and *Petunia*, flavonoid production has been shown, using CHS mutants, to be crucial to successful fertilization. In both species, however, the effects of flavonoids differ from those seen in *I. purpurea* in two important ways (Coe *et al.* 1981). First, in *Petunia* and maize, only when the pollen parent lacks flavonoids is fertilization success reduced. Wild-type pollen exhibits complete fertilization

success on CHS-deficient stigmas (Taylor & Jorgensen 1992; but see Ylstra *et al.* 1994 for contrasting results). By contrast, at high temperatures, not only does pollen from CHS-deficient (*aa*) *I. purpurea* plants exhibit reduced fertilization success, but CHS-deficient pollen recipients also exhibit reduced fertilization success, regardless of the genotype of the pollen parent. Thus, whereas in maize and *Petunia* flavonoids are required only in pollen, in *I. purpurea* they are required in both pollen and pistils for complete pollination success.

Ipomoea purpurea differs from maize and *Petunia* in a second way; in maize and *Petunia* pollen, flavonoids are required at low temperatures (and presumably at high temperatures), whereas in *I. purpurea*, flavonoids are required in fertilization only at high temperatures. One evolutionary interpretation of this difference is that the involvement of flavonoids in reproduction evolved early in angiosperm history as a mechanism to alleviate the adverse effects of heat stress on fertilization, and that in maize and *Petunia* dependence on flavonoids at low temperatures evolved secondarily. Consistent with this interpretation is the fact that in other plants that have been examined (i.e. *Matthiola*, *Dianthus*, *Gerbera*, *Antirrhinum* and *Arabidopsis*), CHS mutants are completely fertile under nonstressful growth temperatures (Taylor & Jorgensen 1992). Unfortunately, it is not known for any of these species whether flavonoids are beneficial at high temperatures, as this evolutionary hypothesis predicts.

Evolutionary dynamics of the *A* locus

In natural populations of *I. purpurea* in the southeastern USA, the frequency of the *a* allele rarely exceeds 0.005. By contrast, the frequency of the *w* allele, which as a homozygote produces flowers with a similar white phenotype, is typically much higher. The detrimental pleiotropic effects associated with the *aa* genotype may account, at least in part, for this difference in white-allele frequency between the two loci.

Current evidence suggests that at both loci, white-flowered individuals, when rare, are visited less by pollinators and have a higher selfing rate than plants with pigmented flowers (Rausher *et al.* 1993). The greater selfing rate of whites, coupled with lack of pollen discounting, is expected to cause the frequency of either white allele to increase when rare. Inbreeding depression in *I. purpurea*, at approximately 25% (Chang & Rausher 1999), is insufficient to prevent this increase.

Convergence experiments involving experimental manipulation of *w*-allele frequencies confirm that the frequency of this allele does increase when rare (Subramaniam & Rausher 2000), indicating that possible detrimental pleiotropic effects associated with this allele are insufficient to counteract the advantageous effects of increased selfing.

Consistent with this result is the failure to detect deleterious pleiotropic effects associated with the *w* allele (Rausher & Fry 1993; Fry & Rausher 1997; Mojonner & Rausher 1997).

By contrast, the failure of the *a* allele to increase when rare, despite ample time for such increase to have occurred, implies that pleiotropic effects of this allele are counteracting the advantageous effects of increased selfing. Two such effects have been identified in our study: a decrease in fertilization success at high temperatures, and a decrease in flower production at low temperatures. Although the following calculations must be viewed only as approximations, it is possible to ask whether the magnitude of these effects might be sufficient to overcome the selfing advantage of the *a* allele.

To do so, we first determine the relative frequency of high- and low-temperature days experienced by *I. purpurea*. Temperature records for the Durham, NC weather station (station #312515) indicate that, on average, 19% of the days during the flowering season have temperature maxima greater than 32 °C (NCDC 2002 database, from 1919 until present, all years for which data are available). For this calculation, we assumed that flower production was constant throughout the flowering season, which runs from approximately July 1 until October 1 (personal observation). This estimate of heat effect on flower production is probably low because, while we assume constant flower production, flowering actually peaks during July and August, the two hottest months of the year.

As a first approximation, and based on the results of this study, we assume that on days on when the temperature exceeds 32 °C, *aa* individuals experience reduced fertilization success, but no reduction in flower production, whereas on days on which the temperature does not exceed 32 °C, they experience no reduction in fertilization success, but a reduction in flower production. When the *a* allele is rare, the ratio of the fitness of an *aa* individual to an *AA* individual can then be represented by

$$W_{aa}/W_{AA} = \{[T_{aa}(1 - \gamma)] + [2(1 - T_{aa})(1 - 2\gamma)\delta] + [T_{AA}(1 - \beta)(1 - \gamma)]\} / \{2[T_{AA} + \delta(1 - T_{AA})]\} \quad (1)$$

where T_i is the outcrossing rate of genotype *i*, γ is the average proportional reduction in fertilization success experienced by *aa* individuals compared to *AA* individuals, β is the average reduction in flower production experienced by *aa* individuals, and δ is 1 - proportional inbreeding depression (see Appendix for derivation of this equation).

Field estimates of inbreeding depression for *I. purpurea* are approximately 0.25 (Chang & Rausher 1999), yielding $\delta = 0.75$. Field estimates of T for the *aa* genotype (C. Fehr, unpublished data) are consistent with those reported for *ww* (Fry & Rausher 1997), but are less extensive. We therefore use the value of 0.31 from Fry & Rausher (1997) for

white-flowered individuals when they are rare. Estimates of T for pigmented (AA) individuals from four different studies (Rausher *et al.* 1993) range between 0.70 and 0.85, with a mean of 0.78, which we use here.

To calculate γ , we recognize that aa individuals experience a reduction in fertilization success of about 25% only on high-temperature days. On low-temperature days there is no reduction. Since high-temperature days are estimated to occur approximately 19% of the time during the flowering season, the average reduction in fertilization success is $\gamma = (0.19)(0.25) + (1 - 0.19)(0) = 0.0475$. Similarly, given a 15% reduction in flower number on low-temperature days, $\beta = (0.19)(0) + (1 - 0.19)(0.15) = 0.12$.

Substituting these values into equation (1) yields $W_{aa}/W_{AA} = 0.99$. The fact that this ratio is less than 1, suggesting that the a allele is at an overall disadvantage, should not be taken too seriously given the crude nature of these estimates. Nevertheless, it seems that in combination with inbreeding depression, the detrimental pleiotropic effects identified in this study appear to be of the order of magnitude needed to offset the selfing advantage enjoyed by the a allele. Moreover, with only the selfing advantage operating (i.e. $\delta = \gamma = \beta = 0$), $W_{aa}/W_{AA} = 1.22$. By contrast, with no inbreeding, but the detrimental effects of the a allele documented here (i.e. $\delta = 0$, $\gamma = 0.0475$, $\beta = 0.12$), $W_{aa}/W_{AA} = 1.10$, which means that the fitness reduction as a result of the pleiotropic effects is approximately 0.12 overall. Conversely, with just inbreeding depression (i.e. $\delta = 75$, $\gamma = 0$, $\beta = 0$), $W_{aa}/W_{AA} = 1.11$, which means that the fitness reduction because of inbreeding depression is approximately 0.11. Thus, it appears that the combined contribution of the detrimental pleiotropic effects documented here is approximately as great as the effect of inbreeding depression, and that taken together, these effects are sufficient to offset the selfing advantage of white flowers. These estimates also suggest that other, unexamined detrimental pleiotropic effects of the a allele, in combination with the effects discussed here, would probably be sufficient to render white-flowered individuals at an overall selective disadvantage when rare.

One question raised by this analysis is why the w allele does not appear to suffer from the same types of detrimental pleiotropy as the a allele. Both alleles are known to eliminate flavonoid production when homozygous. We suggest that this difference lies in the nature of the two loci. As described previously, the A locus encodes the primary CHS enzyme required for flavonoid production throughout the plant. Inactivation of this locus therefore affects flavonoid production not only in the floral corolla, but also in the pistils, anthers and vegetative tissue, and is thus likely to produce widespread pleiotropic effects. By contrast, the W locus appears to encode a transcriptional activator of anthocyanin/flavonoid structural genes (Rausher *et al.* 1999). Similar transcriptional activators in other plants

often have tissue-specific expression, and may only be expressed in the corolla (Ludwig *et al.* 1990; Quattrocchio *et al.* 1999). The observation that ww individuals produce anthocyanins in vegetative tissue and parts of the corolla provides circumstantial evidence supporting a limited expression pattern for the w allele. If this pattern is true then the effects of inactivation may be limited to the loss of pigmentation in the corolla, and there may thus be few deleterious pleiotropic effects of the w allele.

It is of course not certain that the pleiotropic effects of the a allele that we report here would also be manifested under field conditions, nor that their magnitudes would be the same. Nevertheless, our results suggest that it would be profitable to assess these pleiotropic effects under natural conditions in the field, and that pleiotropic effects are likely to be important for understanding the evolution of genes that influence flower colour.

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Caitlin Coberly is interested in the genetic basis of evolutionary change. She is currently studying the pleiotropic effects of mutations in structural genes. She is integrating molecular, field, and controlled experimental studies to understand the current and historical nature of selection on the CHSD mutant in *Ipomoea purpurea*. Dr Mark Rausher is a professor at Duke University where he and his students are examining evolutionary mechanisms involved in plant defence, co-evolution, floral evolution and the evolution of enzyme pathways.

Appendix

In this appendix we derive equation (1), the ratio of the fitness of *aa* individuals to *AA* individuals (see text for definition of symbols). This equation pertains to a situation in which *a* is at low frequency, such that *aa* individuals are rare and most individuals are *AA*. We calculate fitness as the number of copies of an allele (*a* or *A*) an individual transmits to the next generation. Consider first the fitness of *AA* individuals. Let n be the number of seeds produced, on average, by an individual. Because a fraction T_{AA} of ovules produced by an *AA* are outcrossed, on average that individual transmits nT_{AA} copies of an allele to the next generation through outcrossed ovules. The remaining fraction of ovules $(1 - T_{AA})$, are fertilized by selfing. Since each selfed ovule transmits two copies of an allele, the total transmitted by an individual through selfing to fertilized embryos is $2n(1 - T_{AA})$. Because of inbreeding depression, however, only $2n(1 - T_{AA})\delta$ copies are effectively transmitted past the embryo stage. Finally, because *aa* and *Aa* individuals constitute a very small proportion of the population, virtually all outcross pollen produced by *AA* individuals will fertilize *AA* stigmas. This means, essentially, that *AA* individuals compete among themselves for all outcrossed ovules, which means that each individual transmits T_{AA} copies of an allele through outcross pollen. The total number of copies transmitted by an *AA* individual is thus

$$W_{AA} = nT_{AA} + 2n(1 - T_{AA})\delta + nT_{AA}$$

$$\text{i.e. } W_{AA} = 2n[T_{AA} + \delta(1 - T_{AA})] \quad (\text{A1})$$

Next consider the fitness of an *aa* individual. Such an individual produces nT_{aa} ovules that will be outcrossed. However, because a fraction γ of these outcross events will be unsuccessful fertilizations, the total number of copies of an allele transmitted through ovules that are selfed is

$n(1 - \gamma)T_{aa}$. An individual also produces $n(1 - T_{aa})$ ovules that will be selfed, each of which represents $2n(1 - T_{aa})$ copies of an allele. However, some of these fertilizations will also fail. The fraction in this case is 2γ because γ fail because of *aa* pollen being placed on high-temperature stigmas, and another γ fail because of the stigmas having matured at high temperatures (Paternal-Temperature and Maternal-Temperature effects are approximately equal and do not depart significantly from additivity; see Table 2). Of the successful self-fertilizations, only a fraction δ will survive because of inbreeding depression. Consequently, the total number of copies of an allele transmitted by *aa* individuals through selfing is $2n(1 - T_{aa})(1 - 2\gamma)\delta$. Finally, because there is no pollen discounting, each *aa* flower will compete equally with each *AA* flower to fertilize outcrossed ovules, essentially all of which are *AA*. If *aa* plants produced as many flowers as *AA* plants, the per capita number of pollinations through the outcross pollen pool would be the same for the two genotypes, i.e. nT_{AA} . However, *aa* plants produce only a fraction $(1 - \beta)$ as many flowers as *AA* plants, so that the number of successful pollinations through the outcross pollen pool is $n(1 - T_{AA})(1 - \beta)$ (Outcross pollination success in *I. purpurea* is proportional to flower number; Miller and Rausher, unpublished data.). Finally, because of heat stress, only a fraction $(1 - \gamma)$ of outcrossing events involving *aa* pollen will result in successful fertilization. The number of allele copies transmitted in this way is thus $nT_{AA}(1 - \beta)(1 - \gamma)$. The total number of allele copies transmitted by an *aa* individual is just the sum of these three components, or

$$W_{aa} = n(1 - \gamma)T_{aa} + 2n(1 - T_{aa})(1 - 2\gamma)\delta + nT_{AA}(1 - \beta)(1 - \gamma)$$

$$\text{i.e. } W_{aa} = n[(1 - \gamma)T_{aa} + 2(1 - T_{aa})(1 - 2\gamma)\delta + T_{AA}(1 - \beta)(1 - \gamma)] \quad (\text{A2})$$

Dividing equation (A2) by equation (A1) then yields equation (1).