Floral Evolution: Attractiveness to Pollinators Increases Male Fitness

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Because availability of resources often limits seed or fruit set, increased visits by pollinators may not always lead to increases in maternal reproduction. This observation has led evolutionary biologists to hypothesize that a plant's ability to attract pollinators may have its primary impact on male fitness achieved through the fertilization of ovules. This interpretation of angiosperm reproductive ecology is supported by field experiments. Pollinating insects strongly discriminated between two Mendelian petal-color morphs in *Raphanus raphanistrum*, a widespread, self-incompatible crucifer. In experimental populations composed of petal-color homozygotes, color discrimination by naturally occurring pollinators had no statistically significant effect on relative maternal function (fruit and seed production) in the two morphs. In contrast, yellow-flowered individuals were far more successful as fathers (pollen donors) than were the less visited whites. These results suggest that the evolution of floral signals such as petal color may be driven primarily by selection on male function.

INTERACTION BETWEEN PLANTS AND their animal pollinators is thought to have been a driving force in the evolution of the angiosperm flower (*1, 2*). In hermaphroditic species, natural selection favoring floral variants that are more attractive to pollen carriers should operate on both maternal (seed-bearing) and paternal (seed-fertilizing) functions. Individuals with an especially attractive floral display can be expected to receive more pollen from other plants as well as to donate more pollen to other plants through the foraging movements of pollinators. Although the potential importance of both processes is widely understood (*3*), our current knowledge of pollination biology is limited almost entirely to the seed-bearing success achieved through maternal function (*4*).

Resource limitation often constrains an individual's fruit production, and it is not uncommon for only a fraction of a plant's ovules to mature as seeds (*5*). In such cases, enhanced pollination may not increase seed set (*4, 6*). Some studies have shown that enhanced pollination increases short-term fruit production (*4, 7*), but lifetime expenditure on fruits in many of these plants may still be limited by resources (*5, 8*).

Resource limitation may uncouple pollinator visitation rate and seed production. By examining only female reproductive function, pollination biologists might assume that natural selection acts only weakly on floral attractiveness. An accurate picture of plant-pollinator interactions, however, must also include the impact of pollinator behavior on male fitness. Controlled pollination studies with both cultivated and wild species make it clear that an individual's seed-bearing achievement may differ markedly from its potential performance as a pollen donor (*9, 10*). Furthermore, although average male and female fitness must be equal within a sexually reproducing plant population, variation in male reproductive success may exceed that in females. First, successful fruit maturation is costly, and may be limited more by resources than by pollen over the long term. Second, most outcrossing species produce many more pollen grains than ovules, so potential variation for male reproductive success is greater than that for female success (*11*). We thus suspect that the evolution of floral signals may be driven primarily by the process of pollen donation rather than by successful maturation of seeds.

To test this hypothesis, we used genetic markers to compare male and female performance of naturally pollinated floral variants in wild radish (*Raphanus raphanistrum* L.), a self-incompatible, indeterminate annual that is abundant on disturbed, fertile soils on six continents (*12*). In many populations worldwide, wild radish shows a dramatic petal-color polymorphism; white- and yellow-flowered individuals often coexist at relatively high frequencies (*13*). Petal color is controlled at a single locus, with white dominant to yellow (*13*). In our primary study site in Hamden, Connecticut, 80 to 90 percent of the population is yellow.

Raphanus raphanistrum is visited by a variety of generalized pollinators, including honey bees, pierid butterflies, bumble bees, syrphid flies, and solitary bees. Previous studies have shown that, although most of these insects prefer yellow flowers, *Pieris* butterflies and honey bees discriminate strongly in favor of yellow (*13*).

To test the effect of such discrimination on reproductive success in the two petal-color morphs, we established a series of synthetic wild radish populations in New Haven, Connecticut. Plants were grown from seeds that were homozygous for flower color in an insect-free greenhouse, then used in a series of field experiments (*14*). Each population contained 16 potted plants (8 white homozygotes and 8 yellow homozygotes). The eight homozygotes of a given color class were taken from two unrelated lines to minimize incompatibility among related individuals within the arrays. After being trimmed to a constant number of flowers and having all remaining flowers marked, young plants were placed outdoors for 6 hours during the peak of pollinator activity. No other *Raphanus* were growing near the study area, which was an early successional plot at Yale University's Marsh Botanical Gardens. Insect visits to plants of each color were monitored for 1.5 to 2 hours per replicate. After 6 hours of natural pollination, experimental plants were returned to the greenhouse for 2 days, then placed outdoors in another configuration for a second replicate.

Maternal reproductive output for all individuals was determined by counting the fruits and seeds produced by marked flowers on each plant. Relative success of yellow- and white-flowered plants as fathers was determined by rearing the progeny of all yellow-flowered individuals (*15*). Since yellows are recessive homozygotes, seeds fathered by white homozygote fathers give rise to white-flowered plants, while seeds fathered by yellow fathers give rise to yellow-flowered plants. In all, plants used in the experiment produced almost 2000 seeds in 533 fruits. Yellow-flowered mothers produced 863 seeds, of which 621 were successfully reared for paternity analysis.

To examine the potential effect of uneven pollinator visitation to yellow- and white-flowered individuals, we generated expected levels of reproduction on the basis of a null hypothesis of random pollen movement. The impact of genetic compatibility variation within and between homozygote lines on fruit set was determined by a series of 2415 hand pollinations performed in the greenhouse (*16*). The percentage of fruit set from greenhouse pollinations was used to calculate null expectations for maternal and paternal reproduction in each experiment. In no case did expected performance of whites and yellows deviate more than 4 percent from a simple 50:50 ratio. For ease of interpretation, we assumed that yellows and whites will be equally successful if polli-

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nation is random with respect to flower color (*17*).

Pollinator visitation to plants in the study was, as expected, heavily biased toward yellow-flowered individuals (Fig. 1A). Cabbage butterflies (*Pieris rapae*), the most frequent visitors, showed a strong yellow preference, making 75.6 percent of their visits to yellow-flowered individuals (n = 870 visits, G = 239.884, df = 1, $P < 0.0001$). Overall, pollinators made 74.6 percent of their visits to yellow plants (n = 919 visits, G = 222.70, df = 1, $P < 0.0001$). Insect visitors showed significant discrimination against white in all three experimental populations.

Despite strong pollinator discrimination between the two color morphs, white- and yellow-flowered plants were equally successful as mothers (Fig. 1B). Summed over the six replicates, yellow-flowered plants produced 262 fruits (68 percent set), while white-flowered plants produced 271 fruits (70 percent set). For each of the three experimental populations, the relative fruit production of yellows and whites closely approximated the 50:50 expectation (G = 0.377, 0.217, and 0.237; df = 1; $P > 0.5$ in all cases). Total seed production of the two morphs was also similar, yellows producing 863 seeds (3.3 seeds per fruit) and whites producing 876 seeds (3.2 seeds per fruit). If yellows and whites produce seeds of equivalent quality, strong pollinator discrimination against white-flowered plants would have little selective impact on female fitness.

In striking contrast to maternal reproduction, male performance of yellows and whites was strongly correlated with pollinator discrimination. Summed over all experiments, 72.3 percent of the 621 progeny from yellow-flowered recipients were fathered by yellow plants. This observed paternity pattern closely resembles that of pollinator visitation, where 74.6 percent of all visits were made to yellow. In each of the three experimental populations, yellows fathered significantly more than 50 percent of the seeds (G = 48.048, 56.652, and 24.884; df = 1; $P < 0.0001$ in all cases) (Fig. 1C). Over all replicates, the frequency of seed paternity by yellow plants was statistically indistinguishable from the frequency of pollinator visitation to yellow (72.3 percent versus 74.6 percent, G = 1.697, df = 1, $P > 0.1$).

Our results from experimental populations show that pollinator discrimination among floral morphs within a plant population can have profound effects on relative paternal success, even when maternal reproduction is limited by resources (*18*). Additional evidence for this assertion comes from a few previous studies. Pink floral mutants of *Lupinus* (*10*) and *Ipomoea* (*19*) are discriminated against by pollinators and may have lower outcrossing rates than the more abundant blue-flowered individuals. However, paternity success of *Ipomoea* color morphs is apparently dictated by gametic competition rather than by pollinator discrimination (*20*). In milkweeds, larger, more attractive inflorescences are thought to achieve greater success as males (*21*), but the selective impact of variation in inflorescence size remains unclear since the genetic basis for the trait is unknown and genetic markers have not been used to document paternity of seeds.

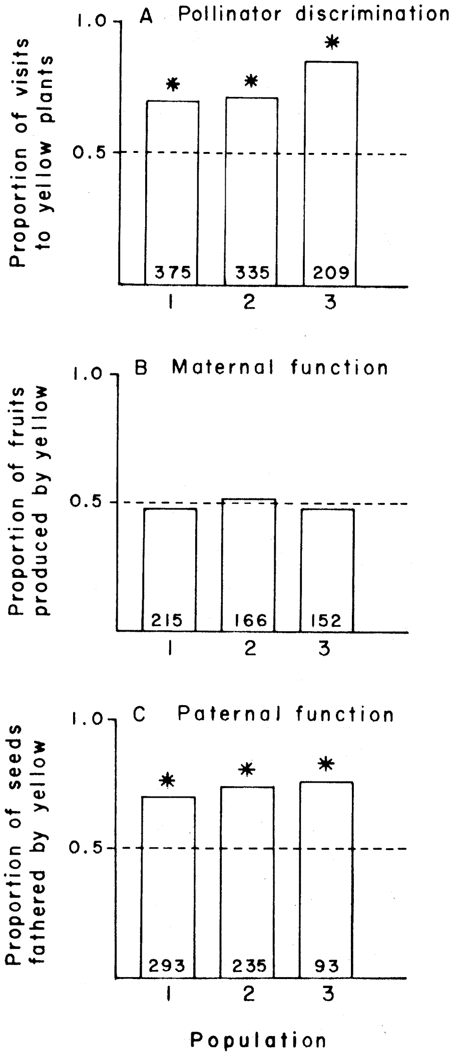

Fig. 1. Relative performance of yellow- and white-flowered homozygotes in three experimental populations. Each population of 16 plants was used in two replicates, which were pooled for this representation. Sample sizes are shown within each bar. For all three populations, yellows were significantly overvisited relative to whites (*, $P < 0.0001$) and fathered significantly more of the seeds produced by yellow (recessive homozygote) mothers (*, $P < 0.0001$), but yellows and whites contributed equal numbers of fruits to total population production.

Our data support the idea that hermaphroditic flowers have evolved primarily as pollen-donating rather than pollen-receiving organs (*22*). Results with *R. raphanistrum* indicate that relative male fitness achieved by genetic floral variants is directly related to the degree of pollinator discrimination between them. In addition, increased visitation may have subtle effects on female reproductive success, even when resources limit the number of seeds a plant can produce. When competition among pollen tubes influences seed quality, maternal plants that attract more pollen-bearing vectors may produce superior offspring (*2*). This effect would complement the paternal advantage of increased floral attractiveness documented here.

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14. Homozygote strains for each petal color variant were developed through several generations of controlled crossing at the University of California, Davis.
15. Seeds produced by white-flowered plants were also collected. Second-generation progeny from test crosses to recessive homozygotes are now being raised for a full analysis of paternity and mating for this study.
16. Not all possible crosses among homozygote lines were equally fertile. Such fertility differences might influence reproduction of yellow- and white-flowered individuals even if pollen movement were random with respect to color. To account for this, hand pollinations were made between and within all lines, and fruit set from each cross was used to calculate expected reproductive outputs for yellows and whites as pollen donors. Details of the calculations will be presented elsewhere (M. L. Stanton, A. A. Snow, S. N. Handel, in preparation). Plants used in this analysis were not the same individuals used in the three experimental populations.
17. While individual donors may produce pollen of differing competitive ability, pollen grains bearing the yellow- and white-petal alleles show no consist-

ent differences in fertilization ability in mixed pollination experiments. Paternity differences seen in this study are probably attributable to differential pollen transfer by insects rather than to gametic competition (S. Mazer, R. Burke, M. Stanton, unpublished data).

18. The patterns of fruit set and pollinator discrimination seen here resemble those observed in naturally occurring *R. raphanistrum* populations. At our primary study site in Hamden, Connecticut, insect visitors in both 1981 and 1983 made 64 to 65 percent of their visits to yellow flowers in color preference tests. In 1983, fruit set by yellows and whites (58.2 versus 55.1 percent) did not differ significantly and could not be increased by additional hand-pollination. In contrast, yellow-flowered plants did have significantly greater fruit set than white-flowered plants in 1981. Apparently, fruit set was limited by pollen in 1981, but not in 1983. These data suggest that pollinator discrimination can influence both maternal and paternal reproductive success, but only when pollen deposition is a limiting factor.
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Acetylcholine Receptor Synthesis in Retina and Transport to Optic Tectum in Goldfish

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Previous studies have suggested that the retinotectal system of the goldfish contains a nicotinic acetylcholine receptor (nAChR) that is sensitive to α-bungarotoxin. Extracellularly recorded field potentials elicited in response to visual stimulation can be blocked by α-bungarotoxin, and α-bungarotoxin can interfere with the maintenance of retinotectal synaptic connections. Whether the transmission between the retinal ganglion cells and the tectal cells is mediated by acetylcholine and whether nAChR's exist on the dendrites of tectal cells are questions that remain. The experiments described in this report were designed to determine the site of synthesis of the nAChR's associated with the goldfish retinotectal projection. Radioactive (^{35}S-labeled) methionine was injected into either the eye or the tectal ventricle, and the incorporation of radioactivity into the nAChR was measured by immunoprecipitation. The use of this technique provides evidence that an nAChR associated with the goldfish retinotectal projection is synthesized in the retina and transported to the optic tectum, which suggests a presynaptic site of acetylcholine action on retinal terminals.

A subset of monoclonal antibodies to nicotinic acetylcholine receptors (nAChR's) purified from the electroplaques of the electric ray *Torpedo californica* and the eel *Electrophorus electricus* (*1, 2*) interacts with antigens from the central nervous systems of the chick (*3*), frog (*4*), and goldfish (*5*). In goldfish, monoclonal antibodies seem to recognize an antigen that binds α-bungarotoxin, and antibody binding colocalizes with α-bungarotoxin binding in the layers of the optic projection (stratum opticum and superficial gray and white). We have used one of these monoclonal antibodies (mAb 47) (*2*) to study the site of synthesis of nAChR in the retinotectal projection of the goldfish *Carassius auratus*.

We found that cholinergic receptors are synthesized in the retina and transported to the optic tectum by rapid axonal transport. This result suggests that the ACh receptor is probably presynaptic and that it performs a modulatory function in retinotectal communication; the result may imply that the retinotectal transmitter is not, as has been suggested, ACh (*6–10*). In addition, this description of the synthesis and transport of an important modulatory molecule in the retinotectal system may advance the molecular characterization of retinotectal synaptogenesis. Finally, the metabolic labeling of neuronal nAChR's and detection by specific immunological precipitation described in this report will, in conjunction with molecular biological methods, allow the characterization of neuronal nAChR's and the definition of their relationship to α-bungarotoxin binding sites and neuromuscular AChR's.

Proteins were labeled by injection of [^{35}S]methionine into either the eye (8 μl, 73 μCi) or the tectal ventricle (2 μl, 18 μCi). After survival times appropriate for incorporation of [^{35}S]methionine into newly synthesized protein, the retina and individual optic tecta were removed and a detergent-solubilized membrane fraction was prepared in 50 m*M* 3-(*N*-morpholino)propanesulfonic acid (MOPS) and 1 m*M* EGTA, *p*H 7.4 (*11*). Aliquots of solubilized membranes were incubated for 1 hour at room temperature with appropriate volumes of mAb 47. The antibody-nAChR complex was precipitated with 35 μl of 10% cell suspension of *Staphylococcus aureus* membranes (incubation period of 1 hour with frequent mixing) by centrifugation for 5 minutes in a Microfuge. The pellet was washed three times and an aliquot of the resuspended pellet was assayed for radioactivity. The mAb 47 (and fixed *S. aureus* membranes) were used to specifically precipitate radioactivity incorporated into proteins with antigenic determinants in common with the nAChR from the *E. electricus* electroplaque. The precipitated radioactivity was identified as nAChR by two criteria (Fig. 1). (i) The precipitation could be inhibited by inclusion of affinity chromatography–purified nAChR from *Torpedo nobiliana*, and (ii) the dilution of antibody that precipitates half of the maximum amount of radioactivity is the same as that which precipitates half of the maximum amount of α-bungarotoxin binding activity from the goldfish retina and tectum and from *Electrophorus* electroplaque.

After radioactive methionine was injected into one eye, radioactivity was found in both tecta; however, the contralateral tectum contained at least four times as much radioactivity as the ipsilateral (Fig. 2). In addition, the specific radioactivity of total protein in the ipsilateral tectum was similar to that of skeletal muscle, which suggests that this represents the proportion of [^{35}S]methionine that leaked into the blood stream. (The projection of the retina to the optic tectum is totally contralateral in the goldfish.) Therefore, the difference in radioactivity between the contralateral and ipsilateral tecta represented the amount of radioactivity specifically transported to the tectum from the retina. Radioactivity was incorporated into nAChR in the optic tectum 6 hours after injection, and the radioactivity incorporated peaked 12 hours after injection (Fig. 2). This time course is compatible with fast axonal transport (*12*). Furthermore, the incorporation of radioactivity into the nAChR in the contralateral tectum

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