DIPLOMARBEIT

Bumblebee vision: Modification of the visual system in terrestrial habitats

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Verfasserin / Verfasser:	Tobias Pamminger
Matrikel-Nummer:	0202061
Studienrichtung /Studienzweig (lt. Studienblatt):	Biologie / Zoologie (A 439)
Betreuerin / Betreuer:	Dr. Johannes Spaethe

Wien, am

Gewidmet

Norma die mir immer zur Seite gestanden ist, auch wenn ich ihr das oft nicht leicht gemacht habe.

Meiner Familie auf die ich mich verlassen kann.

Johannes für die Betretung meiner Diplomarbeit und für die Erkenntnis dass man mit weniger Worten oft mehr ausdrücken kann.

Introduction

The genus *Bombus* represents one of the most species-rich members of the bee family Apidae. Bumblebees are covered with dense and usually black setae giving them a furry appearance. In general, bumblebee species exhibit an annual lifecycle. The fertilized queen is the only individual surviving the winter and she establishes a new nest early the following year (Goulson 2001). Like most Apidae, bumblebees feed exclusively on pollen and nectar. Together with the honeybee (*Apis mellifera* L. 1758), bumblebees are considered the most important pollinators in agriculture. They are widely used for pollination in glass houses e.g. for tomatoes (Dogterom et al. 1998). Visual information plays an important role in the life of a bumblebee, for navigation and detection of potential food sources over long distances (Chittka and Menzel 1992, Goulson and Stout 2001).

Bumblebee distribution

Bumblebees inhabit a diverse range of habitats. They occur in tropical lowlands, for example the species *Bombus transversalis,* as well as in "typical" central European habitats, as the species *Bombus sylvarum* L. (1761). Also, strictly alpine species exist like *Bombus alpinus* L. (1758) which occurs only above 2500 m a.s.l. (Neumayer and Paulus 1999; Williams: http://www.nhm.ac.uk/research-curation/research/projects/bombus/introduction.html). One important aspect discriminating the wide habitat range of bumblebees is the spectral composition of those ecosystems.

The irradiance spectrum of a habitat represents the sum of all light sources, both emitted and reflecting (Endler 1993). Therefore not only abiotic factors like altitude influence the spectral composition and intensity due to the optical properties of the atmosphere, but also biological factors such as the scattered light from leaf surfaces in dense forests. For example, the UV light levels vary not only with altitude - alpine habitats experience a higher level of ultraviolet radiation then low land habitats - but also between open grasslands and dense forests where UV radiation is almost completely filtered out. Therefore, terrestrial habitats differ dramatically not only in light intensity, but also in their spectral composition (Endler 1993).

The visual system of the bumblebee

The compound eye of the bumblebee is composed of several thousand single units, called ommatidia. Each ommatidium consists of a cuticular lens (cornea) followed by a crystal cone and eight major receptor cells called retinula cells (Fig.1.) In addition, a short retinula cell of unknown function is located in the basal part of the

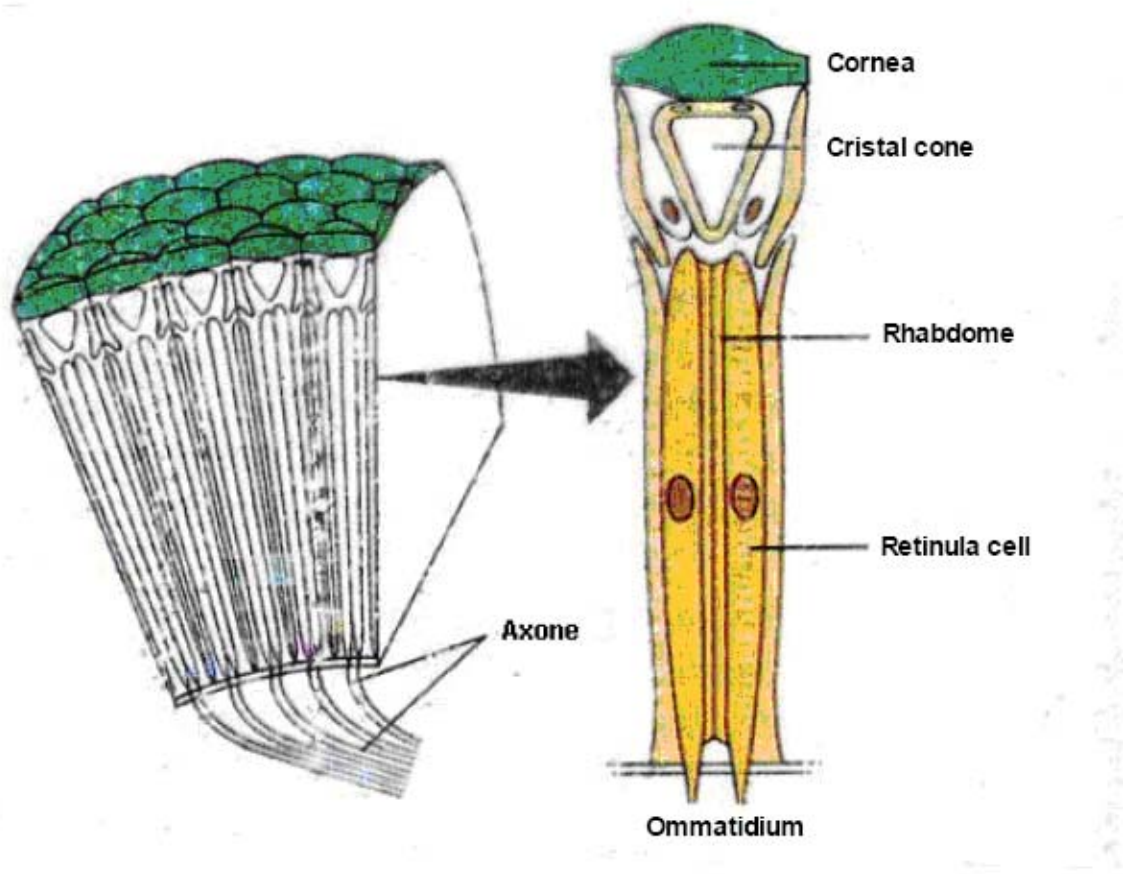

Fig.1. Drawing of the ommatidium morphology (modified after www.sinnesphysiologie.de)

ommatidia. The photosensitive molecules are located in a specialized part of the cell membrane called the rhabdomere. In the core of the ommatidium, they form a light guiding channel called the rhabdome. The microvilli increase the surface available to visual pigments and thereby improve the photon catch of the ommatidium.

The visual pigment consists of two parts: the light sensitive chromophor and the opsin protein. Opsins belong to the G-protein coupled receptor family and are characterized by seven transmembrane (TM) domains forming a binding pocket

holding the chromophor (Fig.2.). The chromophor is bound to the opsin via Schiffs base linkage (Wald 1968, Filipek et al. 2003).

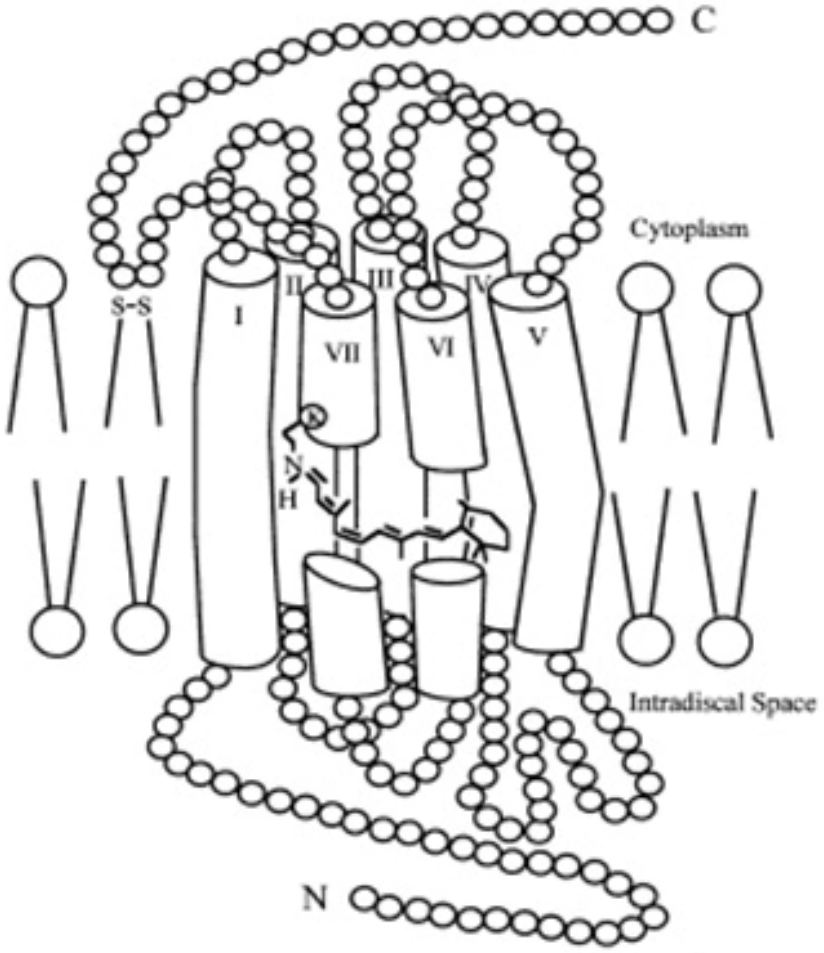

Fig.2. Schematic model of the bovine (P500) visual pigment, illustrating the seven TM containing the chromophor (after Yokoyama 2000).

Intercellular recordings revealed that the eight major retinula cells of bees show different spectral sensitivities (Menzel 1979, Menzel and Blakers 1976, Peitsch et al. 1992). Four of the eight cells are most sensitive in the green part of the light spectrum (~540nm), two in the blue part of the spectrum (~430 nm) and another two in the UV (~340 nm) (Fig.3). The three identified visual pigments of *Apis mellifera* vary not only in their peak spectral sensitivity but also in their relative spectral sensitivity. In dark - adapted honeybees the relative spectral sensitivities values of the three photoreceptor types are about 16 (UV): 2.7 (blue): 1 (green) (Menzel 1979). However, research within the past 5 years revealed a more complex composition of the compound eye. Immunohistochemical studies suggest that there are at least three different types of ommatidia present in *Apis mellifera* and *Bombus impatiens*

(Spaethe and Briscoe 2005, Wakakuwa 2005). All ommatidia of the main retina contain six retinula cells which are the most sensitive in the green part of the light spectrum, while the remaining two cells vary in their composition between ommatidia. In the first type of ommatidia, one retinula cell is most sensitive to blue light, the other one to UV light. In the other two types of ommatidia, the two non-green retinula cells are most sensitive either in the blue or in the UV part of the spectrum. Thus, about 2/3 of all ommatidia express only two out of three spectral receptor types (Spaethe and Briscoe 2005, Wakakuwa 2005).

Levels of spectral modification

From an anatomical point of view the composition of the compound eye is found to be similar across the Hymenoptera. Indeed, variation in the spectral sensitivity of photoreceptors occurs in members of this order (Peitsch et al. 1992). Several mechanisms ranging from changes at the protein level to morphological adaptations have been identified to account for the observed heterogeneous spectral sensitivities (Arikawa and Stavenga 1997, Carleton and Kocher 2001).

Filtering effects of adjacent photoreceptor cells

In ommatidia with closely associated rhabdomes, as in fused rhabdomes containing different opsins with overlapping spectral absorption curves, an effect called optical coupling takes place. The sensitivity curves of visual pigments characterize the part of the spectrum in which the pigment can absorb photons.

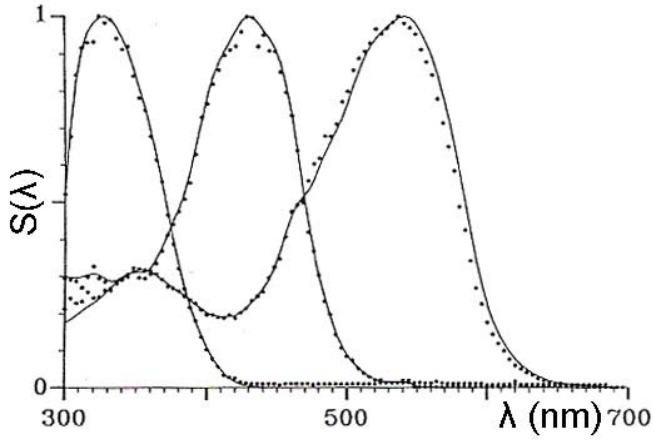

Fig.3. Spectral sensitivity function of the three *Bombus terrestris* opsins (Peitsch et al. 1992)

If the absorption curves of two pigments are overlapping, the two pigments are competing for photons of those wavelengths. In other words, they act as an absorption filter for each other. These filtering effects result in a narrowed sensitivity curve for the involved opsins (Snyder et al. 1973).
This effect can lead to a shift in maximum sensitivity, if the filtering occurs asymmetrically at the short or the long wavelength part of the absorption spectrum of the retinula cell. The resulting sensitivity maximum of the opsin is shifted in the opposite direction of the peak sensitivity of the filtering pigment. For instance in house flies (*Musca*), the UV sensitive photoreceptor cells influence the absorption curves of the proximal located retinula cells, resulting in a modified absorption curve compared to the expected one (Kirschfeld et al. 1978)

Filtering effects of screening pigments

Opsins are not the only photon absorbing pigments found in ommatidia. Screening pigments with variable absorption maxima, spatial distribution and functions have been found in the retina of insects (Arikawa and Stavenga 1997, Stavenga 2002). Many insect eyes contain black screening to prevent stray light from causing background noise in surrounding photoreceptor cells (Stavenga 2002). Some screening pigments have sharp absorption maxima and can produce similar effects to that of the lateral filtering (described above). In the eye of the butterfly, *Papillio xuthus*, a red screening pigment around the rhabdome of the R3-R8 cells was found (Arikawa and Stavenga 1997). Due to the selective absorption of those pigments, the absorption maxima of the photoreceptor are shifted slightly more into the red, enhancing color discrimination in the red part of the color spectrum (Stavenga 2002). Additionally, Arikawa (1999) found a far-UV filtering pigment in the most distal part of the ommatidium of *Papillio xuthus.* This pigment modifies the UV photoreceptor located in that area into a violet receptor shifting its maximum spectral sensitivity towards the longer wavelengths part of the spectrum (Arikawa et al.1999).
The modification of the spectral sensitivity of photoreceptors by means of filtering effects might be more widespread than expected; however, comparative physiological and behavioral data are still lacking (Briscoe and Chittka 2001).

Spectral tuning

The maximum spectral sensitivity of a visual pigment (λ max) is mainly determined by two factors. The first factor influencing the λ max value is depends on the type of chromophor used in the rhodopsin complex. Insects use mainly two types of chromophors, either the 11-*cis* retinal (λ max 383 nm) or the two enantiomers (3R or 3S) of the11- *cis* 3-hydroxyretinal (λ max 379 nm) (Gärtner et al. 1991). The second factor modifying the spectral sensitivity of the visual pigments is the amino acid sequence of the opsin. Various mutagenesis experiments on the bovine opsin P 500 reveal that only some amino acids' substitutions in the opsin sequence have an significant influence on the λ max value of the visual pigment (Yokoyama 2000). Most of the sites (11 out of 12) in which the λ max value of the opsin was changed by more than 5 nm are located in the TM region of the protein where the chromophor is attached to the opsin (Fig. 4).

A modification of the opsin sequence, leading to a shift in the absorption maxima of the visual pigment is called spectral tuning (Kochendoerfer et al. 1999, Britt et al. 1993).

Similar effects have been demonstrated in *Drosophila melanogaster*. The substitution of single amino acids in the TM domains of the Rh2 opsin leads to a shift of the λ max values of the visual pigment. The λ max shifts range between 4 and 11 nm,

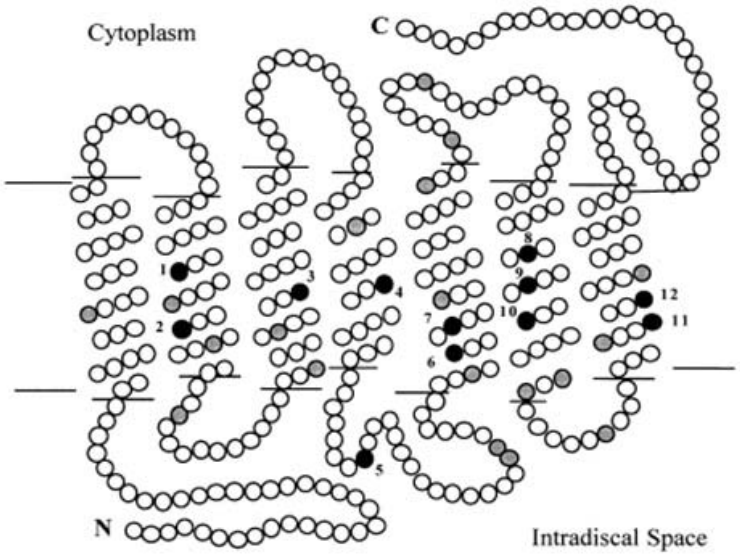

Fig.4. Naturally occurring substitutions in the amino acid sequence of the bovine visual pigment (P500). Black dots indicate a shift >5nm, grey dots <5nm (after Yokoyama 2000).

depending on the amino acid site. The substitution of multiple sites in the TM domains may result in a shift of about 60 nm (Britt et al. 1993).

The small number of amino acid substitutions necessary to shift the maximum sensitivity of a visual pigment in vertebrates' opsins (Yokoyama 2000), suggests that spectral tuning could be a rather fast process in evolutionary terms. In ocean-inhabiting species spectral tuning as well as maximum sensitivity shifts caused by different filtering pigments have been documented (Yokoyama et al. 1999, Briscoe and Chittka 2001, Cronin and Caldwell 2002). Especially mesopelagic fish inhabiting depths of about 200 m to approximately 1000 m seem to have gone through a spectral sensitivity shift towards shorter wavelengths, matching the available light spectrum at that depth (Bowmaker 1995, Yokoyama et al. 1999). Many deep water fish possess pure rod retinas containing visual pigments peaking between 470 and 490 nm only. The pure rod retina increases the spectral sensitivity of these organisms under dim conditions, and the absorption maximum closely matches the available light (470 nm) at that depth (Bowmaker 1995). These findings have been interpreted as adaptations to the specific spectral conditions of this habitat (Bowmaker 1995, Yokoyama 2000).

Retinal composition

The spectral receptor composition in the eye of most animals is heterogenic. Flying insects for example experience different light regimes in the dorsal and the ventral part of their visual system, due to the fact that the light qualities of the sky differ significantly from the reflectance spectrum of the landscape underneath them (Endler 1993).

Honeybee drones (*Apis mellifera*) exhibit a specialized dorsal half of the compound eye which differs significantly from the eye of a worker bee (Menzel 1991). The dorsal region is characterized by an increased facet diameter, a smaller interommatidial angle and the expression of just two receptor types (UV and blue), while the ventral part of the drone eye closely resembles the eye of a typical honeybee worker. This specialized dorsal region probably plays a role in the mating behavior of the drone. In order to copulate during the mating flight a drone has to

identify a small, dark object (the queen) against a bright background dominated by short wavelengths (the sky). The dorsal region seems well adjusted to this task, as the decreased interommatidial angle allows a better spatial resolution (small object). The increased facet diameter results in an improved light sensitivity. The maximum spectral sensitivity of the photoreceptors allows an optimal photon catch, further improving the sensitivity of the dorsal eye of the honeybee drone (Menzel 1991).

Modification of the visual system through differential gene expression

Besides the factors mentioned above, another possibility of modulating the spectral sensitivity of an optical system has been discovered. For instance, several cichlid species possess more opsin genes than actually expressed in the retina. By expressing only three of six opsin genes, an individual can change its spectral sensitivity in the course of its life cycle by changing the composition of the opsin triplet used (Carleton et al. 2008). The peak sensitivities of the receptors actually expressed usually match the spectral properties of the environment inhabited by the cichlids at a given time (Spady et al. 2005, Carleton et al. 2008). Differential gene expression therefore represents a mechanism to shape the visual system of an organism during its entire lifespan allowing it to cope with different photic conditions.

Adaptation of the visual system

It has been argued that the spectral environment shapes the optical system of its inhabitants (Bowmaker 1995). In order to consider a trait (e.g. a shift of the receptor peak sensitivity) as adaptive, it has to be shown that it increases the organism's fitness compared to conspecifics lacking that trait.

In Hymenoptera the general picture of spectral sensitivity is remarkably uniform (Briscoe and Chittka 2001, Peitsch et al. 1992). With the exception of ants, the visual system of the Hymenoptera is composed of an UV, a blue and a green receptor. (Peitsch et al. 1992). The absorption maxima among different species within the three opsin classes show significant variation (Peitsch et al. 1992). Surprisingly, no correlation between the receptors peak sensitivity and the spectral conditions of the habitat has been found, despite the fact that hymenopterans inhabit an extreme

variety of spectral habitats (Peitsch et al. 1992). However, Goldsmith (1990) suggested that phylogenetic and molecular constrains, rather than adaptation to different spectral conditions, could have shaped the maximum sensitivity values of the color receptors. This might be the reason why insects living in spectrally different habitats possess a similar set of photoreceptors (Chittka and Briscoe 2000).

While the λ max values of Hymenoptera seem to have no correlation with the spectral properties of the environment, increasing evidence suggests that the visual system is more flexible than previously assumed (Carleton and Kocher 2001, Spady et al. 2005, Carleton et al. 2008). The development of modern molecular techniques, like real time PCR, enables scientists to take a closer look at a different level of the visual system. Differential gene expression provides a tool enabling organisms to modify their visual system on the level of gene regulation, neither altering the composition of the optical apparatus nor the opsin sequence. This mechanism could represent a fast and effective response for tuning a visual system to a spectrally variable environment.

In bumblebees, workers spend most of their active adult life foraging for pollen and nectar. In an environment where competition for food is high, search efficiency is a limiting factor for maximizing reproductive success (Pyke 1984). Thus, a small decrease in search time could lead to an increase in colony fitness. We hypothesize that an optimally tuned visual system could thus enable a bumblebee to detect and discriminate flowers faster and more reliably and therefore allow her to forage more efficiently. So how do bumblebees adapt to variable spectral habitats?

Modifications of the expression levels of opsins in response to a changing spectral environment could provide evidence for a, so far unknown, mechanism in Apidae for adjusting the sensitivity of the visual system. Such a mechanism could represent a way of accommodation to fast changes in the spectral quality of the habitat. We hypothesize that opsin expression levels correlate with the spectral environment and hence allow bumblebees to react to changes in the spectral composition of ambient light.

Materials and Methods

Laboratory experiments

From February to June, 2008, three colonies of *Bombus terrestris* L. (1758) were purchased from Koppert (The Netherlands) and transferred to wooden breeding boxes (20x20x15 cm) with access to sugar, water and pollen. The colonies were kept under a constant light / dark rhythm (L: D, 12:12) and a constant temperature of 27°C for at least two weeks prior to experiments.

In June, 2008, sixty workers were taken from the colonies, divided into three experimental groups and transferred into separate breeding boxes. Each experimental group was kept under a specific spectral filter. The first experimental group was placed under a short wavelength pass filter (swpf) which allowed only light below 500 nm to be transmitted (Fig. 6). The second group was placed under a long wavelength pass filter (lwpf). Thus they were exposed mostly to light > 450 nm (Fig.5). The last group was placed under a neutral density filter (ndf) to assure similar brightness among the three groups. The light spectra which illuminated the bumblebees were measured using a RPS 900-R (International Light Nist) spectrophotometer. Energy values were converted into photons as eyes are photon counters and cannot measure energy per se (Tab.2., Laughlin 1982). The total number of photons per second and square cm were $3{,}52029*10^{24}$ for the swpf, $6{,}12*10^{24}$ for the lwpf and $5{,}22635*10^{24}$ for the neutral density filter. The three groups were kept under these conditions for six days. To avoid possible diurnal variation in opsin gene expression all workers were collected between 12 am and 2 pm, flash frozen in liquid nitrogen and stored at minus 80°C (Fuller et al. 2004, Sasagawa 2003).

Field sampling

In July, 2007 and 2008, *Bombus lucorum* L. (1761) workers were collected in and around the national park “Hohe Tauern”, Austria. Fifteen workers were collected at the “Hochtor”, approximately 2650 m a.s.l. (47°04’57’’ N 12°50’35’’ E) and fifteen workers near Fusch (47°15’48’’N 12°49’30’’E) 750m a.s.l. In August, 2008, 18 *B. lucorum* workers were caught in Lund (55°42’40’’N 13°11’36’’E), Sweden, at about

50m a.s.l.. All workers were collected between 12 am and 2 pm. The workers were decapitated, their heads cut in two pieces and immediately transferred into RNAlater® for further processing.

RNA extraction

For RNA extraction, one eye of each individual was cut off with a sterile razor blade on a microscope slide kept at approximately 4°C on a coolpack. I performed a transversal cut across the bumblebees' head to avoid possible contamination with opsin mRNA from the ocelli. The whole complex eyes were homogenized in a 1.5 ml Eppendorf tube (Eppendorf Save lock micro test tubes) containing 1 ml of ISOL-RNA lysis reagent™, using a pestle. The homogenized extract was incubated for 5 min at room temperature to promote the dissociation of the nucleoprotein complexes. Afterwards 200 µl Chloroform was added. After shaking the samples for 15 sec the Eppendorf tubes were centrifuged (Sigma 1-15K) at 12 000 x g for 15 min at 4°C. Through centrifugation the samples were separated into three phases, the aquatic phase containing the RNA, a white interphase and the red organic phase containing the DNA and protein components. The aquatic phase was carefully transferred to a 1.5 ml Eppendorf tube. 500 µl isopropanol were added and the samples were vortexed (IKA® Vortex Genius 3). After incubating the samples for 10 min at room temperature they were centrifuged at 12 000 x g for 10 min at 4°C. Then the supernatant was carefully discarded and 1 ml 70% ethanol was added to the RNA pellet sticking to the bottom of the tube. The samples were immediately transferred to the centrifuge and centrifuged at 7500 x g for 5 min at room temperature (RT). The supernatants were removed completely and the RNA pellet briefly air dried.
The RNA was resuspended in 60 µl DNAse/RNAse free water and 15 µl aliquots were stored immediately at -80°C. The RNA content and purity of the sample was measured using an Eppendorf BioPhotometer 6131®, recording the RNA concentration and the 280/260 values.

cDNA synthesis

1 µg of total RNA was utilized to synthesize cDNA, using the Bio Rad iScript cDNA Synthesis Kit. The 20 µl reaction mixture contained:

4 µl	5x iScript Reaction Mix
2 µl	oligo (dT) Primer
1 µl	iScript Reverse Transcriptase
x µl	RNA (1 µg)
x µl	H_2O (to fill the 20 µl depending on the [RNA])

Following the manufacture's protocol the mixture was incubated for 5 min at 25°C, followed by 60 min at 42°C, 5 minutes at 85°C, and finally the hold step at 4°C.

Primer design

The Sequence for the SWRh (UV) and the LWRh (green) opsin of *B. terrestris* were obtained from published sequences (AY655163, AY485306). For the MWRh (blue) opsin an unpublished sequence was used as template (Spaethe, unpublished). Lasergene 7 was employed for the design of sequence specific primers for all three opsins (Tab.1). The products were of comparable size (268-298bp) and contained an intron which allowed me to prove possible DNA contamination of the real-time runs by employing the melting curve analysis. If a DNA contamination is present a second peak at a larger temperature will be visible (Eppendorf Mastercyler® ep realplex manual).

Table 1.: Sequences of the primer used for real time PCR amplification

UV-primer	forward	5' - CCAAAGCGCTGAGATACG - 3'
	reverse	5' - TACTGTCCCGGTTGTGGT - 3'
Blue-primer	forward	5' - TCTGGGGTCGATTCACTA - 3'
	reverse	5' - CACTTTGGCGATTCTCAG - 3'
Green-primer	forward	5' -TCCCGCTGTTCCTCAT - 3'
	reverse	5' - ACCCCAAATTGTGTAAAG - 3'
rp49-primer	forward	5'- TCGTAGACGTTTTAAGGGACAATA - 3'
	reverse	5'- GAGCACGTTCAACAATGGAT - 3'

Primer efficiencies

The PCR efficiency was measured using the same cDNA as was used for the real-time PCR. A dilution series over four orders of magnitude was constructed including the concentration present in the PCR reaction. Five independent RT reactions were set up for each concentration (1:1; 1:10: 1:100; 1:1000; 1:10 000). The data was analyzed utilizing the software provided with the Eppendorf Mastercyler® ep realplex (realplex version 1.5). Reactions which did not show a single peak in the melting curve were excluded from the results. The threshold was chosen using the R^2 max method (user manual Eppendorf Mastercyler® ep realplex).

Real-time PCR

For the real-time PCR triplets of 20µl reactions where set up as follows:

8 µl	5 Prime Real Mastermix
0.5 µl	Forward Primer
0.5 µl	Reverse Primer
1 µl	cDNA (equivalent to 50ng transcribed RNA)
10µl	H_2O

The SYBR-Green florescence was measured over 35 cycles [94°C/2 min initial denaturation 35x (94°C 15sec/44°C 30sec/70°C 30 sec)]. The real-time PCR reaction and florescence measurements were carried out using an Eppendorf Mastercyler® ep realplex following the manufacture's protocol.

Opsin expression quantification

The relative abundance of the three different opsin mRNAs was calculated using a critical threshold, following the methodology of Carleton and Kocher (2001). The relative abundance of the three opsins was calculated as the proportion of the total opsin expression in the sample by the following equation:

$$\frac{T_i}{T_{all}} = \frac{\left(1+E_i\right)^{C_{ti}}}{\sum \frac{1}{\left(1+E_i\right)^{C_{ti}}}}$$

Ti/Tall is the proportional gene expression for gene i, E_i is the primer/probe set efficiency and C_{ti} represents the critical cycle number for each gene (Carlton and Kocher 2001)

Relative quantification of UV mRNA using the $2^{-\Delta\Delta Ct}$ method:

We used the $2^{-\Delta\Delta Ct}$ method to analyze relative changes in mRNA expression between the different experimental treatments. The $2^{-\Delta\Delta Ct}$ method normalizes the Ct values of a target gene, using a control gene known to be expressed at a constant rate in all samples (housekeeping gene). Commonly used control genes include GAPDH, β-actin and rRNA in mammals (Livak 2001). By calculating the ΔCt value as

$$\Delta Ct = (Ct_{target} - Ct_{control})$$

the results become independent of the actual starting amounts of mRNA, representing the relative difference in the amount of target and control gene mRNA in the samples. By subtracting the ΔCt values of the control group from the ΔCt values of the experimental group the difference in target gene expression between the two groups is given as change in target ΔCt, called ΔΔCt.

$$\Delta\Delta Ct = (Ct_{target} - Ct_{control})_{\text{experimental group}} - (Ct_{target} - Ct_{control})_{\text{control group}}$$

By assuming constant expression of the control gene and approximately equal PCR efficiencies for both reactions, the target gene expression can be calculated as n-fold expression relative to the control group:

$$\text{amount of target} = 2^{-\Delta\Delta Ct}$$

The derivation of the $2^{-\Delta\Delta Ct}$ equation is described by Livak (2001). To apply the $2^{-\Delta\Delta Ct}$ method two assumptions must hold (Livak 2001). First, the control gene expression

must be considered constant and unaffected by the experimental treatment. Second, the PCR efficiencies of the target and the control reactions must be about equal.
We used the ribosomal protein 49 (rp49) as a control gene, commonly used as a housekeeping gene for insects (Ben-Shahar et al. 2003, Lehman 2006, Moon et al. 2006, Qurashi et al. 2007, Navajas 2008,). To assure a similar PCR efficiency for both target and control reaction we used the validation experiment suggested by Livak (2001). I plotted the ratio between the ΔCt values at different template dilutions against cDNA concentration. If the slope of a linear regression, including the ΔCt values of all cDNA concentrations used is close to zero (a ≤0.1), the PCR efficiencies can be considered equal and the $2^{-\Delta\Delta Ct}$ calculation can be performed.

Statistics

SPSS 10.0 was used for statistical analysis. The relative percentage values were arcsin transformed and a Kruskal Wallis-test for nonparametric data was used to test for differences of relative UV, blue and green opsin mRNA expression among treatment groups. To account for multiple comparisons a modified Bonferroni correction was used to adjust α-level (Sachs p. 183).

Results

Spectral measurements

The overall light intensity (photons/cm²*s) was comparable among the three experimental groups (Table 2). The two filters reduced the overall light intensity by about one order of magnitude. The absorption of the ndf was calculated as 1/3 of the intensity of the unfiltered spectrum. The absorption effects of the two different filters are presented in Fig. 5 and Fig.6. The strong spikes (~410 nm, ~440 nm, ~550 nm) are caused by the spectral properties of the fluorescence tubes. The lwpf reduced the light intensity in the short wavelength part of the spectrum below ~ 570 nm (Fig. 5), creating a long wavelength dominated spectrum, while the swpf cut off light above 525 nm, (Fig.6).

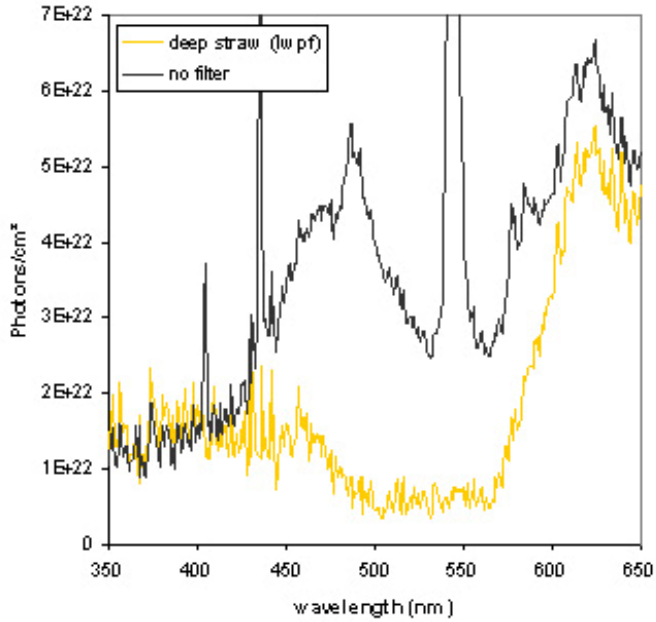

Fig.5. Transmission curve of the long wavelength pass filter (lwpf) compared to the unfiltered spectral environment.

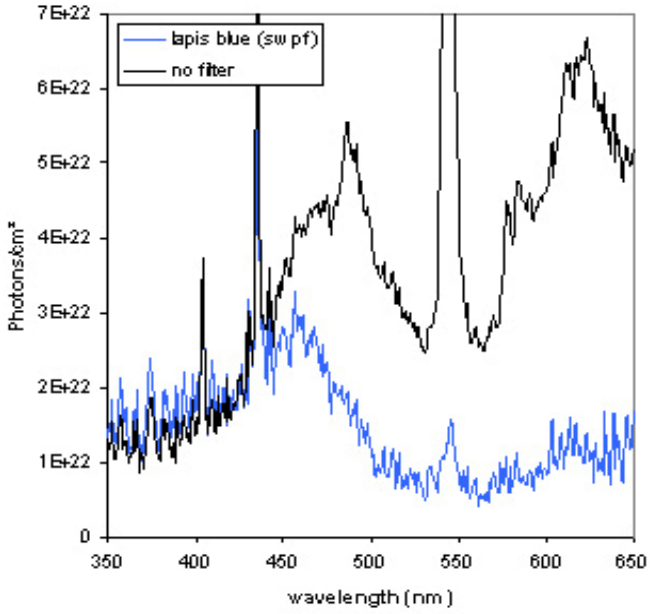

Fig.6. Transmission curve of the short wavelength pass filter (swpf) compared to the unfiltered spectral environment.

Table 2. Light intensity (*hv*/cm²) of the three experimental treatments

Treatment	*h*v/cm²
No filter	1.17343E+25
Short wavelength pass filter (swpf)	3.52029E+24
Long wavelength pass filter (lwpf)	6.12E+24
Neutral density filter (ndf)	5.22635E+24

Primer efficiencies

The analysis of the four different primer efficiencies revealed uneven performance of the four primer/probe systems. The efficiencies of ribosomal protein 49 (rp49) primers as well as the UV-opsin primers were close to 1 indicating 100% primer efficiency over the cDNA concentration range tested. The coefficients of the regression analysis

(R^2) for both primer pairs were close to one (Tab.3). The primer efficiencies for the blue and the green primer systems were 0.91, with R^2 values of 0.98 (Tab.3, Fig.7-9).

Table 3. Efficiencies of the primer used in the real time analysis

Primer-set	Primer efficiencies	Coefficient (R^2)
Green	0.91	0.978
Blue	0.91	0.98
Ultraviolet	1.00	0.997
rp49	1.00	0.996

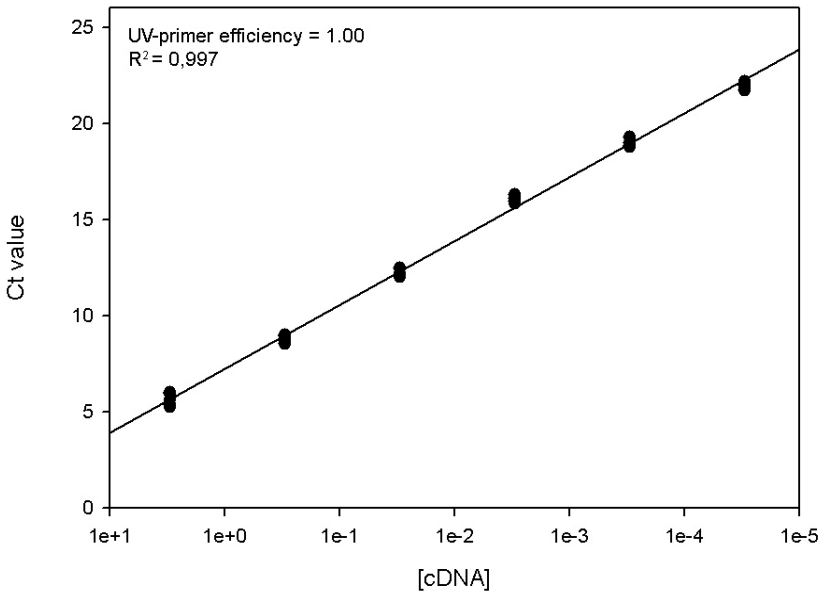

Fig.7. Measuring UV-primer efficiencies by means of a cDNA dilution series over five orders of magnitude including the cDNA concentration used in the real time experiments (N=5).

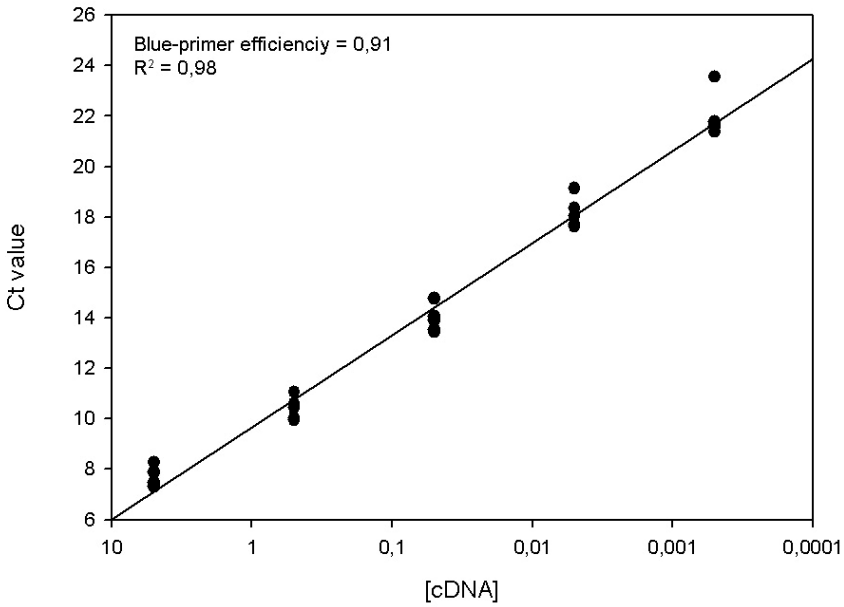

Fig.8. Measuring blue-primer efficiencies by means of a cDNA dilution series over four orders of magnitude including the cDNA concentration used in the real time experiments (N=5).

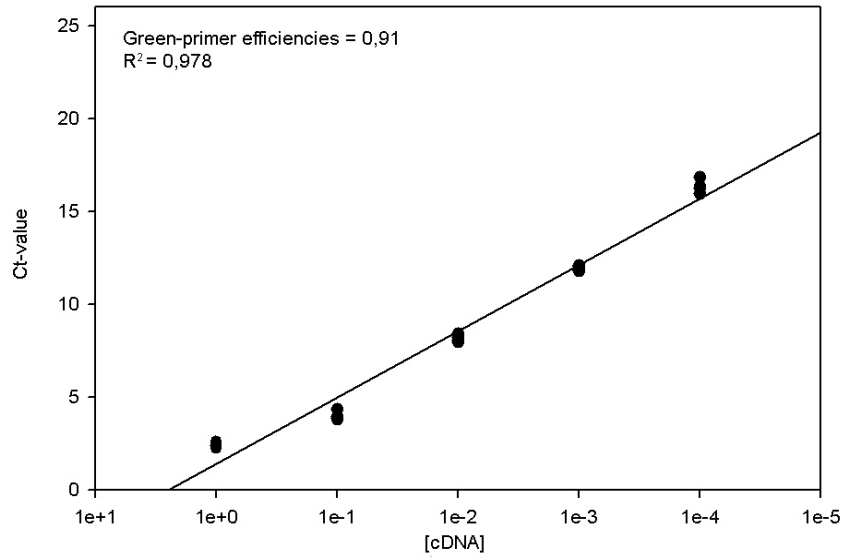

Fig.9. Measuring green-primer efficiencies by means of a cDNA dilution series over four orders of magnitude including the cDNA concentration used in the real time experiments (N=5).

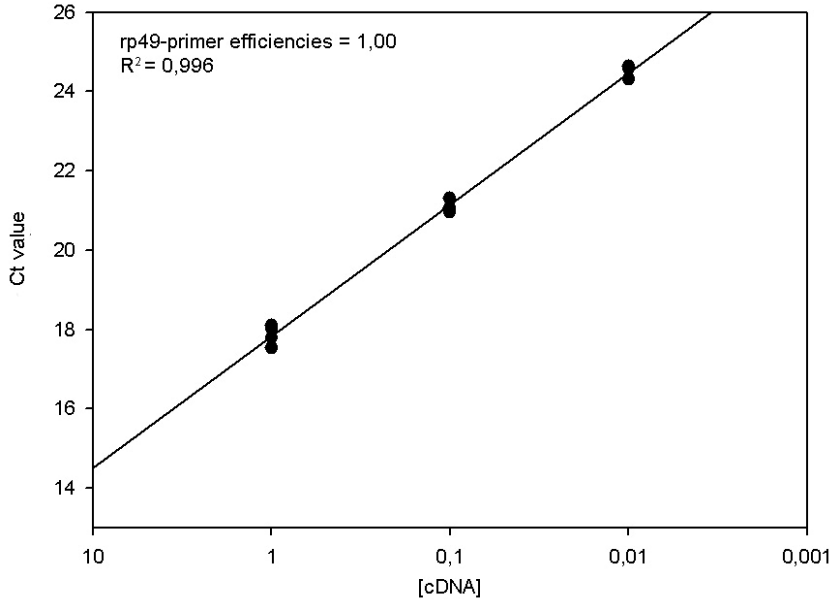

Fig.10. Measuring rp49-primer efficiencies by means of a cDNA dilution series over two orders of magnitude including the cDNA concentration used in the real time experiment (N=4)

Opsin Expression

Laboratory experiments

We found significant differences among the three different experimental conditions with respect to UV and green opsin expressions. The blue opsin expression remained constant in all experimental groups (Fig.11, Tab.4). Relative UV mRNA expression was increased (26% ± 3 SE) in the experimental group kept under the short wavelength pass filter (swpf) and reduced (17% ± 1 SE) under the long wavelength density pass filter (lwpf), in comparison to the control group (neutral density pass filter, 21% ± 2 SE; p=0,02 chi²=7,86). The green opsin expression showed opposite results. While the relative green mRNA opsin expression was reduced (60% ± 3 SE) under the swps filter, I found an increase (70 % ± 2 SE) in mRNA expression under the lwpf. Relative green mRNA expression level of the control group was intermediate (64% ± 3 SE; p=0.02 chi²=7.87). The blue opsin

expression remained unaffected by the experimental treatments (p=0.218 chi²=3.05) (Fig.11, Tab.4).

Table 4. Expression levels of *B. terrestris* opsins shown as percentage of total opsin expression under three different experimental conditions (N=12 for each group)

	treatment 1 (swpf)	treatment 2 (lwpf)	treatment 3 (ndf)
Opsin	$\bar{x}$% ± SE	$\bar{x}$% ± SE	$\bar{x}$% ± SE
UV	26 ± 3	17 ± 1	21 ± 2
Blue	14 ± 1	13 ± 1	15 ± 1
Green	60 ± 3	70 ± 2	64 ± 3

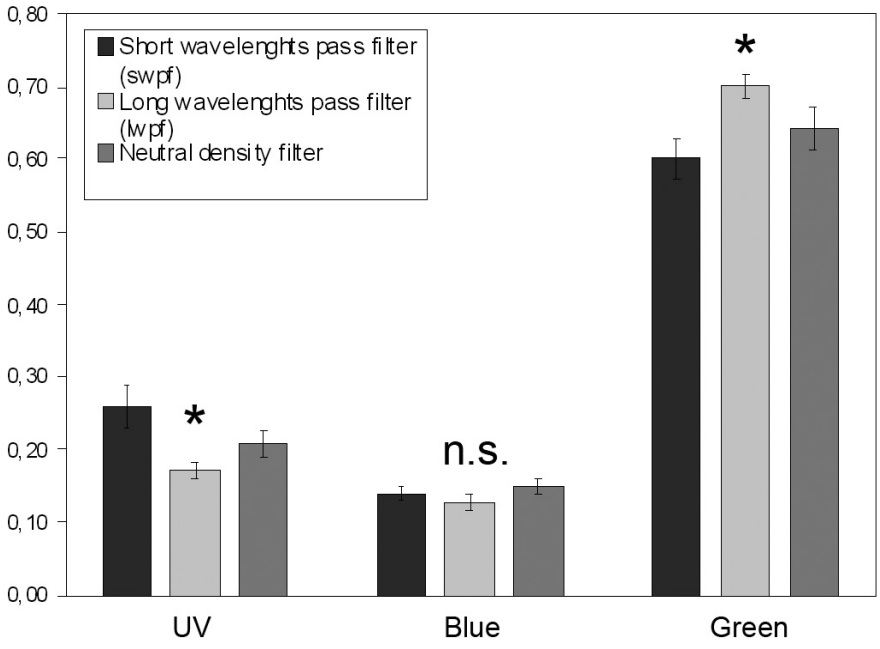

Fig.11. Relative mRNA opsin expression in *B.terrestris* under different spectral filters. Opsin expression is given as percentage of total opsin expression. Error bars indicate SE. * = significant, n.s. = not significant (UV p=0.02 chi²=7.89; Green: p=0.02; chi²=7,87).

$2^{-\Delta\Delta Ct}$ Validation experiment

The validation experiment for the $2^{-\Delta\Delta Ct}$ showed comparable primer efficiencies for the UV opsin and the rp49 control gene. The slope (a) of the linear regression was 0,0161 and thus below the threshold value of 0,1 suggested as acceptable threshold value by the BMI Applied User Bulletin No.2 (P/N 4303859; Fig.12). Both blue (a=0,5954) and green (a=0,6238) primer efficiencies differ significantly from the efficiency of the rp49 primer pair, suggesting that the $2^{-\Delta\Delta Ct}$ method cannot be used for a direct comparison of the rp49 primer pair with these two opsin genes (Fig.13 and Fig.14). I will therefore focus on the UV opsin only.

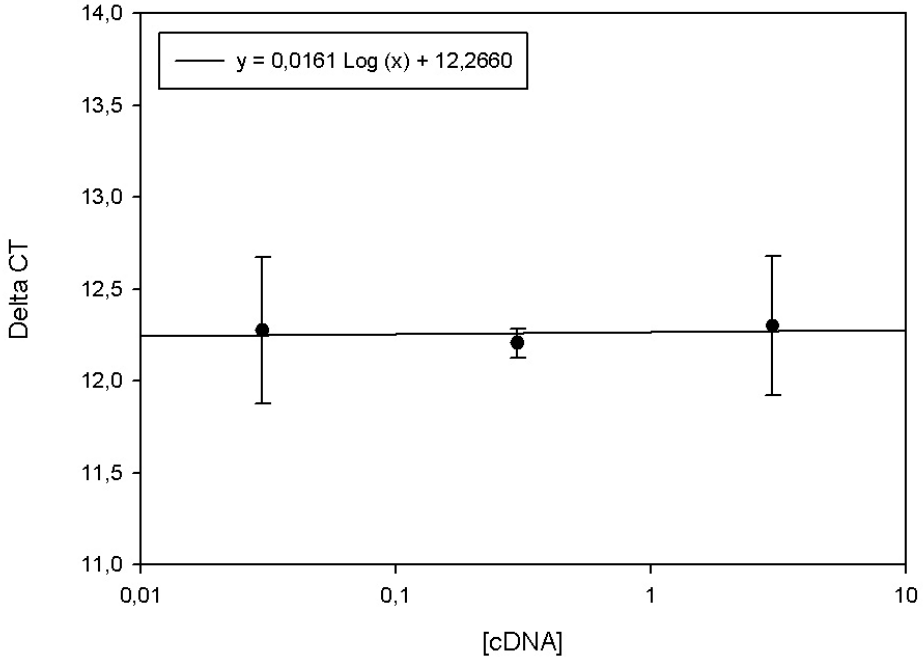

Fig.12. Validation experiment for the UV primer efficiencies. The ΔCt values were calculated for each cDNA concentration ($\Delta Ct = Ct_{UV} - Ct_{rp49}$) and analysed by linear regression (N=4).

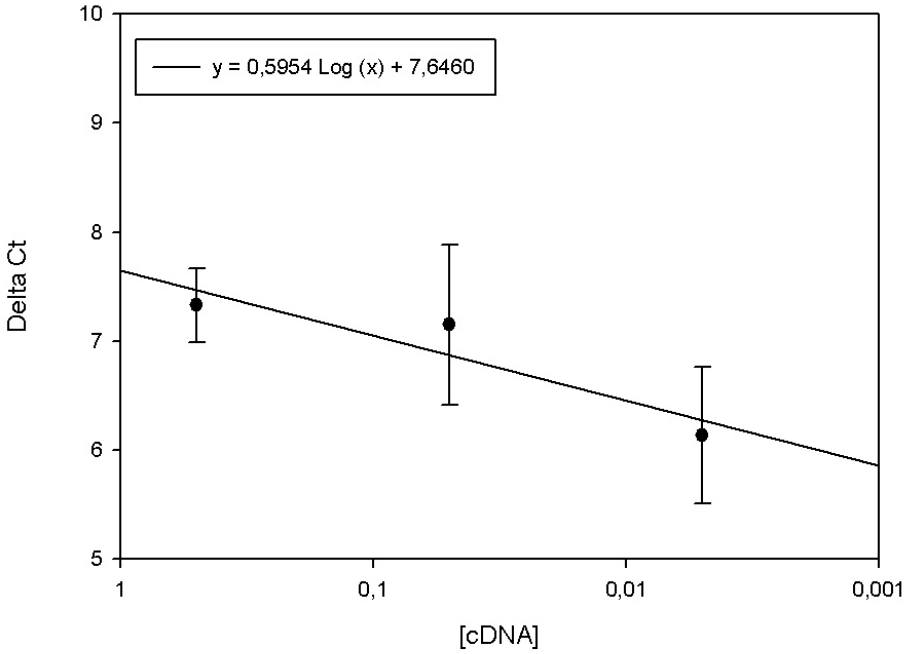

Fig.13. Validation experiment for the blue primer efficiencies. The ΔCt values were calculated for each cDNA concentration ($\Delta Ct = Ct_{blue} - Ct_{rp49}$) and analysed by linear regression (N=4).

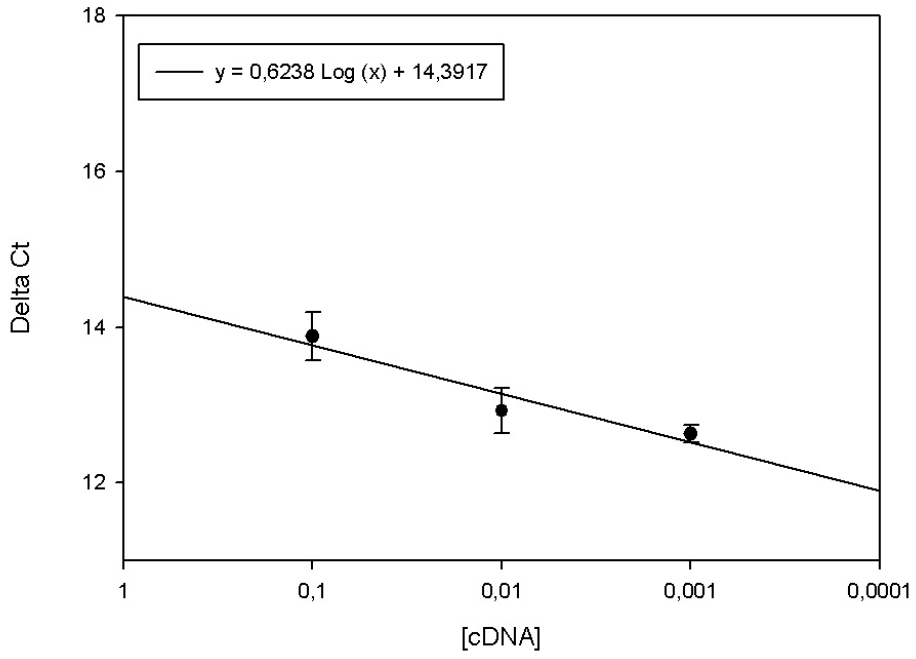

Fig.14. Validation experiment for the green primer efficiencies. The ΔCt values were calculated for each cDNA concentration ($\Delta Ct = Ct_{green} - Ct_{rp49}$) and analysed by linear regression (N=4).

$2^{-\Delta\Delta Ct}$ results

To test if there were actual changes in the amount of opsin mRNA, we normalized the relative expression using the housekeeping gene rp49. The $2^{-\Delta\Delta Ct}$ method revealed high individual variation of UV opsin mRNA within each treatment group (Fig. 15). UV opsin mRNA under swpf was similarly compared to the control group (0.98 fold), whereas under lwpf UV opsin expression was reduced (0.85 fold of the control group; Table 5). However, due to high intra-treatment variation the difference was found not to be significant. Differences between treatment and control groups of the green opsin mRNA was found to be more distinct (Table 5), but probably just reflect different primer efficiencies (Livak 2001).

Table 5. Results of the 2-ΔΔCt analysis of the laboratory experiment. short wavelength pass filter =swpf long wavelength pass filter =lwpf

Opsin	Treatment	n-fold expression	± SE	N
UV	swpf	0.98	0.14	10.00
	lwpf	0.85	0.12	10.00
Blue	swpf	0.80	0.12	10.00
	lwpf	0.90	0.18	10.00
Green	swpf	0.81	0.16	10.00
	lwpf	1.10	0.16	10.00

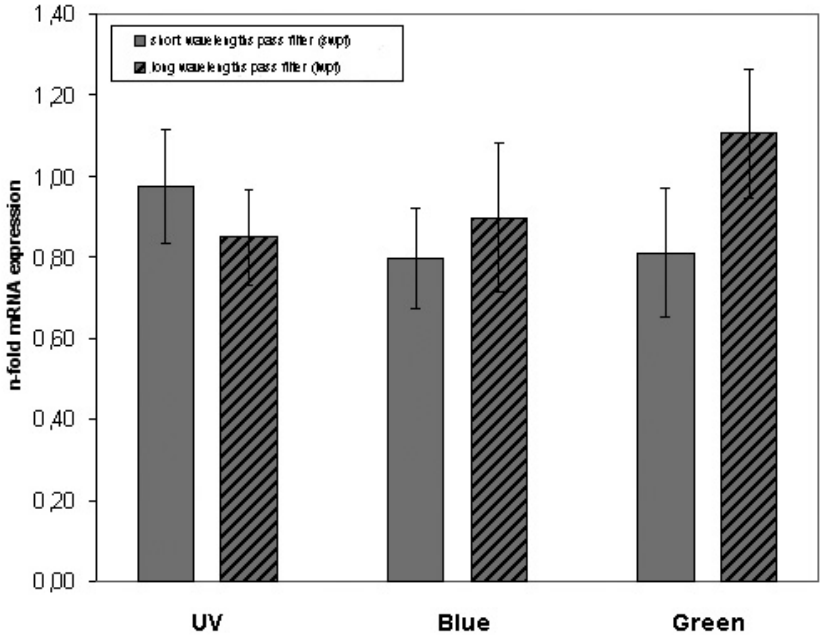

Fig.15. Results of the $2^{-\Delta\Delta Ct}$ analysis of the laboratory experiments. Opsin mRNA expression is given as n-fold expression of the control group. Error bars indicate SE.

Field experiments

The results of the field experiments are shown in Fig.16 and Tab.6. The general picture is similar to the laboratory experiments (Fig.11, Tab.4). The relative green opsin expression is highest, ranging between 68 and 72% of the total opsin expression. Among the populations the highest green opsin mRNA expression was found in bumblebees from low land habitats (30 m a.s.l.) with 72% ± 1 SE. Lowest expression was found in bees from high altitudes (68% ± 2 SE). Bumblebees from 700 m altitude showed an intermediate opsin expression level (70% ± 2 SE; Fig.16 and Tab.6). The UV opsin expression varied between 18 to 21% of total opsin mRNA expression, with highest expression found in animals from high altitudes (21% ± 1 SE) and lowest expression from animals of low altitudes (18% ± 1 SE). The blue opsin expression ranged between 9 and 12 % (±1%SE). However, differences within each group were found not to be significantly different at a α level of 5%.

Table 6. Expression levels of *B. lucorum* opsins shown as percentage of 100% opsin expression at three different altitudes.

	2700m a.s.l. (N=12)	30 m a.s.l. (N=9)	750 m a.s.l. (N=10)
Opsin	$\bar{x}$% ± SE	$\bar{x}$% ± SE	$\bar{x}$% ± SE
UV	21 ± 1	19 ± 1	18 ± 2
Blue	12 ± 1	9 ± 1	12 ± 1
Green	68 ± 2	72 ± 1	70 ± 2

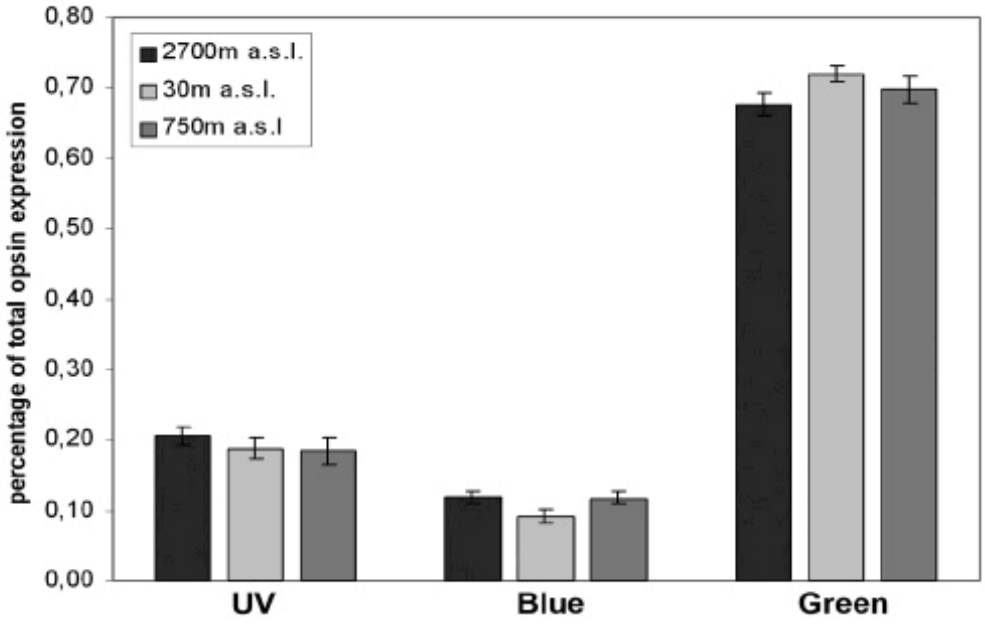

Fig.16. Relative opsin expression of *B. lucorum* in spectrally different habitats. Opsin expression is given as percentage of total opsin expression. Error bars indicate SE.

Discussion

Maximum spectral sensitivity of photoreceptors in Hymenopterans varies significantly (Briscoe and Chittka 2000), but attempts to correlate this variation to ecological factors like spectral quality of the habitat have failed so far (Peitsch et al. 1993). Here I am able to show that the UV, blue and green opsins expressed in the photoreceptors of bumblebees change their relative expression in correlation with spectral changes of ambient light (Experiment 1). Under UV reduced light conditions, the relative UV opsin expression was lower compared to the control. Similarly, in conditions where long wavelengths were reduced, the relative expression of the green opsin was lower. The data clearly show that bumblebees alter their opsin expression in response to changes in the spectral quality of ambient light. Comparable opsin expression levels were documented in the filed study (Experiment 2). Bumblebees from high altitudes (ca. 2700m asl), where the relative amount of UV radiation is high, exhibited the highest relative UV opsin expression level. In contrast, bees from lowland populations (about 50 m a.s.l.) showed the highest relative expression of the green opsin. However, due to a high variation within each population, these differences were not found to be significant at an α level of 5%. In contrast to the laboratory experiments where I could control the major factors of the spectral environment, the spectral composition in the field varied not only with regard to overall intensity, but, in addition the spectral composition of the ambient light changes. It should be remembered that the spectral composition of any particular location will undergo changes on a daily basis, in particular by shifting toward longer wavelengths during the afternoon (Johnsen et al. 2006). Furthermore, mobile animals encounter different photic environments on a regular basis. Bumblebees which forage in both forests and open grasslands can switch between long and short wavelengths at a fast pace (Endler 1993). Unpredictable weather conditions, such as clouds, rain or fog, may constitute additional factors influencing mRNA expression of bumblebees in the field.

What is the mechanism underlying the changes in opsin expression?

Our findings provide evidence that light itself is a factor affecting the expression level of opsin mRNA (Dalal et al. 2003). In mosquitoes, the rhabdome membrane renewal (e.g. turn over rate) together with the synthesis rate of new opsin proteins is increased when the photoreceptor is exposed to light (Stein 1979). In *Limulus*, total opsin expression in the lateral eye is driven by light intensity changes resulting in an increase of opsin mRNA expression levels during the day and a drop in expression levels after sunset (Dalal et al. 2003). In other study systems, G-protein coupled receptors were shown to trigger signaling pathways that modify gene expression (Kiselev et al. 2000, Furgerson 2001). It is tempting to speculate that opsin specific signaling pathways can modify the expression levels of opsins at a molecular level in response to changes in the spectral composition of ambient light.

How do changes in opsin mRNA expression affect visual perception?

The laboratory experiment suggests that the visual system of *Bombus terrestris* is a dynamic one rather than static. The system responds to shifts in the spectral composition of the photic environment by a relative increase in the mRNA expression of the opsin that is most sensitive to the intensified part of the ambient light spectrum (Fig. 11). There are at least three possible explanations for my findings. (1) The changes in relative mRNA expression are the result of a lower expression of the opsin mRNA which is most sensitive to the part of the light spectrum which is removed by the filter. Alternatively, (2) the increased relative mRNA opsin expression is the result of an absolute increase of the opsin mRNA level of the receptor that is most sensitive to the part of the spectrum which passes the filter; (3) or both effects occur, simultaneously.

(1) In rats, the photoreceptor bleaching rate depends on the incident light intensities (Williams et al. 1999). When the light intensity decreases the bleaching rate decreases, and likewise vice versa. In the process of rhodopsin regeneration, a proportion of the visual pigments should is lost due to degeneration and must be replaced by new protein components (Stein 1979). This proportion should be lower in photoreceptors containing a visual pigment that is not sensitive to the stimulating light. Therefore the observed increase in

relative expression rate of opsins could be the result of a lower mRNA expression of those opsins that were not stimulated by the incident light. Overall, total mRNA opsin expression is lower under these conditions.

(2) The alternative possibility involves an active increase of those mRNA opsins that are most sensitive to the stimulating light. This increase in mRNA expression would result in an absolute increase of opsin protein concentration in the microvilli (under the assumption that a higher mRNA level results in a higher protein concentration). The consequence of an increase in visual pigment concentration in the retinula cells is an elevated photon catch (Williams 1998). In an environment dominated by specific wavelengths, most information about objects of interest (e.g. food sources or obstacles) would be coded in the wavelengths available. Therefore an increased sensitivity in that part of the spectrum may allow an improved color discrimination capability. Thus, the increased mRNA expression might constitute a mechanism of optimizing the sensitivity of a visual system under different photic conditions.

(3) Both of these mechanisms apply to the bumblebee system. This would result in an increase of visual pigment concentration in those receptors that are most sensitive to the incident light spectrum combined with a decrease of opsin expression in those photoreceptors experiencing light deprivation. The resulting difference in mRNA expression is expected to be stronger in that kind of regulatory system.

To distinguish between these three explanations, we must examine the absolute change in opsin concentration. If the first explanation is true, the opsin mRNA expression should decrease in photoreceptors that are exposed to light not matching their peak sensitivity and remain constant in receptors that are stimulated by light to which they are most sensitive. Regarding the second explanation, receptors exposed to light that match their maximum sensitivity should exhibit an increase in mRNA concentration and show a constant opsin expression in the other receptor types. According to the third explanation both effects should be observed. An increased expression of the opsin mRNA in one type of the photoreceptors, as well as a decrease of mRNA expression in the other receptor types. The results of the $2^{-\Delta\Delta Ct}$

method where I measured UV opsin expression in relation to a housekeeping gene suggest that, at least, the UV mRNA expression remains constant even when exposed to stimulating light (Fig. 15).

Photoreceptor sensitivity

Overall, the mRNA expression analysis revealed an unexpected picture of opsin expression in the complex eyes of species of *Bombus*. Recent immunohistochemical studies in honeybees and bumblebees have shown that various types of ommatidia exist in the complex eye, which differ in the relative composition of the three photoreceptor types (Spaethe and Briscoe 2005, Wakakuwa et al. 2005). On average six green, one blue and one UV sensitive receptor cell have been found per ommatidium. From these data, I expected the relative mRNA expression of the green, blue and UV opsin to be 0.75: 0.125: 0.125, assuming an equal opsin expression in each photoreceptor. While the blue opsin and the green expression turned out to closely match the expected ratio (blue: 11% ± 3 SD, green: 70% ± 6%), the UV expression was found to be significantly higher than expected (19% ± 4%; $p < 0{,}001$; $T=8.60$; $df=30$; t-test). Considering the fact that in the compound eye, the green retinula cells are six times more frequent than the UV and blue ones, the elevated UV opsin expression becomes even more obvious. Assuming that a higher mRNA opsin expression also results in a higher protein concentration, I conclude that the visual pigment concentration in the microvilli membrane of the UV-sensitive retinula cells is higher compared to the concentration in the blue or green receptors. As a consequence, the higher pigment concentration increases the photon catch of the retinula cell and thus increases the overall sensitivity of the photoreceptor. Interestingly, my findings nicely match the results of electrophysiological studies by Menzel (1979) who measured spectral sensitivity of photoreceptors in *Apis mellifera* by means of intracellular recordings. He reported that UV receptors exhibited the highest sensitivity compared to the green and blue photoreceptor (relative sensitivity for the UV, blue and green receptors was found to be 16:2.7:1). Therefore, the higher concentration of mRNA opsin per ommatidium found in the UV photoreceptor may, at least, partially explain the differences in absolute sensitivity of the different photoreceptor types found by electrophysiology (Menzel 1979).

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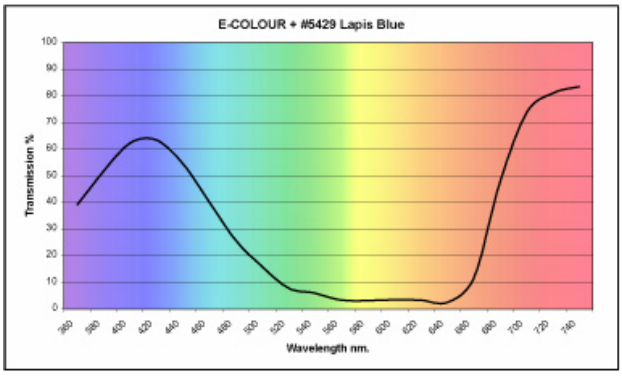
Appendix

Technical data sheets

COLOR FILTER TECHNICAL DATA SHEET

SWATCHBOOK: E-COLOUR +
COLOR FILTER: #5429 Lapis Blue
DESCRIPTION: Color Lighting Filter. Enhances Blue by Three Stops.
TRANSMISSION = 15% or -2.7 stop loss
MIRED SHIFT = Not Applicable.
CC EQUIVALENT = cc 90 Blue

COLORIMETRIC DATA
OBSERVER: CIE 1964 10°
SOURCE: • 'A' (tungsten)
° 'D65' (daylight)

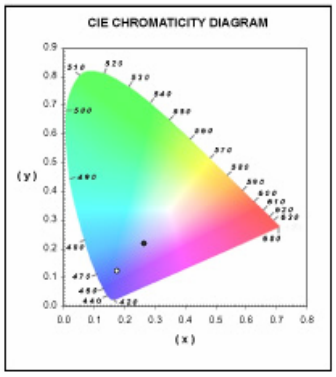


HUNTER LAB SOURCE A	
L*	29.973
A*	5.257
B*	-69.828

HUNTER LAB SOURCE D65	
L*	35.057
A*	31.31
B*	-65.044

CIE 1964 SOURCE A	
Y	6.225
(x)	0.265
(y)	0.220

CIE 1964 SOURCE D65	
Y	8.527
(x)	0.175
(y)	0.124



nm.	360	380	400	420	440	460	480	500	520	540	560	580	600	620	640	660	680	700	720	740
trans %	39	52	62	63	54	40	26	16	8	6	3	3	3	3	3	11	48	74	81	83

MATERIAL SPECIFICATIONS:
General Description: Deep-dyed polyester film
Substrate: PET (Polyethylene Terephthalate)
Thickness: 2.0 mil (.002" or 50 micron)

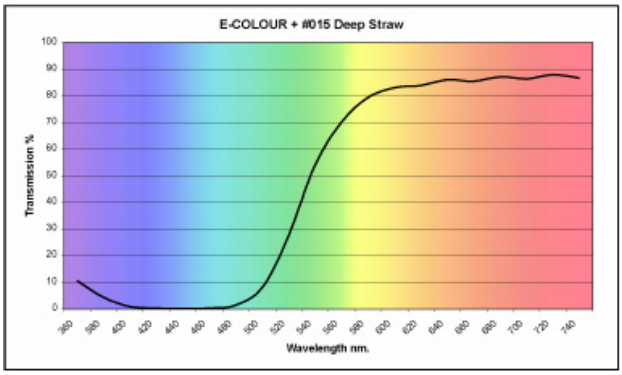
Manufactured in: U.S.A.

AVAILABLE SIZES:
20 in. x 24 in. sheets (50cm x 60cm)
√ 21 in. x 24 in. sheets (53 x 60cm)
√ 24 in. x 25 ft. rolls (60cm x 7.62m)
√ 48 in. x 25 ft. rolls (121cm x 7.62m)
60 in. x 20 ft. rolls (152.4cm x 6.10m)
13.5 in. Diameter Glass (34.3cm) - Cut to order

COLOR FILTER TECHNICAL DATA SHEET

SWATCHBOOK: E-COLOUR +
COLOR FILTER: #015 Deep Straw
DESCRIPTION: Color Effects Lighting Filter.
TRANSMISSION = 61% or -0.7 stop loss
MIRED SHIFT = Not Applicable.
CC EQUIVALENT = Not Applicable.

COLORIMETRIC DATA
OBSERVER: CIE 1964 10°
SOURCE: • 'A' (tungsten)
° 'D65' (daylight)

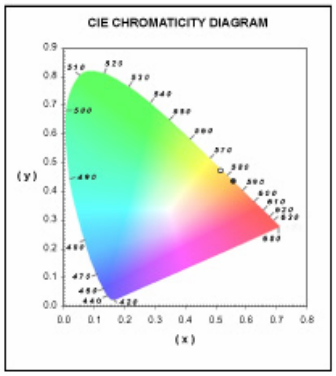


HUNTER LAB SOURCE A	
L*	84.207
A*	22.960
B*	119.797

HUNTER LAB SOURCE D65	
L*	78.374
A*	19.731
B*	118.606

CIE 1964 SOURCE A	
Y	64.464
(x)	0.559
(y)	0.436

CIE 1964 SOURCE D65	
Y	53.850
(x)	0.517
(y)	0.472



nm.	360	380	400	420	440	460	480	500	520	540	560	580	600	620	640	660	680	700	720	740
trans %	11	4	1	0	0	0	2	8	28	54	70	79	83	84	86	86	87	86	88	87

MATERIAL SPECIFICATIONS:
General Description: Dye-Coated Polyester Film
Substrate: PET (Polyethylene Terephthalate)
Thickness: 3.0 mil (.003" or 75 micron)

Manufactured in: U.K.

AVAILABLE SIZES:
20 in. x 24 in. sheets (50cm x 60cm)
√ 21 in. x 24 in. sheets (53 x 60cm)
24 in. x 25 ft. rolls (60cm x 7.62m)
√ 48 in. x 25 ft. rolls (121cm x 7.62m)
60 in. x 20 ft. rolls (152.4cm x 6.10m)
13.5 in. Diameter Glass (34.3cm) - Cut to order

Curriculum vitae

Tobias Pamminger July 2008

PERSONAL DETAILS

Date of birth: 21.09.1981

Place of birth: Vienna, Austria

Address:
Department of Evolutionary Biology
University of Vienna
Althanstrasse 14
1090 Vienna, AUSTRIA
Tel.: 0043 (0) 650 822 40 59
E-mail: tobias_pamminger@yahoo.com

EDUCATION

2002 – 2008	Biology / Zoology at the University of Vienna
2001 – 2002	Civil Service
2000 – 2001	Graduation School (Externistenmatura in the AHS Mariannum)
1987 – 2000	Private School in Vienna

STUDIES ABROAD

2005 – 2006	Erasmus year at University Bergen (NOR)
1998 – 1999	Senior year in High School, Grass Valley CA (USA)

PROFESSIONAL EXPERIENCES

2008	Poster presentation at the International conference on invertebrate vision (SWE): "Adaptation of the visual system in terrestrial habitats"
2007/2008	Hohe Tauern/AUSTRIA National Park "Hohe Tauern" Pollination and foraging stategies on *Aconitum napellus*.
2007	Fethiye/Turkey (5 week) Sea Turtle Project (http://www.seaturtlecourse.com)