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**Correspondence** and requests for materials should be addressed to J.Tovar (j.tovar@rhul.ac.uk).
The sequence of *GiiscU* has been deposited with GenBank under accession number AY040612.

# Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers

**H. D. Bradshaw Jr**[1] **& Douglas W. Schemske**[2]

[1]*Department of Biology, University of Washington, Seattle, Washington 98195, USA*
[2]*Department of Plant Biology, Michigan State University, East Lansing, Michigan 48824, and W. K. Kellogg Biological Station, Hickory Corners, Michigan 49060, USA*

**The role of major mutations in adaptive evolution has been debated for more than a century[1,2]. The classical view is that adaptive mutations are nearly infinite in number with infinitesimally small phenotypic effect[3], but recent theory suggests otherwise[4]. To provide empirical estimates of the magnitude of adaptive mutations in wild plants, we conducted field studies to determine the adaptive value of alternative alleles at a single locus, *YELLOW UPPER*[5–7] (*YUP*). *YUP* controls the presence or absence of yellow carotenoid pigments in the petals of pink-flowered *Mimulus lewisii*, which is pollinated by bumblebees[5,8–10], and its red-flowered sister species[11] *M. cardinalis*, which is pollinated by hummingbirds[5,8–10]. We bred near-isogenic lines (NILs) in which the *YUP* allele from each species was substituted into the other. *M. cardinalis* NILs with the *M. lewisii YUP* allele had dark pink flowers and received 74-fold more bee visits than the wild type, whereas *M. lewisii* NILs with the *M. cardinalis yup* allele had yellow-orange flowers and received 68-fold more hummingbird visits than the wild type. These results indicate that an adaptive shift in pollinator preference may be initiated by a single major mutation.**

Where their ranges overlap, the monkeyflowers *M. lewisii* and *M. cardinalis* are >99% reproductively isolated by the difference in their pollinator guilds[8,9]. In previous studies of artificial $F_2$ hybrids between *M. lewisii* and *M. cardinalis*, we showed that flower colour has marked effects on pollinator visitation[8], and that yellow pigment concentration is controlled in part by the major quantitative trait locus (QTL; reviewed in ref. 12), *YUP*[6,7]. Although $F_2$ populations are useful for mapping QTLs controlling differences in floral traits between species[6,7,13], they are less than ideal for assessing the adaptive effect of a single mutation. The many intermediate flower phenotypes in an $F_2$ population[6–8,13] may provide a bridge for pollinators to develop learned visitation patterns completely unlike those that would occur as a result of a single-locus mutational step in an adaptive walk.

By substituting one allele for another using repeated backcrosses, NILs more closely mimic the effect of a single mutation likely to be part of an adaptive pollinator shift; that is, from bumblebee-pollinated to hummingbird-pollinated, or vice versa. The dominant *M. lewisii YUP* allele prevents carotenoid deposition, so the petals show only their pink anthocyanin pigments. The recessive *M. cardinalis yup* allele allows carotenoid deposition in the petals and produces red flowers when present in conjunction with a high concentration of anthocyanins[5–7]. Although phylogenetic evidence suggests that the hummingbird pollination syndrome of *M. cardinalis* is derived from a bee-pollinated ancestor similar to *M. lewisii*[11], we constructed *YUP* NILs in both species (Fig. 1). The wild-type *M. lewisii* NIL is pink-flowered (Fig. 1a), whereas the 'mutant' NIL homozygous for the introgressed *M. cardinalis yup* allele has pale yellow-orange flowers (Fig. 1b). The wild-type *M. cardinalis* NIL is red-flowered (Fig. 1c), but the presence of a dominant *M. lewisii YUP* allele produces a dark-pink-flowered NIL (Fig. 1d).

Pollinator visitation rates were determined by field observation of NIL experimental arrays near a zone of sympatry between *M. lewisii* and *M. cardinalis*[5,8] to ensure that pollinators were familiar with both species in their natural habitat. Bumblebees strongly prefer pink-flowered NILs carrying the *YUP* allele (Fig. 1a, d) in both the *M. lewisii* and *M. cardinalis* genetic backgrounds (Table 1). Hummingbirds prefer yellow-orange- or red-flowered NILs homozygous for the *yup* allele (Fig. 1b, c) in both backgrounds (Table 1).

The striking effect of flower colour on pollinator specificity is evidence for the adaptation of both monkeyflower species to their current pollinators (Table 1). A wild-type pink *M. lewisii* flower (Fig. 1a) is >700 times more likely to be visited by a bumblebee than by a hummingbird, whereas the yellow-orange-flowered 'mutant' (Fig. 1b) is only 1.8 times as likely to be visited by a bumblebee. In the *M. cardinalis* background, a wild-type red flower (Fig. 1c) is >1,200 times more likely to be visited by a hummingbird than by a bumblebee, but the pink-flowered 'mutant' (Fig. 1d) is visited only 15 times as frequently by hummingbirds.

When these visitation rates are compared with the results from our previous $F_2$ QTL mapping population[8], we find that the $F_2$ experiments accurately predict pollinator visitation when we consider only bumblebees visiting *M. lewisii* NILs, and hummingbirds visiting *M. cardinalis* NILs. In *M. lewisii* NILs and the $F_2$, the wild-type pink flowers were visited by bumblebees at about a fivefold higher rate than were the 'mutant' yellow-orange flowers (Table 1 and ref. 8). In *M. cardinalis* NILs, hummingbirds showed a slight 1.1-fold preference for wild-type red flowers over the pink-flowered 'mutants', similar to that found in the $F_2$ population (Table 1 and ref. 8). The close correspondence of the results from these indepen-

  


**Figure 1** Near-isogenic lines of *M. lewisii* and *M. cardinalis* with alternate alleles at the *YUP* locus. **a**, **b**, *M. lewisii*; **c**, **d**, *M. cardinalis*.The wild-type allele at the *YUP* locus (**a**, **c**) has been substituted by introgression with the allele from the other species (**b**, **d**). Flowers in each NIL pair (**a** and **b**, **c** and **d**) are full siblings.

dent experiments suggests that they address the same question: what is the effect of a *YUP* mutation on visitation by the current pollinator?

From an evolutionary perspective, it is perhaps more illuminating to ask a different question: what is the effect of a *YUP* mutation on the attraction of a novel pollinator guild, as would be the relevant scenario for a new mutation leading to an adaptive shift from one pollinator guild to another in the common ancestor of *M. lewisii* and *M. cardinalis*? Our NIL experiments reveal that hummingbirds visit yellow-orange-flowered 'mutants' of *M. lewisii* at 68 times the rate of the pink-flowered wild type, and bumblebees visit pink-flowered 'mutants' of *M. cardinalis* at 74 times the rate of the red-flowered wild type (Table 1). The large and symmetrical effect of the *YUP* allele substitution on the attraction of a new pollinator guild implies that a mutation at the *YUP* locus has the potential to alter the pollinator assemblage dramatically in the common ancestor of *M. lewisii* and *M. cardinalis*.

As 'mutations' at the *YUP* locus decrease visitation by the current pollinator guild, and simultaneously increase visitation by a new pollinator guild, are there plausible ecological circumstances in which the mutant might be favoured by natural selection? The combined rate of bumblebee and hummingbird visitation to the yellow-orange-flowered 'mutants' of *M. lewisii* is just 26% of that to the wild-type pink flowers, and the combined rate for dark-pink-flowered 'mutants' of *M. cardinalis* is 95% of the wild type. This implies that a change in the relative abundance of bumblebees and hummingbirds, compared with the pollinator assemblage present during our field experiments, would be required for the mutant to be favoured by natural selection in the common ancestor of

Table 1 **Pollinator visitation rates to NILs of *M. lewisii* and *M. cardinalis***

| | Bumblebees ($10^{-3}$ visits per flower per hour) | Hummingbirds ($10^{-3}$ visits per flower per hour) |
|---|---|---|
| *M. lewisii* NILs | | |
| Wild-type (pink; Fig. 1a) | 15.4 | 0.0212 |
| 'Mutant' (yellow-orange; Fig. 1b) | 2.63 | 1.44 |
| *M. cardinalis* NILs | | |
| Wild-type (red; Fig. 1c) | 0.148 | 189 |
| 'Mutant' (dark pink; Fig. 1d) | 10.9 | 168 |

Note that the visitation rates estimated for bumblebees to red-flowered *M. cardinalis* NILs and for hummingbird visits to pink-flowered *M. lewisii* NILs are likely to be less accurate owing to the small absolute number of visits ($N = 2$ and $N = 1$, respectively).

*M. lewisii* and *M. cardinalis*. The change in relative abundance of pollinators necessary to produce equal visitation to both flower colour phenotypes can be estimated from our data. A ninefold decrease in the relative abundance of bumblebees would produce equal combined visitation rates in the wild-type pink-flowered and 'mutant' yellow-orange-flowered *M. lewisii* NILs. At the equilibrium point, 99% of visitors to wild-type *M. lewisii* flowers would be bumblebees, whereas 87% of visitors to 'mutants' would be hummingbirds. In the *M. cardinalis* NILs, a twofold increase in the relative abundance of bumblebees would produce equal visitation rates to pink and red flowers. At the equilibrium point, hummingbirds would be virtually the only visitor to the wild-type red *M. cardinalis* flowers, and remain the major visitor (89% of visits) even to the dark-pink 'mutants.'

The evolution of hummingbird-pollinated flowers from insect-pollinated ancestors is a recurring theme in the flora of western North America[14]. A molecular phylogenetic analysis of *Mimulus* indicates that hummingbird pollination has evolved independently twice within the section Erythranthe, in one of these cases leading to the evolution of *M. cardinalis* from an insect-pollinated ancestor likely to have resembled the extant *M. lewisii*[11]. We have shown that an adaptive divergence in pollinator preference, as might be expected at the speciation event that occurred in the common ancestor of *M. lewisii* and *M. cardinalis*, could in principle be initiated by a single mutation with a large effect on flower colour.

To understand in greater detail the dynamics of an adaptive pollinator shift, it will be necessary to more closely replicate the appearance of a new mutation. First, it must be demonstrated that the recessive allele at the *YUP* locus can be produced by a single loss-of-function mutation, ruling out the possibility that the *YUP* locus contains more than a single gene. Second, a null mutant at the *YUP* locus in *M. lewisii* could be established at a realistic (that is, low) frequency in a natural setting, and its evolutionary trajectory observed. In addition, NILs could be developed carrying 1, 2,…*N* allele substitutions at major QTLs, in various combinations, to test alternative hypotheses for the trajectory of floral evolution and speciation in response to pollinator choice. □

## Methods

### NIL construction

Near-isogenic lines were derived from two backcross (BC) populations: *M. lewisii* × (*M. lewisii* × *M. cardinalis*) and *M. cardinalis* × (*M. lewisii* × *M. cardinalis*). All NILs were produced by single-seed descent. Ten first-generation backcross ($BC_1$) plants with *M. lewisii* as the recurrent parent were chosen as the founders of NILs on the basis of their inheritance of a dominant random amplified polymorphic DNA (RAPD) marker (AG13_108; ref. 6) linked in coupling to the recessive *yup* allele (H.D.B. and D.W.S., unpublished work). A single plant from each of these ten *M. lewisii* NILs was backcrossed to a series of unrelated *M. lewisii* recurrent parents for three additional generations, maintaining selection for the AG13_108 marker. After four generations of backcrossing, each NIL is expected to share 97% of its genome with the recurrent parent. For each of the ten lines, a single $BC_4$ plant was self-pollinated to produce ten $BC_4S_1$ families segregating at the *YUP* locus. The $BC_4S_1$ families yielded both wild-type pink and 'mutant' yellow-orange flowers. Ten *M. cardinalis* NILs were constructed using the same mating design, except that selection for the presence of the dominant *YUP* allele was done visually (dark-pink flowers), and self-pollination of the $BC_4$ generation was unnecessary because each generation segregated 1:1 for red:pink flowers.

### Recurrent parent similarity index

Six characters (upper petal reflexing, lateral petal reflexing, pistil length, stamen length, lateral petal width and nectar volume) for which QTLs have been mapped[6,7] were measured on two flowers from each plant. There was a significant difference between the multivariate flower phenotypes of wild-type and 'mutant' NILs in both the *M. lewisii* (multiple analysis of variance, MANOVA, $F = 18.18$, Wilks' $\lambda = 0.32$, $P < 0.0001$) and *M. cardinalis* (MANOVA, $F = 11.00$, Wilks' $\lambda = 0.56$, $P < 0.0001$) genetic backgrounds (PROC GLM, SAS Institute). Least-squares means for each trait within each NIL genotypic class were normalized to the difference between trait means of the two parental species[7], setting the recurrent parent trait value at 100% and the nonrecurrent parent at 0%. Lower recurrent parent similarity (RPS) values are evidence of linkage drag, whereas values larger than 100% represent measurement error or heterosis. In the *M. lewisii* genetic background, the wild-type plants had a mean RPS index across all traits of 91% (range 66–108%), whereas their 'mutant' sibs had a value of 80% (range 51–103%). In the *M. cardinalis* genetic background, the wild-type plants had a mean RPS index of 95% (range 49–129%), whereas their 'mutant' sibs had a value of 80% (range 46–155%). Although 'mutant' NILs show more linkage drag than the wild type, we judge the difference to be small. Nectar volume, which is known from our $F_2$ experiments to have a marked effect on hummingbird visitation[8], has RPS index values that are very close to one another in the NILs: 105% and 103% in the *M. lewisii* background, and 46% and 53% in the *M. cardinalis* background. This suggests that differences in nectar production between pairs of NILs did not affect pollinator visitation patterns.

### Pollinator visitation

For each of two field experiments conducted to measure pollinator visitation, 50 pink or dark pink (*YUP*/___) and 50 yellow-orange or red (*yup/yup*) plants were drawn at random from five $BC_4S_1$ (*M. lewisii*) or $BC_4$ (*M. cardinalis*) NIL families. Assessments of pollinator visitation were performed at Mather (California, USA), the site where much of the previous work on these two species of *Mimulus* has been done[5]. Pollinator observations were carried out from dawn to evening, with a 1–2 h break at midday when pollinators were least active. Dates of observation were 18–30 August 1999 for *M. cardinalis* NILs, and 18–27 July 2000 for *M. lewisii* NILs. These dates correspond closely to the peak flowering times of natural populations of the two *Mimulus* species. We chose to do the experiments in different years so that pollinators were faced with a binary choice of flower phenotypes, as would be the case for a newly arisen mutation. Plants were placed at random on a 1 m × 1 m grid to produce the experimental arrays (a black bear visit reduced the total sample size in the *M. lewisii* NIL array from $N = 100$ to $N = 99$). A pollinator visit was counted if it appeared that the pollinator probed the flower and contacted the anthers or stigma. Bumblebees and hummingbirds were the only pollinators observed. We observed 1,090 bumblebee visits to the *M. lewisii* NILs, 180 bumblebee visits to the *M. cardinalis* NILs, 201 hummingbird visits to the *M. lewisii* NILs, and 3,738 hummingbird visits to the *M. cardinalis* NILs. The number of flowers on each plant was recorded daily, along with the number of hours spent observing. Visitation rates were calculated by dividing the total number of pollinator visits across all days by the aggregate number of hours in which visits could have occurred to each flower (flower-hours). For the *M. lewisii* NILs, both bumblebee and hummingbird pollinator observations were carried out simultaneously, with 47,159 flower-hours for the wild-type NILs and 138,648 flower-hours for the 'mutants'. For the *M. cardinalis* NILs, separate pollinator observation periods were required to keep track of the large number of hummingbird visits. During the bumblebee observation periods, there were 16,291 flower-hours for the 'mutant' NILs and 13,556 flower-hours for the wild-type. During the hummingbird observation periods, there were 11,505 flower-hours for the 'mutant' NILs and 9,520 flower-hours for the wild type.

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**Correspondence** and requests for materials should be addressed to H.D.B. (toby@u.washington.edu).

# Light-induced hormone conversion of $T_4$ to $T_3$ regulates photoperiodic response of gonads in birds

**Takashi Yoshimura[1], Shinobu Yasuo[1], Miwa Watanabe[1], Masayuki Iigo[3], Takashi Yamamura[1], Kanjun Hirunagi[2] & Shizufumi Ebihara[1]**

[1]*Division of Biomodeling, Graduate School of Bioagricultural Sciences, Nagoya University, and* [2]*The Nagoya University Museum, Furo-cho, Chikusa-ku, Nagoya, 464-8601, Japan*
[3]*Department of Applied Biological Chemistry, Faculty of Agriculture, Utsunomiya University, Mine-Machi, Utsunomiya, Tochigi 321-8505, Japan*

**Reproduction of many temperate zone birds is under photoperiodic control. The Japanese quail is an excellent model for studying the mechanism of photoperiodic time measurement because of its distinct and marked response to changing photoperiods. Studies on this animal have suggested that the mediobasal hypothalamus (MBH) is an important centre controlling photoperiodic time measurement[1–8]. Here we report that expression in the MBH of the gene encoding type 2 iodothyronine deiodinase (Dio2), which catalyses the intracellular deiodination of thyroxine ($T_4$) prohormone to the active 3,5,3′-triiodothyronine ($T_3$), is induced by light in Japanese quail. Intracerebroventricular administration of $T_3$ mimics the photoperiodic response, whereas the Dio2 inhibitor iopanoic acid prevents gonadal growth. These findings demonstrate that light-induced *Dio2* expression in the MBH may be involved in the photoperiodic response of gonads in Japanese quail.**

The molecular mechanism of photoperiodic or seasonal time measurement is not well understood in any organism studied so far. In birds, the MBH—which includes the nucleus hypothalamicus posterior medialis (NHPM), the infundibular nucleus and the median eminence—is an important centre controlling photoperiodic time measurement (Supplementary Figs 1 and 2). For example, introduction of a lesion to the nucleus hypothalamicus posterior medialis and/or the infundibular nucleus resulted in loss of photoperiodic response of the gonads[1–3] even though the gonadotrophin-releasing hormone (GnRH) system of the lesioned animal had been left intact[4]. Electrical stimulation of this area increases luteinizing hormone secretion[5] and induces testicular growth[6]. Furthermore, c-Fos expression has been reported in these structures as a result of photostimulation for one long day (20/4 h light/dark cycle)[7,8] and deep-brain photoreceptors are thought to be localized in the infundibular nucleus[9]. Recently, we have also observed the expression of circadian clock genes in the MBH, and proposed that the clock in the MBH may function as the 'photoperiodic clock'[10]. These observations indicate that all of the essential machinery for photoperiodic time measurement is localized in the MBH.

Single light pulses within the photo-inducible phase increase

  