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ORIGINAL RESEARCH PAPER

Mutations perturbing petal cell shape and anthocyanin synthesis influence bumblebee perception of *Antirrhinum majus* flower colour

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Abstract We wished to understand the effects on pollinator behaviour of single mutations in plant genes controlling flower appearance. To this end, we analysed snapdragon flowers (*Antirrhinum majus*), including the *mixta* and *nivea* mutants, in controlled laboratory conditions using psychophysical tests with bumblebees. The *MIXTA* locus controls petal epidermal cell shape, and thus the path that incident light takes within the pigment-containing cells. The effect is that *mixta* mutant flowers are pink in comparison to the wild type purple flowers, and mutants lack the sparkling sheen of wild type flowers that is clearly visible to human observers. Despite their fundamentally different appearance to humans, and even though bees could discriminate the flowers, inexperienced bees exhibited no preference for either type, and the flowers did not differ in their detectability in a Y-maze—either when the flowers appeared in front of a homogeneous or a dappled background. Equally counterintuitive effects were found for the non-pigmented, UV reflecting *nivea* mutant: even though the overall reflectance intensity and UV signal of *nivea* flowers is several times that of wild type flowers, their detectability was significantly *reduced* relative to wild type flowers. In addition, naïve foragers preferred wild type flowers over *nivea* mutants, even though these generated a stronger signal in all receptor types. Our results show that single mutations affecting flower signal can have profound effects on pollinator behaviour—but not in ways predictable by crude assessments via human perception, nor simple quantification of UV signals. However, current models of bee visual perception predict the observed effects very well.

Keywords Epidermis · Evolution · Mixta · Nivea · Pollination · Psychophysics · Snapdragon

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Introduction

Several different plant species represent important models to develop our understanding of flower signal evolution and plant-pollinator relationships. Much previous work has focussed on species such as *Mimulus* (Bradshaw and Schemske 2003) and *Aquilegia* (Hodges et al. 2003), but our primary research system is the snapdragon, *Antirrhinum majus. Antirrhinum* is an extremely powerful model for such studies because numerous mutations in genes controlling petal colour, floral morphology and petal epidermal cell shape have been identified (e.g. Luo et al. 1996; Glover and Martin 1998; Whibley et al. 2006). The ability to compare isogenic lines, differing in only a single phenotypic characteristic controlled by a single molecularly characterised locus, eliminates much of the complexity

arising from the multiple other variables which differ between two species or even two varieties within a species. It also offers an advantage over the use of near isogenic lines (NILs), as used in *Mimulus* and *Aquilegia.* Bradshaw and Schemske (2003) estimated that the NILs with which they elegantly demonstrated pollinator preferences for the *Mimulus YUP* locus (controlling yellow carotenoid deposition) were 97% identical genetically, despite being extensively backcrossed. If a plant genome contains around 20,000 genes then this means a difference in 599 genes besides the one controlling the trait of interest, and it is likely that at least some of those loci may control other factors which influence pollinator behaviour. The use of single mutant lines from molecular model systems provides data free from these potential complicating variables. *Antirrhinum* is a particularly useful model system because its flowers are pollinated by a relatively specific group of insects, large bees (particularly bumblebees).

It has previously been observed that some mutant lines of *Antirrhinum* differ in fitness from wild type lines, indicating that single mutations in genes controlling flower appearance can influence fitness via the attraction of pollinators (Glover and Martin 1998). However, we cannot truly understand the role of individual floral traits in the manipulation of pollinator perception and behaviour, unless the influences of such traits are tested under carefully controlled laboratory conditions, and in situations where the previous experience of pollinators is known. Our goal here is to test the detectability and discriminability of wild type and mutant flowers of *Antirrhinum*, and to examine innate biases of bumblebees towards flowers with varying visual appearances.

One well characterised mutation is found in *MIXTA*, the gene that controls whether flower petals will produce conical epidermal cells (Fig. 1). Molecular genetic analysis has shown that the *MIXTA* locus encodes a MYB transcription factor and that the gene is expressed only in the petal epidermal cells and cannot therefore function in any other tissue (Noda et al. 1994; Glover et al. 1998). Loss of function of this gene results in plants that differ from the wild type only in the shape of their petal epidermal cells, producing flat rather than conical cells, and the effects of the gene are cell autonomous, meaning that the protein cannot be transported to act in other cells (Noda et al. 1994). Approximately 80% of angiosperms produce petals with similar conical epidermal cells (Kay et al. 1981), and numerous suggestions as to their function have been made. These include the possibilities that they enhance petal colour, act as a direct tactile cue, increase the temperature of the flower, influence scent production or release, or influence the wettability of the flower surface (Kay et al. 1981; Kevan and Lane 1985).

When the *mixta* mutant line was isolated, it was observed that flowers lacking conical cells appeared paler for human vision than wild type flowers (Noda et al. 1994). It has since been shown that the presence of conical epidermal cells affects petal colour by increasing the proportion of incident light that enters the pigment-containing vacuoles of epidermal cells, so enhancing petal colour saturation for human observers (Gorton and Vogelmann 1996). This finding appeared to support the hypothesis that the function of conical petal cells is to facilitate better colour detection or discrimination by pollinators like bumblebees (*Bombus terrestris*) (Glover and Martin 1998; Waser and Chittka 1998), but the bee subjective appearance of these flowers remains to be quantified.

The *nivea* mutation also affects petal colour, as it consists of a deletion of the single *Antirrhinum* gene encoding chalcone synthase. This enzyme is necessary for the first committed step of the flavonoid biosynthetic pathway (Wienand et al. 1982). Therefore this

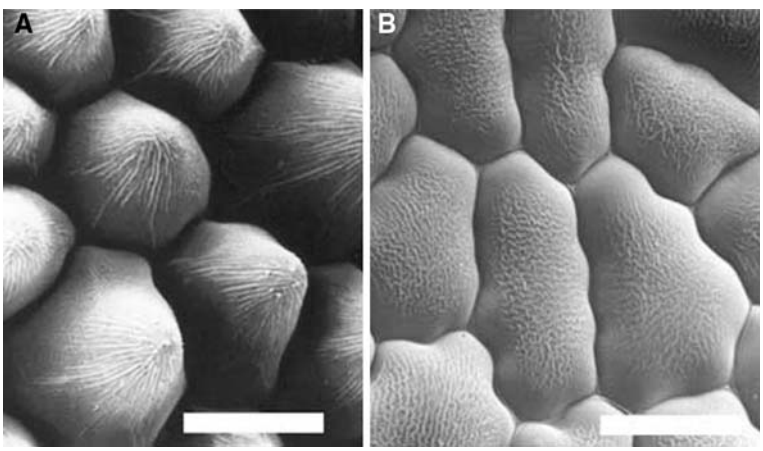

Fig. 1 Scanning electron microscope images of *Antirrhinum* petal surfaces (**A**) Conical shaped cells of the wild type. (**B**) Flat cells of a *mixta* mutant petal (Scale bar = 20 μm)

mutant line is lacking not only the purple anthocyanins visible to the human eye but also all of the flavonoid pigments capable of absorbing light of any wavelength, including ultraviolet (UV) A radiation (Glover and Martin 1998). These flowers not only appear white to human eyes, but will also be 'bee-white', as a result of the reflection of all incident light from cell walls (Kevan et al. 1996).

Both the *mixta* and *nivea* mutations in *Antirrhinum* have been found to decrease fruit set in open-pollinated field grown plants (Glover and Martin 1998). Both mutant lines, and wild type plants, were grown outside in mixed plots and flowers emasculated to prevent self-pollination. Emasculated flowers were tagged and scored according to the presence or absence of fruit. Both mutant lines produced significantly fewer fruit, although all lines produced the same amount of fruit when hand pollinated (Glover and Martin 1998). Further field experiments showed that wild bumblebees presented with arrays of different flowers were more likely to reject *nivea* and *mixta* mutant flowers than wild type ones, both before and after landing (Comba et al. 2000).

Here we address the question of whether the differential success of wild type and these mutant flowers is mediated via the efficiency with which flowers exploit the features of the bee visual system. The colour vision of bumblebees is based on three classes of photoreceptors maximally sensitive in the ultraviolet (UV) at 350 nm, in the blue at 440 nm and in the green at 540 nm (Peitsch et al. 1992). Bumblebees have compound eyes which are only able to resolve relatively low spatial frequency information (Spaethe and Chittka 2003), and bees see flowers very differently to humans (Kevan et al. 2001; Vorobyev et al. 1997). How bees perceive flower colour is potentially relevant to several avenues of investigation for understanding plant pollinator relationships. Firstly, when searching for a flower, colour contrast is relevant to the reliability and speed with which an insect can detect a flower (Spaethe et al. 2001). Secondly, the way in which insects discriminate between different colours is dependent upon their level of experience and the spatial arrangement of the stimuli (Dyer and Chittka 2004c; Giurfa 2004; Neumeyer 1980). Thirdly, if bees can reliably discriminate between different colours, then it is important to know whether they have any colour preferences for the respective colours (Giurfa et al. 1995; Lunau and Maier 1995; Lunau et al. 1996; Chittka et al. 2004). This study tests bumblebee colour perception in controlled laboratory conditions with wild type, *mixta* mutant, *nivea* mutant and *mixta/nivea* double mutant flowers to understand whether colour vision of insect pollinators might explain why plant petals have evolved certain features, including pigments and conical epidermal cells.

Materials and methods

Experimental conditions

Experiments were conducted indoors at 20°C with bumblebees (*Bombus terrestris*) housed in a plastic nesting box. Bee colonies were supplied by Koppert Ltd (UK) as special research colonies. Prior to an experiment bees had not had any experience with colour stimuli, thus allowing for the collection of data independently of learnt preferences. To identify individual bees, a coloured mark was placed on the thorax whilst they were at a glass feeder in a flight arena.

Illumination was provided by six Sylvania 36W Professional Activa 172 tubes mounted 1.8 m above the floor of a foraging arena. The frequency of the tubes was controlled with special ballasts (Philips HF-B 236 TLD) and was greater than 200 Hz. This illumination closely simulates natural daylight illumination for bee vision (Dyer and Chittka 2004a).

Plant growth conditions

All four lines were grown from seed. The generation of self seed from plants previously genotyped by Southern blotting as homozygous for the mutant or wild type alleles of the two genes was described previously (Glover and Martin 1998). Plants were grown under greenhouse conditions at 23°C in 4-inch pots in Levingtons (UK) M3 compost. During the growth period plants received supplemental lighting from 400 Osram (Osram, München, Germany) lamps on a 16 h light/8 h dark photoperiod.

Measurement of flower spectral properties and colorimetry

The spectral reflectance function (SRF) of flower petals was measured with an Ocean Optics (Dundedin, Fl., USA) spectrophotometer (S2000) relative to a white reflection standard. A minimum of 15 different flowers of each type were measured to obtain a mean spectral reflectance function, and an indication of the variance within petals of the same flower type. We also measured dorsal and ventral petals, and both lobe and tube tissue for each flower, but these data showed no indication of being different and so the data were pooled. The green foliage of a wild type plant, Humbrol (UK)

number 2 green enamel paint (closely matched foliage), and Humbrol number 41 cream enamel paint (closely matched white *Antirrhinum*) were also measured with the spectrophotometer.

The relative amount of light absorbed by each photoreceptor class (UV, blue, green) is given by *P*:

$$P = R\int_{300}^{650} S(\lambda)I(\lambda)D(\lambda)\mathrm{d}\lambda \quad (1)$$

where $S(\lambda)$ is the spectral sensitivity function of the respective photoreceptor, $I(\lambda)$ is the reflectance function of the object in question, $D(\lambda)$ is the spectral power distribution of the illuminant, $\mathrm{d}\lambda$ is the wavelength step size, and the variable R is the coefficient of adaptation to the green background stimulus (I_B). This coefficient is determined by:

$$R = 1/\int_{300}^{650} S(\lambda)I_B(\lambda)D(\lambda)\mathrm{d}\lambda \quad (2)$$

where $I_B(\lambda)$ is the reflectance function of the background to which the eyes are assumed to be adapted. The transduction of photoreceptor absorption (P) into receptor excitations (E) is given by:

$$E = P/(P + 1) \quad (3)$$

Colour loci of the stimuli were calculated in a hexagon colour space model (Chittka 1992). Coding is performed by two unspecified colour opponent mechanisms and colour distance is calculated as the Euclidean distance between stimuli loci in colour space (Chittka 1992). We also evaluated green contrast (i.e. the difference in green receptor signal generated by the background and respective flower type), since this contrast has previously been shown to aid long range detection of flowers (Giurfa et al. 1996).

Experiment 1A: Evaluation of flower detection by bumblebees

To determine if colour cues provided by the four different plant lines might be important for the detection of the flowers by bumblebees, we used a Y-maze apparatus of the dimensions described by Giurfa et al. (1996), see their Fig. 1. The Y-maze was covered with UV-transparent Plexiglas. The Y-maze allows a bee to fly into the decision chamber and simultaneously view both arms of the Y-maze.

A bee colony was connected to the Y-maze with Plexiglas tubes containing a series of gates to control the movement of individual bees. Prior to experiments, a glass feeder containing 10% sucrose solution was placed in an arm of the Y-maze so that bees would become familiar with flying within the apparatus and returning to the colony. The position of the feeder was pseudo-randomised every half hour to prevent bees learning a side preference. Bees had a minimum of 2 days experience with the apparatus before participating in an experiment.

In tests, each arm contained a homogeneously painted green background, and each arm also contained a laboratory clamp (painted green) to hold a flower. The correct arm presented a single flower. To control for floral scent, the incorrect arm had a laboratory clamp which presented a flower behind a small green screen. The visual signal of a flower was presented in pseudo-randomised alternating arms of the Y-maze. Between choices the arena floor and the clamps were cleaned with 30% ethanol, and fresh flowers were provided. The flower presenting a visual signal had a 20 μL drop of 40% (vol.) sucrose solution placed on top of the flower as a reward, whilst the incorrect arm contained no reward. The bee had to choose the correct arm in order to receive a reward, and when a bee flew into one arm of the Y-maze, a decision was counted. If the arm contained the flower, this was counted as a correct choice. If the bee flew into the arm without a flower, this was counted as an incorrect choice and a subsequent correct choice was not counted (Giurfa et al. 1996). The dependent variables considered in this experiment were frequency of correct choice and response time within the decision chamber. Response time is an important measure of performance in psychophysical studies on human subjects (Rival et al. 2003), and has recently been recognised to be equally important for understanding perception in insects (Chittka et al. 2003; Dyer and Chittka 2004b).

Bees were introduced to the visual detection task by first making this task relatively easy: both wild type and *mixta* flowers were presented at a distance of 21 mm from the decision chamber, so that they appeared at a large visual angle of 25°. Once bees performed well in this task, and had been tested in it, bees were tested with flowers at a distance of 37 mm from the decision chamber. This means that the flowers' projection subtended 15° on the bee eye, which approximately matches the minimum visual angle at which honeybees can reliably use their colour vision (Giurfa et al. 1996).

Experiment 1B: Flower detection with complex backgrounds

An additional group of bees ($N = 15$) was tested using wild type and *mixta* mutant flowers in a potentially

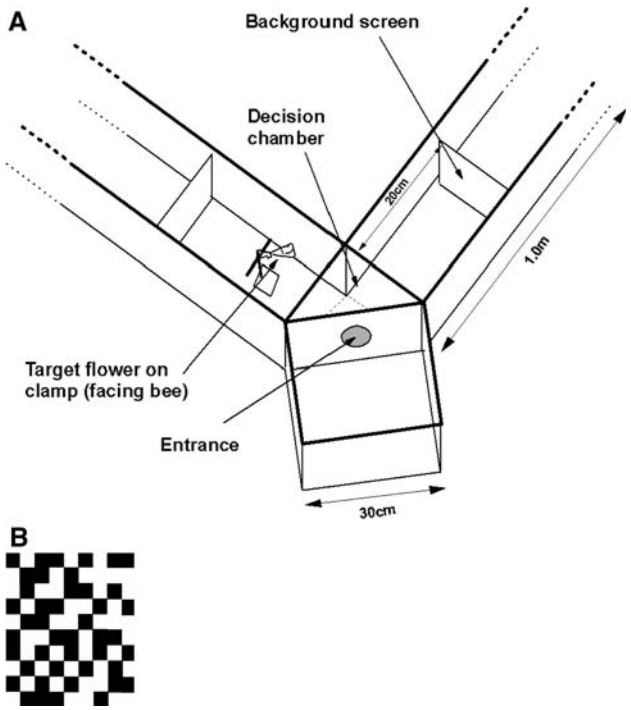

Fig. 2 Apparatus used to test bumblebee detection of *Antirrhinum* flowers. (**A**) A Y-maze arena that allows individual bees to enter a decision chamber and choose the arm that contained a flower. The colour of the background screen was either green or a dappled Julesz pattern of green and cream colours (see text). A stand (painted green) was presented in each arm of the Y-maze; in the correct arm the stand presented a flower rewarded with sucrose, whilst the incorrect arm contained a non-rewarding flower (not visible) facing away from the bee, to equalise olfactory cues in both arms. In some experiments a distractor flower, facing the bee, was placed in incorrect arm (see text). (**B**) An example of a patterned Julesz type background (b/w rendition) that required bees to detect flowers on a noisy background

more challenging visual test in the Y-maze, where the background was not homogeneous. The background was a 2 cm × 2 cm random Julesz checkerboard pattern (Srinivasan et al. 2000) constructed of Humbrol number 2 (green) and Humbrol 41 (ivory) paints. The background was at a distance of 20 cm from the decision point, equivalent to a spatial frequency of approximately 0.1 cycles/degree (Fig. 2). As the maximum visual acuity of bees is approximately 0.23 cycles/degree (Srinivasan and Lehrer 1988), the Julesz checkerboard thus had the effect of creating a noisy background on which the targets had to be detected. The target *Antirrhinum* flower (wild type or *mixta*) was placed in one arm of the Y-maze and rewarded with a drop of sucrose, whilst in the second arm, a white *nivea* flower was placed to act as a distractor. The rationale behind this test was to evaluate whether wild type conical-celled flowers allowed for easier detection under competing information from a complex background and a similarly shaped but differently coloured flower.

The white flower acted as a control for detection of a visual signal occurring via motion parallax of a 3D stimulus against the background (Zhang and Srinivasan 1994), or the bee simply detecting the presence of a signal independent of the requirement for colour processing. The *nivea* flower contained no reward. Thus in this test, bees had to correctly detect a flower on a noisy background and ignore an irrelevant distractor that was identical in all properties except colour.

Experiment 2: Can bumblebees discriminate between differently coloured *Antirrhinum* mutants?

Experiment 2 was conducted in the same illumination using a bee flight arena that was a box with a wooden frame (dimensions: L = 110 cm, W = 70 cm, H = 100 cm) having wire mesh on 3 of the 4 sides, and UV transparent Plexiglas screens in the top and front panels (Dyer et al. 2006). The arena floor was painted green (Humbrol No. 2; UK). Ten individual bees were trained to wild type flowers by placing a 20 μL drop of 40% (vol.) sucrose solution on top of the flower as a reward. The reason for this placement was that it took bees an extended period to locate the natural location of nectar in the flowers, and we were interested here only in detectability. Eight flowers were presented in the arena at spatially randomised positions, and if a bee had filled its honey stomach, it was allowed to return to the nesting box and fresh flowers were placed in the arena. This training continued until a bee had received a reward on 15 flowers. Each bee was then given a non-rewarding test where five wild type and five *mixta* flowers were presented in the arena and we scored the first 20 choices made by each bee. The bee was then satiated on one wild type flower to ensure continued motivation to forage. When the bee subsequently returned to the arena, it was given a second non-rewarding test with wild type and *nivea* flowers.

Experiment 3: Do bumblebees exhibit an innate preference for flowers depending upon colour?

To evaluate whether bees exhibit an innate colour preference for any particular flower type, a separate group of 10 bumblebees was first allowed to collect 40% (vol.) sucrose solution from clear 2 cm × 2 cm glass squares presented on 8 cm high clear Sterilin tubes (Bibby Sterilin Ltd., Stones, Staffordshire, UK) for 32 h over 4 days. Every hour, the artificial flowers

were cleaned with 30% ethanol and spatial positions within the arena were randomised; every 4 h the arena was cleaned with ethanol. After this pre-training (Lunau et al. 1996), which was to encourage a high level of motivation in bees to forage, each bee was individually tested in a single non-rewarding test. In a non-rewarding test, four clear plastic vases containing the four types of flowers were randomly placed in the arena. In each vase there was a cluster of approximately 20 flowers that were wrapped in black netting (hole diameter 2 mm, thread thickness 0.1 mm), permitting the bees to see and smell flowers, but the netting prevented bees from physically landing on the flowers. A test for each of the first 5 bees lasted 4 min, and each minute the position of the vases was re-randomised. For the second group of 5 bees, the spatial positions were pseudo-randomised in a counterbalanced fashion (e.g. so that wild type and *mixta*, or *nivea* and *mixta/nivea* spatial position were counterbalanced). During a 4 min test, the flight behaviour was scored as the number of approach flights where a bee flew directly towards a cluster to within 10 cm, and the number of times a bee physically tried to land on flowers by touching the netting.

Results

Measurement of flower spectral properties and colorimetry

Wild type *Antirrhinum* flowers have a reflectance peak in the violet near 430 nm, a reflectance minimum in the green/yellow (around 550 nm) and a steep increase in reflectance in the red, above 600 nm (Fig. 3A). The combination of violet and red reflectance causes a purple perception in human observers, but the red component is of little relevance for bees, whose visual spectrum extends only slightly into the red (Chittka and Waser 1997). Wild type flowers are lower in overall reflectance than all other types, including the *mixta* mutants that are similar in the shape of their reflectance curves, but reflect higher intensities at all wavelengths except near 650 nm. *nivea* mutants reflect all wavelengths above 360 nm, and their reflectance declines steadily at lower wavelengths. This is interesting because the reflectance curve includes a significant portion of UV light, and will stimulate the bees' UV, blue, and green receptors roughly equally (Table 1), and therefore appear as achromatic (spectrally neutral) to bees. This is in contrast to the vast majority of white flowers which cut out all light below 400 nm, and thus appear blue-green to bees (Chittka et al. 1994). Combined *nivea/mixta* mutants (which are also white to the human eye) are similar in reflectance curve shape to plain *nivea* mutants, but reflect less light overall (Fig. 3A).

Overall intensity (as measured by the sum of relative quantum fluxes in all three photoreceptor types, calculated according to Eq. 1; Backhaus et al. 1987) was lowest in wild type flowers (2.8); this was exceeded by *mixta* mutant flowers (3.5). Non-pigmented flowers had much higher intensities (*nivea/mixta*: 16.1; and *nivea* had by far the highest intensity at (20.3), thus ca. 6 times more than the wild type flowers, and intensity contrast to the background was very high in *nivea* (575%) but not wild type flowers (11%; Table 1). The UV receptor quantum catch in these mutants (6.7) was much higher than in the wild type flowers (0.79) and UV receptor signal contrast to the background was much stronger in the *nivea* mutants (0.37—where the maximum contrast is 0.5) than in the wild type flowers (0.06). Green receptor signal contrast was also substantially higher in the *nivea* mutants (0.32) than in the wild type flowers (–0.19).

The hexagon colour space allows us to predict the bee subjective colours of the flower phenotypes used. The angular position in the hexagon (as measured from the centre) informs us about bee subjective hue, whereas distance to the uncoloured point (centre) designates saturation, which, in our tests, corresponds to the contrast that the flower makes with its backdrop. If the maximum distance from centre to any of the corners of the hexagon is defined as 1, colours with a distance of below 0.1 from the centre have been defined as having low colour saturation for bees (Chittka et al. 1994). The colour distances from the hexagon centre of the *nivea* and *mixta/nivea* mutant flowers were 0.07 and 0.08 units, respectively, which means that they will be perceived as perceptually similar to the background colour for bees. The colour contrasts of the wild type and *mixta* flowers were 0.23 and 0.25 units, respectively, which means that they are predicted to be equally easily detectable (and equally saturated) in front of the green background. Both wild type and *mixta* mutants are predicted to appear blue to bees (i.e. stimulating the bees' blue receptors substantially more than their UV and green receptors; Table 1).

Experiment 1A: Flower detection with homogeneous background

When flowers were tested near the entrance of the Y-maze arms (25° visual angle) detection accuracy was near 90% (see Fig. 4). At this large angle, it is perhaps unsurprising that there was no significant difference

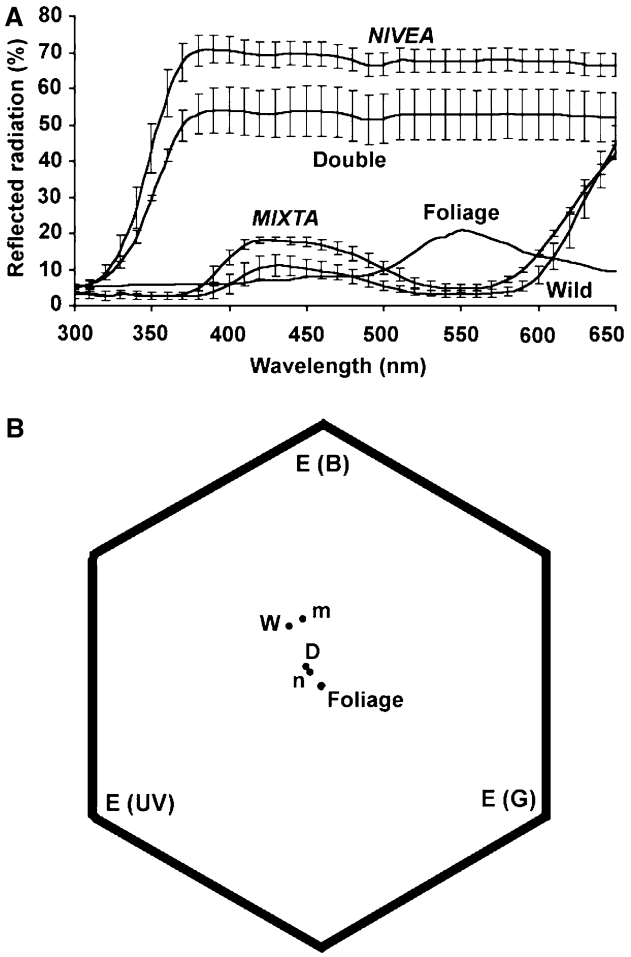

Fig. 3 **(A)** Mean spectral reflectance of *Antirrhinum* flowers (± SD for >15 individual measurements) and foliage. **(B)** Plots of spectral reflectance functions [wild type (W), *mixta* (M), *nivea* (N), double mutant *mixta/nivea* (D)] in a colour hexagon representing opponent colour space for bumblebee vision, where receptors are assumed to be adapted to the green foliage background

Table 1 Receptor quantum catch values P and receptor excitation values E in the bumblebees' UV (U), blue (B) and green (G) receptors, calculated for the four *Antirrhinum majus* genotypes under consideration

Genotype	P_U	P_B	P_G	Σ_P	E_U	E_B	E_G
background	1	1	1	3	0.5	0.5	0.5
wild type	0.79	1.43	0.45	2.67	0.44	0.59	0.31
mixta	0.85	2.03	0.61	3.49	0.46	0.67	0.38
nivea	6.69	9.00	4.56	20.25	0.87	0.90	0.82
double mutant	5.25	7.33	3.54	16.12	0.84	0.88	0.78

Note that by definition (Eqs. 1–3), the receptor quantum catch for the adaptation background is 1 for each receptor, and the excitation value is 0.5 for the background. Intensity is the sum of the three receptor quantum catch values—this is 3 for the background by definition

between the detectability of wild type and *mixta* mutant flowers (paired samples *t*-test, $t = 0.488$, df = 9,

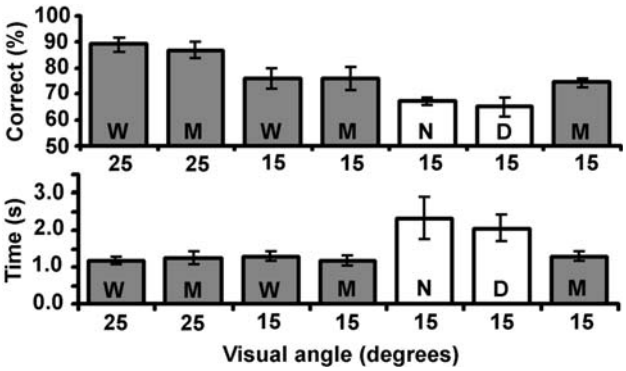

Fig. 4 Detection frequency and response times for four different lines of *Antirrhinum* flowers (W = wild type, M = *mixta*, N = *nivea*, D = double mutant *mixta/nivea*) presented on a homogeneous green background. Bees were tested on all flower types in the order from left to right, and then re-tested on wild type to control for sequential effects (see final column). The *X*-axis shows the visual angle that the flower subtended when the bee was at the decision point in the Y-maze. (**A**) Frequency with which bees chose the correct arm of the Y-maze depending upon visual angle flower subtended or flower type (W, M, N, D). (**B**) Response time for bees detecting the flowers; white flowers (*nivea* or *mixta/nivea*) took a significantly longer time to detect than wild type or *mixta* (pigmented) flowers. Bee performance on wild type or *mixta* mutant flowers was almost identical considering either the dependent variables of detection or response time

$P = 0.64$). At the more challenging visual angle of 15° (distance of 37 mm), detection rate was lower (near 75%) but still not significantly different between the flower types ($t = 0.074$, df = 9, $P = 0.943$); neither were the decision times ($t = 0.777$, df = 9, $P = 0.457$. However, bees detecting white non-pigmented (nivea) *Antirrhinum* flowers at this distance were substantially poorer both in terms of detection rate (paired samples *t*-test, $t = 3.020$, df = 9, $P = 0.014$) and decision time (paired samples *t*-test, $t = 2.598$, df = 9, $P = 0.029$) compared to the pigmented flowers. From the perspective of a human observer this is counterintuitive, since white flowers appear much brighter and therefore easily detectable to humans.

There was no significant difference between flowers with or without the *MIXTA* locus active (i.e. wild type vs. *mixta* or *nivea* vs. *mixta*/*nivea*) in terms of detection accuracy ($t = 0.293$, df = 9, $P = 0.776$) or detection time ($t = 1.239$, df = 9, $P = 0.247$). Thus the only apparent effect of the *mixta* or *nivea* mutations on the colour perception of bees that might directly affect foraging performance was that non pigmented white flowers are not detected as well as pigmented purple or pink flowers. The deteriorating performance of bees from early to late tests (shown from left to right in Fig. 4) is not a sequence effect (e.g. caused by fatigue) since when bees where tested on mixta mutants at a

visual angle of 15° at the end, their performance reverted to ca. 75% correct choices, as exhibited earlier in the same test.

Experiment 1B: Flower detection with a complex background and distractor flowers

We tested whether a more complex visual environment produced by a Julesz pattern background and white distractor flower in the opposite Y-maze arm might affect the ability of bumblebees to detect either wild type or *mixta* flowers. In this experiment, the detection rates were practically identical with mean detection of wild type flower = 72.7% (SD 12.5) and *mixta* mean = 72.7% (SD 11.9); results were not significantly different (paired samples *t*-test, $t = 0.004$, d.f. = 14, $P = 0.997$). This experiment further extends the findings of experiment 1A, by showing that colour difference in *Antirrhinum* due to the role of the *MIXTA* locus in regulating petal cell shape does not affect the ability of bees to detect flowers even if the background is noisy.

Experiment 2: Discrimination between differently coloured *Antirrhinum* mutants?

The colour distance between wild type and *mixta* flowers was 0.059 hexagon units. Earlier work shows that this colour distance is discriminable by bees, but only with difficulty (Dyer and Chittka 2004c). Conversely, the colour distance between wild type and white *nivea* flowers was 0.175 hexagon units, which makes it about three times larger than the distance between wild type and *mixta* flowers. Colours with such a distance in colour space are predicted to be well distinguishable (Dyer and Chittka 2004a). After training to the wild type flowers, bees discriminated these flowers from the *mixta* mutants with 69.2% accuracy (choices significant from chance; $\chi^2 = 17.6$, df = 1, $P < 0.01$). This result indicates that whilst bees *can* discriminate between wild type and *mixta* flowers, the colours are perceptually similar so that bees make a substantial number of errors (approx. 30%). When these bees were required to discriminate between the wild type and white *nivea* flowers, all bees chose the wild type 100% of the time, confirming the colorimetric prediction that this is a perceptually easy task for bee vision.

Experiment 3: Innate preferences for *Antirrhinum* genotypes

We evaluated the flower colour preferences of 10 naïve bumblebees tested individually in a 4 min trial. The bees made relatively few attempts to land on flowers (wild type 10, *mixta* 8, *nivea* 7, *mixta*/*nivea* 8) and thus bee approaches to flowers were used to quantify innate preferences (wild type 26.9% ± 7.9 SD, *mixta* 27.6% ± 5.5 SD, *nivea* 20.1% ± 5.3 SD, *mixta*/*nivea* 25.4% ± 7.3 SD). There was no significant difference in colour preference between wild type and *mixta* flowers (paired sample *t*-test, $t = 0.251$, df = 9, $P = 0.807$). There was also no significant difference in colour preference between *nivea* and *mixta*/*nivea* flowers (paired sample *t*-test, $t = 2.120$, df = 9, $P = 0.063$). However, consistent with previous reports on bumblebee colour preferences (Lunau et al. 1996), these bees did prefer the pigmented flowers over the white flowers (pigmented 27.2% ± 2.9 SD, unpigmented 22.8% ± 2.9 SD, paired samples *t*-test, $t = 2.421$, df = 9, $P = 0.039$; Fig.5).

Discussion

Our study provides empirical data to show that single mutations have profound effects on flower attractiveness and detectability, though not necessarily in ways that are intuitively apparent when flowers are inspected by human observers. On the other hand, the rate at which bees detected different *Antirrhinum* flowers (Fig. 3) fits well with colorimetric predictions of how colours would be perceived by bumblebees. We will discuss the effects of the various mutations on bee visual perception in turn.

For bumblebees, unpigmented *nivea* flowers, despite their exceptionally bright, and (for humans) highly contrasting appearance, were detected with lower speed and accuracy than wild type flowers. Why do bees have difficulty in detecting flowers that produce a strong contrast in all of the three receptor channels, the bees' UV, blue and green receptors (Table 1)? Before there were any data on the detectability of different colours by bees, Chittka et al. (1994) made a somewhat counter-intuitive prediction: that white, UV reflecting flowers

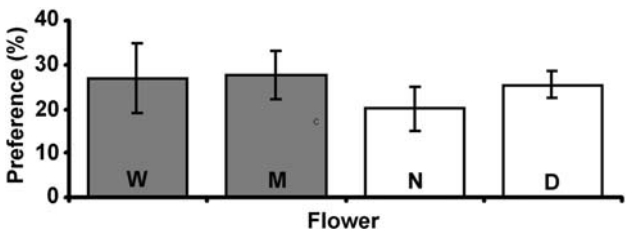

Fig. 5 Innate preferences of naïve bumblebees for different types of *Antirrhinum* flowers (W = wild type, M = *mixta*, N = *nivea*, D = Double mutant *mixta/nivea*) during a 4 min non-rewarding test. Error bars: SD

should be hard to detect for bees on a green foliage background, and that such flowers might therefore be rare in nature (see also Kevan et al. 1996). At the time, this prediction was based on the observation that, in colour discrimination tests, bees attached relatively little importance to brightness (Chittka et al. 1992), and the fact that such UV-reflecting, white flowers would differ from green foliage more in brightness than in colour (Chittka et al. 1994).

Giurfa et al. (1996) found empirical support for this prediction, by experimentally measuring detectability of artificial flowers presented to bees in a Y-maze: the authors found that a target that stimulated the bees' UV, blue and green receptors roughly equally (''bee-white'') was exceptionally difficult to detect for bees—only 2 out of 19 bees learnt the task at all, and even those two only after very extended training. At that time, the implication that the low detectability of bee-white flowers might reduce such flowers' fitness remained hypothetical (Kevan et al. 1996). The first empirical evidence to support this hypothesis followed when Glover and Martin (1998) described in *Antirrhinum majus* a unique system which allowed the rigorous testing of the function in pollination of a single floral feature, using isogenic mutant lines. They found that white *nivea* mutants, when exposed to bees in a field plot, had lower fruit set than wild type flowers (see also Waser and Chittka 1998). Comba et al. (2000) found that this effect was likely mediated through pollinator behaviour, because the mutation reduces pollinator visits. However, until this study, experimental support for the notion that different natural flowers differ in detectability remained outstanding. Our study places *Antirrhinum* flowers and their pigment-less mutants directly into an established laboratory paradigm to study bee vision, the Y-maze setup, and therefore shows directly that the lower reproductive success of *nivea* mutant flowers is likely mediated through their reduced detectability.

It is worth pointing out that the detectability of *nivea* mutants is low despite generating a strong contrast in each individual receptor channel. It has become fashionable in recent years to analyse UV signals without reference to contributions by (and interaction with) other receptors (see Kevan et al. (2001) for a critique of this approach). Our results show that, despite a high UV contrast, *nivea* mutants are poorly detectable. This is because bees do not evaluate the UV receptor signal in isolation when analysing flowers, but instead process output from all three photoreceptor types. Our findings clearly demonstrate that a casual assessment of UV signal or UV contrast is not even useful for basic predictions about target conspicuousness or crypsis (Chittka et al. 1994; Kevan et al. 2001).

A signal in a different visual channel, the green receptor, could potentially be of more importance. White *nivea* mutant flowers produce a strong contrast with foliage in the green receptor, and this receptor signal has been shown to be fundamental in target detection and tasks related to motion vision (Lehrer et al. 1990; Giurfa et al. 1996; Spaethe et al. 2001; Chittka and Tautz, 2003). It appears, however, that there are complex interactions between the green receptor channel and the colour vision channel, so that in targets with near-zero colour contrast, even a strong green contrast cannot rescue the target's detectability. This was found by Giurfa et al. (1996) using artificial flowers, and is confirmed here for natural flowers. In conclusion, our findings support the notion that white flowers with UV reflectance (such as the *nivea* mutants of *Antirrhinum*) are selected against because of their poor detectability for bees (Chittka et al. 1994), and are therefore rare in nature (Kevan et al. 1996). Most flowers that appear white to humans absorb light in the UV range, and therefore appear coloured to bees (Kevan et al. 1996).

The comparison of detectability of purple conical-celled wild type flowers and pink flat-celled *mixta* mutant flowers provides another example where human vision cannot usefully predict the bees' behaviour. To human observers, *mixta* mutants appear paler and less sparkling, and one might therefore conclude that they might be less attractive to naïve foragers, and less easily detectable. The restriction of conical cells to the petal epidermis, and the frequency with which they are found on petals, has led several authors to conclude that they must function to enhance attractiveness of the corolla to pollinating animals. It was not possible to determine in any more detail how conical petal cells functioned until the identification of the *mixta* mutant of *Antirrhinum* allowed comparisons of conical and flat cells within a single species (Noda et al. 1994). The use of isogenic lines differing only in the shape of the petal epidermal cells allows accurate and sensitive dissection of the function of these specialised cells, without the complication of multiple other variables. Similarly, mutant lines of *Antirrhinum* are available with alterations in characters such as floral symmetry, landing platform development, floral organ development and petal pigment patterning, all of which may provide useful tools for analysis of the adaptive significance of such traits through their interactions with pollinators.

To bees *mixta* mutant and wild type flowers are predicted to have very similar colour contrast (Table 1) and the accuracy and speed with which these flowers were detected by bees was almost identical,

even in simulated complex foraging environments where the background is noisy and an irrelevant distractor must be ignored. This is not because bees could not discriminate the flowers. Despite their relative similarity in the bees' colour space, bees could learn to distinguish the two flower types, if specifically trained to do so. Taken together, our results show that, despite the profoundly different appearance of wild type and *mixta* mutants, bees are unlikely to discriminate against *mixta* mutants based on colour alone.

However, in a field environment, bees prefer visiting wild type flowers over *mixta* mutants (Comba et al. 2000) and such flowers also have higher fruit set when open-pollinated (Glover and Martin 1998). Our results above indicate that colour itself is not responsible for this difference—but colour could be used as a cue to reward value. Because the conical cells found in the epidermis of wild type flowers focus incident light into the pigment-containing vacuoles, not only the colour but also the floral temperature may be affected by the *MIXTA* locus (Comba et al. 2000). Wild type flowers may, therefore, be sometimes warmer than *mixta* mutants—and bees prefer warmer flowers (Dyer et al. 2006). Moreover, since bees can learn to use colour as a cue to flower temperature (Dyer et al. 2006), bees might learn to discriminate against cooler *mixta* mutant flowers by using flower colour as a signpost. Alternatively, differences in petal cell shape may influence the way a petal feels to a potential pollinator (Kevan and Lane 1985), and thus affect the time taken to efficiently handle the flower. Again, colour could be used as a cue to distinguish between flowers with different handling properties. Approximately 80% of the approximately 200 species of angiosperms analysed to date have petal epidermal surfaces composed exclusively, or almost exclusively, of conical cells (Kay et al. 1981). The fact that this innovation is so widespread may indicate a general function in increasing floral attractiveness through a number of routes, any of which can be learned using the linked colour difference. In conclusion, our results show the power of combining a plant genetic model with bee visual psychophysics to provide an integrated understanding of the evolution of floral traits.

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