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ORIGINAL PAPER

Photoreceptor spectral sensitivity in island and mainland populations of the bumblebee, *Bombus terrestris*

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Abstract Most species of flower-visiting Hymenoptera are trichromatic, with photoreceptor spectral sensitivity peaks in the UV, blue and green regions of the spectrum. Red flowers, therefore, should be relatively difficult to detect for such insects. Nevertheless, in population biological studies in the bumblebee, *Bombus terrestris*, the Sardinian island population (*B. t. sassaricus*) displayed significantly higher responses to red artificial flowers (in tests of innate colour choice and detectability) than several mainland populations of the same species (Chittka et al. in Cognitive ecology of pollination, pp 106–126, 2001; Popul Ecol 46:243–251, 2004). Since there is relatively little physiological data on population differences in sensory systems, we used intracellular recording to compare photoreceptor spectral sensitivity in *B. t. sassaricus* and the southern European and Mediterranean population, *B. t. dalmatinus*. The results show both populations to be UV–blue–green trichromats, but with a small but significant increase in long-wave sensitivity in island bees. Spectral peaks were estimated at 348, 435 and 533 nm (*B. t. dalmatinus*) and 347, 436 and 538 nm (*B. t. sassaricus*) for UV, blue and green receptors, respectively. There were no significant differences in UV and blue receptor sensitivities. We found no photoreceptors maximally sensitive to red spectral light in the Sardinian population and model calculations indicate that the behavioural population differences in colour responses cannot be directly explained by receptor population differences.

Keywords Photoreceptor · Spectral sensitivity · Colour vision · Island population · Wavelength discrimination

Introduction

Most species of flower-visiting Hymenoptera are trichromatic, with photoreceptor spectral sensitivity peaks in the UV, blue and green regions of the spectrum (Menzel et al. 1988; Peitsch et al. 1992). The long wave sensitivity of such species peaks in the region of 530–570 nm, i.e. in the green–yellow region of the spectrum from the human point of view. UV-absorbing, red flowers should therefore be relatively difficult to detect for such insects (especially when viewed against a green background). At wavelengths greater than the spectral peak of the long-wave receptor, and where the response of the adjacent middle-wave (blue) receptor is negligible, changes in the spectral composition of reflected light can only be translated into changes in the intensity of the long-wave receptor signal; that is to say, detection would have to be mediated mainly or wholly by intensity rather than chromaticity.

Despite the above considerations, in a detailed population biological study of island and mainland subspecies of the bumblebee, *Bombus terrestris*,

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Chittka and colleagues found a remarkable behavioural preference for red flower colour in Sardinian island bees that had never seen flowers before (*B. t. sassaricus*). Chittka et al. (2001) compared innate colour preference across seven subpopulations of *Bombus terrestris* from various European and Mediterranean mainland and island locations, by assessing behavioural choice of laboratory-raised, colour-naive bees for artificial flowers of various colours. All populations tested show a strong preference for blue and violet flowers over white, orange or yellow ones. However, the Sardinian population (*B. t. sassaricus*) exhibited a striking additional preference for red (UV-absorbing) flowers, which was significant in all colonies tested, and which in some individual colonies even exceeded the blue–violet preference. Even more suggestively, detection times for small red flowers were significantly shorter for Sardinian (*B. t. sassaricus*) compared with mainland (*B. t. terrestris*) bees (Chittka et al. 2004).

These results do not in themselves provide evidence for differences in colour vision between island and mainland bees, but nevertheless, they prompt the question whether island bees are better at seeing red than their mainland conspecifics. The available data suggest that receptor-based colour vision in the Hymenoptera is rather conservative, with almost all species bearing very similar sets of UV, blue and green receptors (Peitsch et al. 1992). However, in many comparative studies, data comes from only a few individuals, leaving open the possibility that subtle differences between individuals, or between closely related species, could be missed (Chittka et al. 2001, 2004). In addition, island populations are especially interesting in this context because of the greater possibility for chance processes leading to the spread of evolutionary innovation. Finally, there is relatively little data on population differences in sensory systems, and no data on insect colour vision with sufficient sample sizes to allow detailed comparison between populations. With this in mind we asked whether any difference in photoreceptor spectral sensitivity could account for the behavioural preference for red of the Sardinian bees. We therefore used intracellular recording to measure and compare photoreceptor spectral sensitivity in *B. t. sassaricus* and a mainland population from southern European and the Mediterranean area, *B. t. dalmatinus*. We find no evidence for the presence of an additional, red receptor in the island bees. However, our results show that the green receptor of the island bees is displaced in the long wavelength direction by about 5 nm relative to their mainland conspecifics.

Materials and methods

Animals

Worker bumblebees used in these experiments were obtained from commercially available colonies (*B. t. dalmatinus*: Koppert BV, Netherlands; *B. t. sassaricus*: Biobest, Belgium). To make sure that any differences in spectral sensitivity of island bees were not simply a result of selection in a breeding regime by commercial breeding, an additional eight *B. t. sassaricus* workers used in this study were caught in the wild in Sardinia. Prior to electrophysiological recordings, bees were placed in the freezer compartment of a refrigerator for 10–14 min to immobilize them, then fixed with sticky wax to a platform mounted on a ball joint. The head and thorax were firmly immobilized with wax, but care was taken to leave the abdomen free for ventilation movements. A small incision, covering a few facets of the compound eye, was made in the dorsal cornea for microelectrode insertion. The hole was sealed with a viscous silicon grease (Dow Corning). We used electrodes with a long taper so that, by varying the angle of attack, we could sample cells from all regions of the retina apart from the most dorsal extreme.

Recording and stimulation

Recordings were made with microelectrodes of 100–150 MΩ resistance (Flaming/Brown P-97, Shutter Instruments) when filled with 2 M potassium acetate and measured in the retina. The reference electrode was a chlorided silver wire inserted into the contralateral eye. Signals were amplified using an Axoclamp 2B (operating either in bridge or discontinuous current clamp mode), displayed on a digital oscilloscope, and simultaneously digitized at 5 kHz with a CED micro1401 (CED, Cambridge UK) and written to disk.

Light was delivered from a 300 W tungsten–halogen lamp via a monochromator (M300, Bentham, UK). The monochromatic beam, whose output was controlled by a set of neutral density filters to vary light intensity over 4 log units, was then focused onto one end of a liquid light guide (3 mm core diameter). The other end of the light guide was mounted on a Cardan arm perimeter device. The bee was positioned at the centre of rotation of the Cardan arm, 6 cm from the opening of the light guide. Stimulus duration was controlled using a Uniblitz LS2 shutter (Vincent Associates, NY, USA) with a 0.3 ms rise time, and was usually set at 10 ms. The system was calibrated by measuring irradiance with a spectrophotometer (Avaspec-2048; Avantes, Eerbek, NL) using a calibrated

UV–Vis light source (DH 2000-CAL, Ocean Optics, Dunedin, Florida). Measurements were repeated at 10 nm intervals over the range 320–650 nm for all neutral filter settings. Energy spectra were converted to quantum flux spectra.

Once the electrode was in the retina, and its approximate angular position in the eye ascertained by rotating the Cardan arm to maximize the field response (ERG) to flashes of white or green light, the preparation was left in complete darkness for at least 30 min. On commencement of a recording session, exposure of the preparation to light was restricted by minimizing flash duration and intensity during the search for a photoreceptor. The dissecting lamp was not normally switched on again unless it became necessary to change the electrode; on occasions when this occurred, the preparation was again dark adapted for a minimum of 30 min. Once stable recordings from a photoreceptor were obtained, the light source was carefully centred using the Cardan arm. The preparation was then again left in complete darkness for at least 4 min (but more usually up to 10 min) before collecting any data (cells which remained stable for 10 min following impalement had a relatively high likelihood of yielding consistent recordings for 60–90 min). During the course of an experiment, the integrity of an intracellular recording was constantly assessed by monitoring membrane responses to brief hyperpolarizing current pulses (0.1 nA, 20 ms), timed to appear 50–60 ms before flash onset. Any change in apparent input resistance was usually correlated with changes in spectral response amplitude (and therefore, spectral sensitivity), and often also with changes in resting membrane potential. Such recordings were immediately discontinued.

Measurement and analysis

We used the flash method for measuring spectral sensitivities (DeVoe et al. 1997). First, the cell was calibrated by recording responses to flashes of monochromatic light varying in intensity over 4 log units (V/log I function) at a fixed wavelength (Fig. 1a). These data are well fitted by a hyperbolic function of the form

$$V/V_{\max} = (RI)^n/((RI)^n + 1) \quad (1)$$

where V is the response amplitude (in mV) to a flash of intensity I (in quantal flux), $V_{\max}$ is the saturated response amplitude, R is the reciprocal of the intensity giving a response of 50% saturation, and n is a constant determining the slope of the function (Naka and Rushton 1966; Laughlin 1981; Menzel et al. 1986). In some experiments, we compared directly the spectral sensitivities by recording the V/log I function at different wavelengths. However, this is time consuming and in practice only a limited number of wavelengths can be measured. In most cells we measured the V/log I function at a wavelength close to the presumed peak spectral sensitivity and then scanned the spectrum with moderate intensity flashes in 10 nm increments. The spectral sensitivity ($S(\lambda)$) function was then estimated by sliding the V/log I function along the wavelength axis to fit the measured response. This method assumes that the intensity-response functions at different wavelengths are parallel, as required by the principle of univariance (Naka and Rushton 1966).

Previous studies have established limitations in the accuracy of the flash method (Menzel et al. 1986). However, by adhering to the following precautions, we found we were able to obtain reasonably consistent spectral data from different cells of a given class. First, we only accepted data from cells where maximal responses to spectral light of >40 mV could reliably be evoked. Second, we allowed each cell at least 4 min in complete darkness, allowing recovery from any adaptation effects from flashes delivered during the searching and initial characterization of a cell. Third, we kept the flash duration very short (10 ms), minimized exposure to high intensity flashes, and allowed 2–3 min recovery from such flashes when necessary in V/log I measurements. During spectral scans we limited light intensity so as to limit response amplitudes to less than half maximal, with 8 s between flashes. Finally, we only accepted data where responses to flashes at a series of wavelengths (scans) were repeated at least four times, with no significant change in cell resting potential and response amplitude (at a given wavelength).

To estimate the peak sensitivity ($\lambda_{\max}$) for a given cell, we fitted the normalized spectral data with a simple exponential function as described by Stavenga et al. (1993), using the lognormal function with no more than a third degree term in the exponent, which reliably describes the main peak of the absorbance spectrum. Where $x = \log(\lambda/\lambda_{\max})$, the sensitivity S is described by

$$S = A \ \exp[-a_0 x^2(1 + a_1 x)]. \quad (2)$$

By fitting this equation to the spectral data we were able to calculate the peak, $\lambda_{\max}$. Curve fitting was done using the program FigSys (Biosoft, Cambridge, UK).

Our aim was to search for differences in photoreceptor spectral sensitivities between island and mainland bees. In order to model the effects of any such

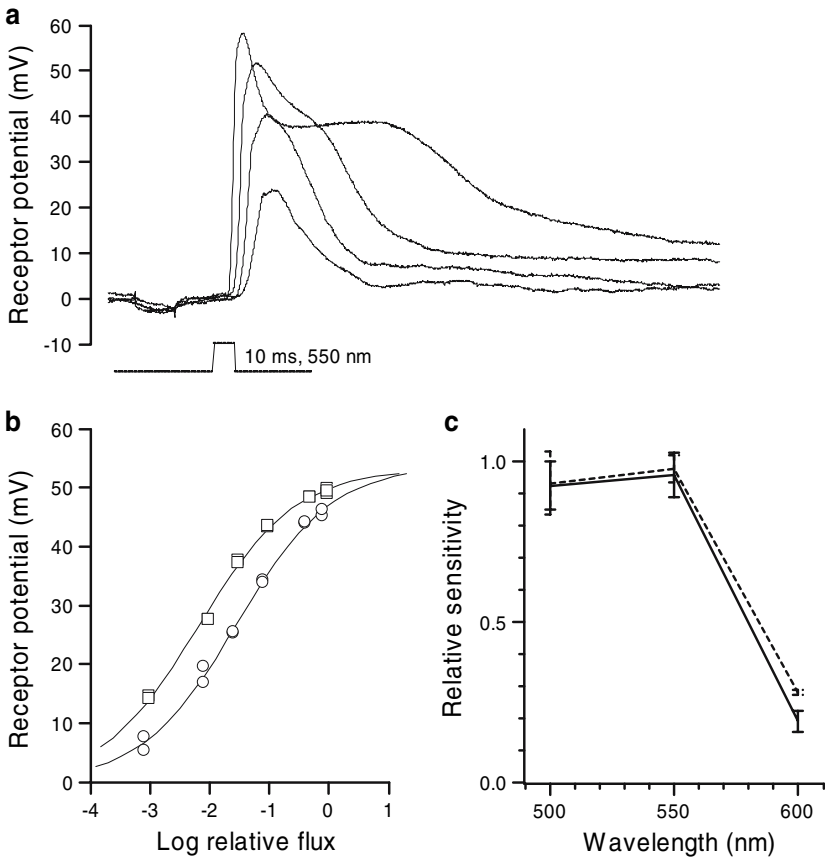

Fig. 1 Spectral sensitivity as measured by intensity-response functions (V/log I function). **a** Responses of a photoreceptor to 10 ms flashes of 550 nm monochromatic light varied in intensity over 3 log units. **b** V/log I functions for the same cell plotted at 550 (*squares*) and 600 (*circles*) nm. The data were fitted with the self-shunting equation (see "Materials and methods"), and the sensitivity R estimated using an iterative curve-fitting process. **c** Spectral sensitivity was computed from V/log I functions measured at 500, 550 and 600 nm. Mean normalized values (±SD) plotted for 12 cells from *B. t. sassaricus* (*dashed lines*) and 6 cells from *B. t. dalmatinus* (*solid lines*). Error bars: ±1.0 SD

differences on colour discrimination capabilities in the two populations, we used the colour hexagon method described by Chittka (1992). This is a chromaticity diagram in the form of an equilateral hexagon where the trichromatic photoreceptor excitations are plotted at an angle of 120° from each other. It has the advantage of coding chromaticity in a generalized colour opponency space, without commitment to any particular colour opponent coding processes.

The relative amount of light, P, absorbed by each photoreceptor type is given by

$$P = R \int_{300}^{700} I_s(\lambda) S(\lambda) D(\lambda) \mathrm{d}\lambda \tag{3}$$

where $I_s(\lambda)$ is the stimulus intensity, $S(\lambda)$ is the spectral sensitivity function of the receptor in question, and $D(\lambda)$ is the spectral function of the illuminating daylight. R is the range sensitivity; it is determined by

$$R = 1 \Big/ \int_{300}^{700} I_B(\lambda) S(\lambda) D(\lambda) \mathrm{d}\lambda \tag{4}$$

where $I_B(\lambda)$ is the spectral reflectance of the background to which the photoreceptors are adapted. We assume the receptors are adapted to a green foliage background and we use the standard daylight function D65 (Wyszecki and Stiles 1982) for the illumination spectrum. To calculate the spectrum locus, we adjust the intensity of each spectral light so that it produces a sum of photoreceptor quantum catches of 3. This is done following formulae 5.1–5.3.

$$P_U = 3 \times R_U S(\lambda)_U / (R_U S(\lambda)_U + R_B S(\lambda)_B + R_G S(\lambda)_G) \tag{5.1}$$

$$P_B = 3 \times R_B S(\lambda)_B / (R_U S(\lambda)_U + R_B S(\lambda)_B + R_G S(\lambda)_G) \tag{5.2}$$

$$P_G = 3 \times R_G S(\lambda)_G / (R_U S(\lambda)_U + R_B S(\lambda)_B + R_G S(\lambda)_G) \tag{5.3}$$

where $P_{U,B,G}$ are the quantum fluxes in the UV, blue and green receptors, $R_{U,B,G}$ are the range sensitivities for the three receptor types (see Eq. 4) and $S(\lambda)$ is the spectral sensitivity of the photoreceptor at the wavelength in question. The quantum catch, P, for each receptor can then be converted to relative

excitation V, by using Eq. 1 above, assuming $n = 1$ (see Backhaus and Menzel 1987 for details) and $V_{max} = 1$. It follows that for quantum flux $P = 1$, the relative voltage signal is V is 0.5. The excitation values, V, (the normalized receptor potentials) are then plotted for each photoreceptor as vectors 120° apart. Vector addition of any triplet of photoreceptor activations then yields a point in colour space. The x, y coordinates of this point are determined by simple trigonometry

$$x = \sin 60^{\circ}(E_L - E_S) \quad (6)$$

and

$$y = E_M - \cos 60^{\circ}(E_L + E_S) \quad (7)$$

where E_S, E_M and E_L are the excitations of the long, medium and short wave receptors, respectively, which in the present study correspond to the UV, blue and green photoreceptors. The location of any spectral light in the colour hexagon can thus be determined, and the Euclidean distance between any two such spectral stimuli gives their relative discriminability. We modelled spectral stimuli 10 nm apart to plot the difference sensitivity function (inverse $\Delta\lambda/\lambda$) as in Fig. 5. See Chittka (1992) for further details.

A two-tailed Student's t-test was used to evaluate differences in spectral sensitivity or other values. All quantitative results in the text and figures are expressed as mean ± SD.

Results

Stable intracellular recordings were readily obtained from *Bombus terrestris* photoreceptors, from both island and mainland bees. Following impalement, a cell was initially characterized by a sequence of 10 ms flashes of spectral light ranging from 350 to 650 nm, delivered in 50 nm increments with a 20 s delay between flashes. All photoreceptors generated peak responses to spectral light either at 350, 450 nm, or either 500 or 550 nm. On the basis of these raw voltage responses, then, photoreceptors could unambiguously be divided into three classes. Spectral analysis confirmed that these 3 classes conformed to typical hymenopteran photoreceptors with peak sensitivities in the UV, blue and green regions of the spectrum (see below). Although cells generating peak voltage responses to 500 or 550 nm also generated large responses to 600 nm, no cells were recorded with larger responses to 600 or 650 nm than 500 or 550 nm. This was true for both island and mainland bees (from over 160 intracellular recordings). Therefore our results provide no evidence for the existence of an additional long-wave receptor in island bees (although of course it cannot entirely be ruled out that such receptors exist, but are very rare).

Green receptors

Green receptors, as in previous studies (e.g., Peitsch et al. 1992), were the most common spectral class encountered in all experiments. In initial experiments, where we scanned the spectrum from 350 to 650 nm in 50 nm increments, we noted a small tendency for increased long-wave sensitivity of the *B. t. sassaricus* green receptor at 600 nm. To confirm this, we directly compared the spectral sensitivity of *B. t. sassaricus* and *B. t. dalmatinus* green receptors, by computing sensitivity R from V/log I functions measured at 500, 550 and 600 nm. This also provided direct estimates of the maximal response amplitude, V_{max}, and the slope parameter n at different wavelengths, thus confirming the principle of univariance for the bumblebee (Fig. 1b). We found a significant increase in relative sensitivity of the *B. t. sassaricus* green receptor at 600, but not 500 or 550 nm (Fig. 1c).

Although the above result provides direct evidence for an increased sensitivity of the *B. t. sassaricus* green receptor to longer wavelengths beyond its spectral peak, the spectral resolution is too low to determine whether the effect is due to a small long-wave shift in peak sensitivity, or a change in shape of the spectral function (or both). Therefore we next analysed the $S(\lambda)$ function of the green receptor from the two populations in more detail, by means of spectral scans in the region of 500–600 nm in 10 nm increments. Figure 2a shows the $S(\lambda)$ functions for 28 cells from *B. t. sassaricus* and 22 from *B. t. dalmatinus*. Green receptors from both populations show a peak in the region of 530–540 nm, in agreement with previous reports (Autrum and Zwehl 1964; Menzel 1979; Menzel et al. 1986; Peitsch et al. 1992). However, while the two populations showed no significant difference in sensitivity to wavelengths on the short wavelength limb of the spectral sensitivity function, green receptors from *B. t. sassaricus* were significantly more sensitive to light on the long wavelength limb; differences between the two populations were significant at wavelengths between 570 and 610 nm at $P < 0.001$ (580, 600 nm) or $P < 0.05$ (570, 590, 610 nm; two-tailed t-test). When we fitted spectral data from individual cells with simple exponential functions of the form described by Stavenga et al. (1993; see "Materials and methods"), in order to estimate the peak wavelength sensitivity for each cell, the range of λ_{max} values was relatively broad (526–555 nm), with overlap between the two popula-

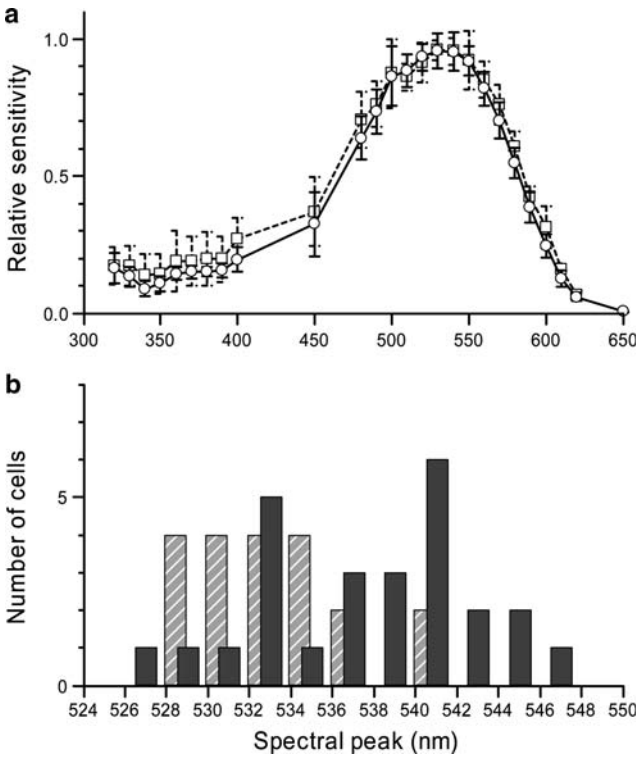

Fig. 2 Comparison of green receptor spectral sensitivity in *B. t. dalmatinus* and *B. t. sassaricus*. **a** Spectral sensitivity functions for *B. t. dalmatinus* (*solid lines*, *circles*) and *B. t. sassaricus* (*dashed lines*, *squares*). Data points between 500 and 600 nm are means of 22 cells from 9 animals (*B. t. dalmatinus*) and 28 cells from 11 animals (*B. t. sassaricus*); for remaining data points *n* ranges from 8 to 18 cells. Error bars: ±1.0 SD. **b** Distribution of λ_{max} values for green receptors from *B. t. dalmatinus* (*light bars*) and *B. t. sassaricus* (*dark bars*). Data were fitted with exponential functions as described by Stavenga et al. (1993) in order to estimate peak sensitivity (λ_{max}) (see text for further details)

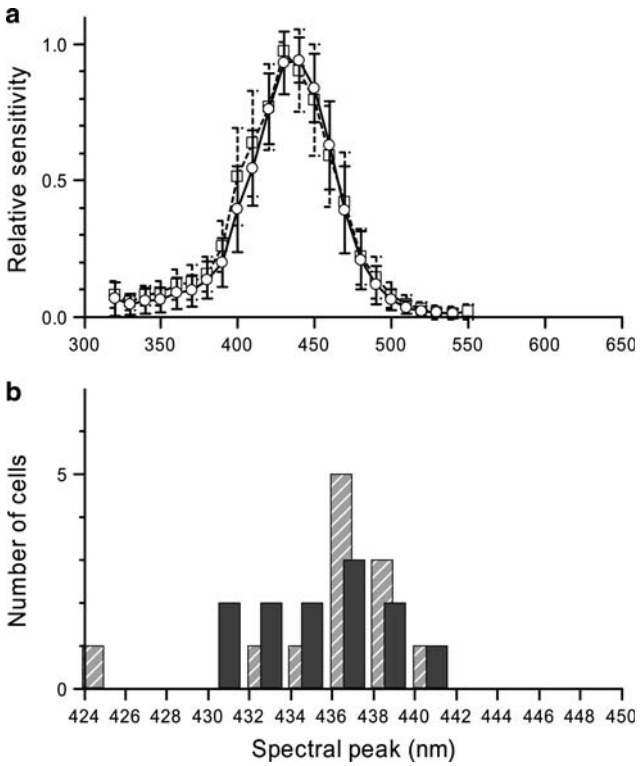

Fig. 3 Blue receptors. **a** Spectral sensitivity functions for *B. t. dalmatinus* (*solid lines*, *circles*) and *B. t. sassaricus* (*dashed lines*, *squares*). Error bars: ±1.0 SD. **b** Distribution of λ_{max} values for all blue receptors from *B. t. dalmatinus* (*light bars*) and *B. t. sassaricus* (*dark bars*)

tions (Fig. 2b); nevertheless, there was a statistically significant difference between the two means (*B. t. sassaricus*: 538.23 ± 6.11 nm; *B. t. dalmatinus*: 533.44 ± 3.58 nm, $P < 0.001$).

Blue receptors

Of recordings from cells responding most strongly to blue light, 25 were of sufficient quality and duration for detailed spectral analysis (*B. t. sassaricus*: n = 13; *B. t. dalmatinus*: n = 12). Spectral sensitivity was calculated from the *V*/log *I* function measured at 450 nm (or occasionally, both 400 and 450 nm), with the estimated parameters *n* and V_{max} used to solve for the sensitivity *R* over a range of wavelengths from 320 to 600 nm. (To increase the accuracy, within the limitations of the flash method, by averaging spectral responses, in many experiments we restricted these from 380 to 500 nm in 10 nm increments). We found similar sensitivity functions for both *B. t. sassaricus* and *B. t. dalmatinus* (Fig. 3a). Estimates of the spectral peaks yielded values of 434.87 ± 4.80 nm (*B. t. sassaricus*) and 436.43 ± 3.86 nm (*B. t. dalmatinus*); $P = 0.37$ (Fig. 3b). These figures are slightly higher than the peak of 428 nm reported by Peitsch et al. (1992) for the German population of *Bombus terrestris terrestris*.

We noted some variability of the responses of blue receptors to longer wavelength light. In many cells the response amplitude declined rapidly as the stimulus wavelength increased beyond about 500 nm, and the receptor potential became bi or multiphasic, with hyperpolarizing as well as depolarizing components. Five cells responded with 'pure' hyperpolarizations to spectral light of 540 or 550 nm. These results, which were also obtained in recordings from UV receptors, will be presented separately (Skorupski and Chittka in preparation).

UV receptors

Spectral analysis was done on 17 cells identified as UV receptors (seven from *B. t. sassaricus* and nine

from *B. t. dalmatinus*). Again, we found no significant sensitivity differences at any wavelength tested over the range 320–600 nm (typically 320–450 in 10 nm increments). Spectral peaks were estimated at 347.89 ± 8.08 and 346.86 ± 6.83 for *B. t. sassaricus* and *B. t. dalmatinus*, respectively (Fig. 4). These λ_{max} values are rather higher than that for the German population, *B. t. terrestris*, where Peitch et al. (1992) reported a peak sensitivity of 326 nm, one of the shortest wavelength sensitivity peaks for any hymenopteran photoreceptor.

Electrophysiological properties of photoreceptor classes

For each class of photoreceptor, we compared the parameters fitted to the *V*/log *I* function to test for population differences that might be indicative of more subtle differences in photoreceptor electrophysiological properties between populations. There was no between-population difference in the values estimated for the parameters *n* and V_{max} for any photoreceptor class. However, we did find that the *V*/log *I* function was markedly steeper for blue and UV receptors than for green receptors, as indicated by the estimated values for the slope parameter *n*, which were 0.47 ± 0.08 (n = 65) for green receptors and 0.66 ± 0.12 (n = 26) for blue (two-tailed *t*-test, $P < 10^{-7}$; island and mainland data combined). The mean slope parameter for UV receptors was 0.68 ± 0.13 (n = 17), which was not significantly different from that for blue receptors, but the difference from green receptors was highly significant ($P < 0.0001$). Since we have here used a *t*-test to compare measurements from three receptor classes, the rejection threshold should be adjusted accordingly: in this case a significance level set at 0.05 would actually require a *P*-value of approximately 0.0167 (the Bonferroni correction). However, both UV and blue receptors differed from green with *P*-values very much lower than this.

Photoreceptor spectral sensitivity and wavelength discrimination

To assess the implications of the small long-wave shift of the *B. t. sassaricus* green receptor, we plotted wavelength discrimination functions (inverse $\Delta\lambda/\lambda$ functions) according to the method of Chittka (1992). We modelled the receptors by fitting exponential templates of the form described by Stavenga et al. (1993) to the spectral data for receptors with λ_{max} values corresponding to those determined above for the three spectral receptor classes in *B. t. sassaricus*, and *B. t. dalmatinus*. The spectral loci of monochromatic lights 10 nm apart, over the range 300–650 nm, were then determined as described in "Materials and methods", and the reciprocals of the intervals between spectral stimuli plotted against wavelength (Fig. 5). This exercise reveals only very slight predicted differences in wavelength discrimination ability between the two populations, with both exhibiting maxima in the spectral regions around 400 and 490 nm. In the region of the second discriminability peak, the curve for *B. t. sassaricus* (Fig. 5, dashed line) is shifted very slightly to longer wavelengths, but this small effect is only evident up to about 530 nm, and at wavelengths >585 nm discriminability is effectively zero for both populations.

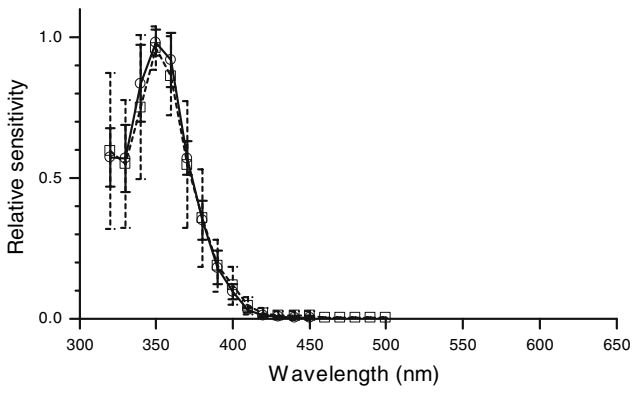

Fig. 4 Spectral sensitivity functions of UV receptors from *B. t. dalmatinus* (*circles*) and *B. t. sassaricus* (*squares*). Error bars: ±1.0 SD

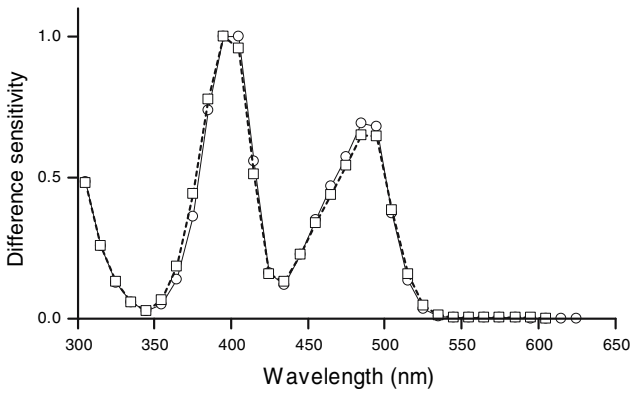

Fig. 5 Spectral discrimination curves for *B. t. dalmatinus* (*circles*) and *B. t. sassaricus* (*squares*). Discrimination is plotted as the reciprocal of $\Delta\lambda/\lambda$ (normalized to unity), calculated from the relative distances in the colour hexagon of 10 nm steps of monochromatic lights (see "Materials and methods" for further details)

Discussion

Sardinian island bumblebees of the species *Bombus terrestris* exhibit a remarkable innate preference for red floral colour, which is absent from their mainland (and some other island) conspecifics. This observation led Briscoe and Chittka (2001) to hypothesise that the incremental changes in spectral sensitivity required to produce a new colour receptor type may be highly unlikely to accumulate in a large population, due to simple inertia associated with classical population genetic factors. However, the chances of a small change in spectral sensitivity, with only a minor adaptive advantage, spreading through island populations, which may be exposed to repeated bottlenecks, is substantially higher. In addition, it remains possible that much of the apparent conservatism in arthropod colour receptor tuning could be due to the fact that physiological comparisons between a few individuals of different orders may simply be too coarse-grained.

Between-population comparison of photoreceptor spectral classes

In the present paper we have undertaken a detailed study of photoreceptor spectral tuning in individuals of the two bumblebee subspecies, *B. t. sassaricus* and *B. t. dalmatinus*. We find no evidence for an additional, 'red' receptor in *B. t. sassaricus*; therefore a simple explanation for the behavioural colour choice data in terms of photoreceptor evolution seems unlikely. Nevertheless, the question arises whether it is possible that we missed a relatively rare red receptor, or one restricted to particular eye regions. This question is especially pertinent in view of increasing evidence for spectral heterogeneity of ommatidia in the insect retina (Arikawa and Stavenga 1997; Wakakuwa et al. 2004, 2005; Spaethe and Briscoe 2005). While we cannot be entirely certain that *B. t. sassaricus* lacks a red receptor, we consider it unlikely. First, we recorded from many cells which we did not hold long enough for full spectral analysis, but during initial characterization (with 10 ms flashes at 50 nm increments) it was always possible to assign a cell broadly to a spectral class with peak voltage response occurring either in the UV, blue or green. For example, blue and UV receptors were more difficult to obtain stable recordings from, and we often briefly recorded such cells with high apparent input resistance and large amplitude responses, but which were then lost before full spectral analysis could be done. However, on no occasion did we even briefly record from a cell that exhibited larger voltage responses to >600 nm than 500–550 nm. Second, we do not believe we have systematically failed to sample any region of the retina other than the extreme dorsal rim, since this is where we made the incision for electrode access. However, we would not expect this region to be involved in recognition of floral colour (Giurfa et al. 1999), and where data are available, this region has been found to contain a preponderance of short-wave receptors. In the bumblebee *Bombus impatiens*, for example, it contains a high density of expression of UV-sensitive opsin (Spaethe and Briscoe 2005).

Red receptors are rare in the Hymenoptera, but they appear to have arisen more than once in other groups (see Briscoe and Chittka 2001). Many species of Lepidoptera, for example, have one or more photoreceptor class maximally sensitive to wavelengths >600 nm. This can be accounted for both by the presence of opsins with absorption maxima in the longer wavelength range, and also by the effect of optical filtering via screening pigments (e.g., Wakakuwa et al. 2004). However, our electrophysiological measurements from two populations of *Bombus terrestris* have failed to reveal any cells with spectral peaks beyond the 'typical' trichromatic hymenopteran complement of UV, blue and green receptors, regardless of the question of optical pathway or photopigment tuning.

Nevertheless our results show subtle differences between the green receptors of the two populations, in particular a small but significant red-shift in peak spectral sensitivity of about 5 nm in *B. t. sassaricus*. The basis for this long-wave shift is not yet known. In principle, it could be caused by a photopigment mutation. For example, a single amino acid substitution in the human long-wave opsin can shift spectral sensitivity by 3–4 nm (Merbs and Nathans 1992). However, a change in protein structure is not the only possible explanation for a change in spectral sensitivity, since it is well known that screening pigments and other filtering effects can significantly affect both the shape and the peak spectral sensitivity of insect photoreceptors (Stavenga 2002). An additional question concerns the variability in spectral peaks from individual green receptors around the two population means. Is this due to noise inherent in our experimental methods, or is there really such variability between receptors and individuals within a population? Another possibility is that there are two sensitivity peaks within a population, but differentially distributed between populations. Although we cannot exclude such a scenario, we believe the most conservative explanation, taking into account the limits with which the accuracy of a receptor potential can be measured, is that the distribution of sensitivity values around each population mean is due to noise inherent in electro-

physiological measurements. However, these possibilities are not mutually exclusive. It remains possible that genuine variation within populations or even individuals could be superimposed upon variation due to electrophysiological noise. Ultimately this question may be resolved by comparing sequence data for long-wave opsins from the two subspecies studied here.

Comparison with previous studies

The sensitivity peaks of green receptors of the two populations of *Bombus terrestris* studied here are very close to the value of 536 nm reported by Peitsch et al. (1992) for the German population, *Bombus terrestris terrestris*, and somewhat lower than the value of 548 nm reported by Meyer-Rochow (1980) for *Bombus hortorum* (although it should be noted that this figure is the wavelength of the interference filter eliciting the maximum response, and from the data presented it appears that the actual spectral sensitivity peak in that study is somewhat lower than this). The shapes of the sensitivity functions around the main peak in all three populations of *Bombus terrestris* (Fig. 5c in Peitsch et al. 1992, Fig. 2a in present paper) are also broadly similar in their asymmetry, due to a ''shoulder'' of sensitivity in the region between 450 and 500 nm. However, our data differ significantly in the UV region, where the German population shows a significant secondary peak ($S(\lambda)$ of about 0.3 at 350 nm), which was absent from our recordings of both island and mainland bees (where the relative sensitivity in the equivalent region is only about 0.15). Similarly with the blue receptor, we find a lower secondary sensitivity in the UV compared to the Peitsch et al. data. Finally, there is also a discrepancy in the case of the UV receptor, where Peitsch et al. (1992) report a spectral peak of 326 nm, which is among the shortest wavelength sensitivity maxima of known hymenopteran photoreceptors (Briscoe and Chittka 2001), whereas we find spectral peaks close to 350 nm for both populations. Although it cannot be excluded that this represents a population difference between *Bombus terrestris terrestris* and the two subspecies studied here, we must also consider the possibility that methodological differences may underlie the discrepancy. First, although Peitsch et al. (1992) compared an impressively large number of species, sample sizes for UV and blue receptors within a species were rather low (55 cells from 26 species for UV receptors, 77 from 30 for blue, implying 2 and 2–3 cells per species, respectively). In addition, Peitsch et al. (1992) used a fast scanning method, which measures the light intensity required to depolarize a photoreceptor by a criterion voltage level, which has the advantage of being very fast, but which necessarily results in the light-adaptation of the receptor (to an extent dependent upon the criterion voltage selected). In our study, light-adaptation was kept to a minimum by restricting flash duration to 10 ms, which is much shorter than the 100's of ms used in previous investigations with the flash method (Menzel et al. 1986). Our own observations suggest such effects could be quite significant: for example, even with 10 ms flashes, lights sufficient to depolarize a receptor by 50–60 mV also generated prolonged after effects, typically in the form of an after-hyperpolarization followed by a depolarization (data not shown) which could last for several 10's of seconds. In support of this it has been observed that the secondary sensitivity of honeybee green receptors in the UV is increased by adaptation to white light (Menzel and Blakers 1976). Similarly, in green receptors in the bumblebee, *Bombus hortorum*, the height of the secondary UV peak depends on light intensity used in the spectral scan, with higher light intensities generating a higher secondary peak (Meyer-Rochow 1980)

Photoreceptor spectral sensitivity and wavelength discrimination

Regardless of the above considerations, the small population difference in the spectral peak of the green receptor does not by itself lead to significantly greater wavelength discrimination in the red region of the spectrum in island compared to mainland bees. Our model calculations indicate no significant changes in wavelength discriminability across the spectrum resulting from a 5 nm shift in the spectral peak of the *B. t. sassaricus* green receptor; both populations exhibited enhanced wavelength discriminability in the regions of 400 and 490 nm (Fig. 5). These values are fairly typical for trichromatic hymenopterans (Chittka 1992; Peitsch et al. 1992). The only noticeable difference between the two curves of Fig. 5 is that predicted wavelength discrimination in *B. t. dalmatinus* is slightly more acute in the region of 490 nm. This effect is most likely due to the slight broadening of the sensitivity function of the *B. t. sassaricus* green receptor.

The present results leave us with an apparent conundrum, where we have a rather dramatic between-population behavioural difference in colour choice on the one hand, but only a subtle difference in photoreceptor spectral tuning on the other. Since relatively little is known about higher-order mechanisms of chromatic processing in the insect brain, the question of whether differences in colour discrimination could arise from differences in neural wiring upstream from

the photoreceptors remains open. In addition, further behavioural studies may be required to investigate the extent to which the difference in colour preference is related to differences in wavelength discrimination ability, as opposed to being based on achromatic cues.

Our conclusion, then, is that the clear behavioural differences between *Bombus terrestris* populations are probably not explained by any simple change at the receptor level. The Sardinian island population, *B. t. sassaricus*, is distinguished by a striking innate preference for red, and also performs more quickly in tests involving the detection of small red flowers. But despite these behavioural differences, the two populations appear essentially to be UV–blue–green trichromats, with very similar spectral classes of photoreceptor.

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Electronic supplementary material

Relative spectral sensitivities of UV, blue and green receptors of Bombus terrestris dalmatinus and B. t. sassaricus. Sensitivity was calculated in 4nm steps after fitting exponential templates of the form described in Stavenga et al. (1993) to the normalised, electrophysiologically measured sensitivity data for each class of receptor from each population.

	Relative sensitivity					
Wavelength	*B.t. dalmatinus*			*B.t. sassaricus*		
(nm)	UV	blue	green	UV	blue	green
300	0.099121	0.016162	0.120754	0.099121	0.063057	0.140553
304	0.144706	0.02154	0.123795	0.144706	0.066464	0.145011
308	0.204246	0.027982	0.126182	0.204246	0.070004	0.148987
312	0.278691	0.035444	0.127909	0.278691	0.073665	0.152405
316	0.367595	0.043796	0.129001	0.367595	0.077421	0.155206
320	0.46869	0.05281	0.129515	0.46869	0.081232	0.157354
324	0.577671	0.062177	0.129544	0.577671	0.08505	0.158851
328	0.6883	0.071517	0.129209	0.6883	0.08882	0.159744
332	0.792895	0.080417	0.128651	0.792895	0.09249	0.160138
336	0.883165	0.08848	0.128021	0.883165	0.096021	0.160185
340	0.951299	0.095369	0.127469	0.951299	0.099407	0.160081
344	0.991084	0.100869	0.127128	0.991084	0.102698	0.160049
348	0.99885	0.104934	0.127114	0.99885	0.10603	0.160311
352	0.974032	0.107736	0.127518	0.974032	0.109662	0.161069
356	0.919227	0.109688	0.128404	0.919227	0.114008	0.162488
360	0.839746	0.111472	0.129819	0.839746	0.119681	0.16468
364	0.742769	0.114029	0.131789	0.742769	0.127509	0.167709
368	0.636284	0.118551	0.134331	0.636284	0.138551	0.171588
372	0.528025	0.126437	0.13746	0.528025	0.154072	0.176295
376	0.424602	0.139241	0.141188	0.424602	0.175483	0.181783
380	0.330945	0.158585	0.145532	0.330945	0.204239	0.187997
384	0.250093	0.186044	0.150517	0.250093	0.241688	0.194883
388	0.183293	0.223004	0.156177	0.183293	0.288871	0.202398
392	0.130322	0.270484	0.162553	0.130322	0.34631	0.210519
396	0.089919	0.328941	0.169696	0.089919	0.413782	0.219239
400	0.060225	0.398068	0.177667	0.060225	0.490139	0.228572
404	0.039168	0.476622	0.18653	0.039168	0.573192	0.238551
408	0.024743	0.562305	0.196361	0.024743	0.659716	0.249222
412	0.015187	0.651747	0.207236	0.015187	0.745575	0.260643
416	0.00906	0.740612	0.219238	0.00906	0.826001	0.272878
420	0.005255	0.82386	0.232452	0.005255	0.89599	0.285999
424	0.002964	0.896141	0.246966	0.002964	0.950782	0.300077
428	0.001627	0.952305	0.262869	0.001627	0.986367	0.315185
432	0.000869	0.98796	0.280246	0.000869	0.999934	0.331392
436	0.000452	1	0.299184	0.000452	0.999699	0.348767
440	0.000229	0.987012	0.319761	0.000229	0.997996	0.367373
444	0.000113	0.949495	0.34205	0.000113	0.994828	0.387266

448	5.42E-05	0.889842	0.366112	5.42E-05	0.990205	0.408498
452	2.54E-05	0.812078	0.391995	2.54E-05	0.957592	0.431108
456	1.16E-05	0.721393	0.419724	1.16E-05	0.904158	0.455128
460	5.16E-06	0.623543	0.449302	5.16E-06	0.833376	0.48057
464	2.24E-06	0.524231	0.4807	2.24E-06	0.749734	0.507431
468	9.5E-07	0.428534	0.513852	9.5E-07	0.65825	0.535686
472	3.93E-07	0.340492	0.548644	3.93E-07	0.563957	0.565279
476	1.59E-07	0.262874	0.584915	1.59E-07	0.471448	0.596123
480	6.27E-08	0.197137	0.62244	6.27E-08	0.384521	0.628092
484	2.42E-08	0.143563	0.660933	2.42E-08	0.305966	0.661017
488	9.12E-09	0.101494	0.700032	9.12E-09	0.237503	0.694678
492	3.36E-09	0.069638	0.739305	3.36E-09	0.179838	0.728798
496	1.21E-09	0.04636	0.778238	1.21E-09	0.132828	0.763046
500	4.27E-10	0.029937	0.816247	4.27E-10	0.095692	0.797027
504	1.47E-10	0.018748	0.852674	1.47E-10	0.06724	0.830282
508	4.97E-11	0.011384	0.886803	4.97E-11	0.046082	0.862296
512	1.64E-11	0.0067	0.917868	1.64E-11	0.030801	0.892495
516	5.31E-12	0.003822	0.945078	5.31E-12	0.020079	0.920261
520	1.68E-12	0.002112	0.967636	1.68E-12	0.012765	0.944937
524	5.23E-13	0.001131	0.984768	5.23E-13	0.007915	0.965851
528	1.59E-13	0.000587	0.995756	1.59E-13	0.004786	0.982329
532	4.75E-14	0.000295	0.999972	4.75E-14	0.002823	0.993721
536	1.39E-14	0.000143	0.99691	1.39E-14	0.001623	0.999427
540	3.98E-15	6.74E-05	0.986221	3.98E-15	0.000911	0.998927
544	1.12E-15	3.07E-05	0.967743	1.12E-15	0.000498	0.991807
548	3.09E-16	1.36E-05	0.941521	3.09E-16	0.000266	0.977787
552	8.38E-17	5.78E-06	0.907823	8.38E-17	0.000138	0.956749
556	2.23E-17	2.39E-06	0.867145	2.23E-17	7.02E-05	0.928751
560	5.83E-18	9.54E-07	0.820199	5.83E-18	3.47E-05	0.894045
564	1.49E-18	3.69E-07	0.767898	1.49E-18	1.68E-05	0.85308
568	3.77E-19	1.38E-07	0.711319	3.77E-19	7.9E-06	0.806494
572	9.33E-20	4.99E-08	0.651664	9.33E-20	3.63E-06	0.755102
576	2.27E-20	1.74E-08	0.590207	2.27E-20	1.63E-06	0.699867
580	5.44E-21	5.89E-09	0.52824	5.44E-21	7.11E-07	0.641864
584	1.28E-21	1.93E-09	0.467013	1.28E-21	3.03E-07	0.58224
588	2.96E-22	6.09E-10	0.407688	2.96E-22	1.26E-07	0.522166
592	6.74E-23	1.86E-10	0.351281	6.74E-23	5.12E-08	0.462784
596	1.51E-23	5.5E-11	0.298637	1.51E-23	2.03E-08	0.405163
600	3.33E-24	1.57E-11	0.250396	3.33E-24	7.85E-09	0.350254
604	7.21E-25	4.34E-12	0.206987	7.21E-25	2.96E-09	0.298853
608	1.54E-25	1.16E-12	0.168627	1.54E-25	1.09E-09	0.25158
612	3.24E-26	2.99E-13	0.135338	3.24E-26	3.91E-10	0.208866
616	6.71E-27	7.46E-14	0.106971	6.71E-27	1.37E-10	0.170944
620	1.37E-27	1.8E-14	0.083235	1.37E-27	4.69E-11	0.137869
624	2.75E-28	4.19E-15	0.063737	2.75E-28	1.57E-11	0.109531
628	5.45E-29	9.45E-16	0.048015	5.45E-29	5.11E-12	0.085684
632	1.06E-29	2.06E-16	0.035571	1.06E-29	1.63E-12	0.065976
636	2.05E-30	4.33E-17	0.025908	2.05E-30	5.06E-13	0.049985
640	3.89E-31	8.81E-18	0.018544	3.89E-31	1.54E-13	0.037247
644	7.28E-32	1.73E-18	0.013041	7.28E-32	4.55E-14	0.02729

648	1.34E-32	3.3E-19	0.009007	1.34E-32	1.32E-14	0.019651
652	2.45E-33	6.08E-20	0.006108	2.45E-33	3.72E-15	0.013904
656	4.4E-34	1.09E-20	0.004066	4.4E-34	1.03E-15	0.009662
660	7.8E-35	1.89E-21	0.002656	7.8E-35	2.77E-16	0.006592
664	1.36E-35	3.2E-22	0.001702	1.36E-35	7.3E-17	0.004415
668	2.36E-36	5.4E-23	0.001069	2.36E-36	1.88E-17	0.002901
672	4.02E-37	9.23E-24	0.000659	4.02E-37	4.72E-18	0.00187
676	6.78E-38	1.67E-24	0.000398	6.78E-38	1.16E-18	0.001182
680	1.13E-38	3.35E-25	0.000235	1.13E-38	2.78E-19	0.000732
684	1.85E-39	7.7E-26	0.000136	1.85E-39	6.52E-20	0.000444
688	3.01E-40	2.01E-26	7.74E-05	3.01E-40	1.5E-20	0.000264
692	4.82E-41	5.7E-27	4.3E-05	4.82E-41	3.35E-21	0.000154
696	7.65E-42	1.69E-27	2.34E-05	7.65E-42	7.34E-22	8.78E-05