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Visual sensitivity in the crepuscular owl butterfly *Caligo memnon* and the diurnal blue morpho *Morpho peleides*: a clue to explain the evolution of nocturnal apposition eyes?

Rikard Frederiksen* and Eric J. Warrant

Lund University, Department of Cell and Organism Biology, Helgonavägen 3, S-22362 Lund, Sweden

*Author for correspondence (e-mail: rikard.frederiksen@cob.lu.se)

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SUMMARY

Insects active in dim light, such as moths and many beetles, normally have superposition compound eyes to increase photon capture. But there are nocturnal and crepuscular insects – such as some species of bees, wasps and butterflies – that have apposition compound eyes. These are likely to have adaptations – including large eye and facet size and coarsened spatial and temporal resolution – that improve their sensitivity and thus their visual reliability. Is this also true for crepuscular insects that are active at intermediate intensities? To test this hypothesis, the visual performance of two closely related butterflies, the diurnal blue morpho *Morpho peleides* and the crepuscular owl butterfly *Caligo memnon*, were compared. Compared to the diurnal *M. peleides*, the crepuscular *C. memnon* does not appear to be adapted to a nocturnal lifestyle in terms of spatial resolution: the interommatidial angle $\Delta\phi$ is similar in both species, and acceptance angles, $\Delta\rho$, are only marginally larger in *C. memnon*. Moreover, temporal resolution is only a little coarser in *C. memnon* compared to *M. peleides*. Using a model for sensitivity, we found that the eyes of *C. memnon* are about four times as light-sensitive as those of *M. peleides* in the frontal visual field, much of this difference being due to the larger facet diameters found in *C. memnon*. In summary, greater visual sensitivity has evolved in *C. memnon* than in *M. peleides*, showing that adaptations that improve sensitivity can be found not only in nocturnal apposition eyes, but also on a smaller scale in crepuscular apposition eyes.

Key words: visual sensitivity, crepuscular vision, apposition eye, Nymphalidae.

INTRODUCTION

Competition and predation are two of the main selective forces that have driven the evolution of animal senses and have led to adaptations that improve the detection of food, mates and predators (Land and Nilsson, 2002). In terms of lost resources, and time and energy spent in defence, competition is costly, and evolution should therefore favour every strategy that reduces it. One such strategy is to develop a nocturnal or crepuscular lifestyle (Wcislo et al., 2004; Smith et al., 2003).

Visual systems are known to show considerable flexibility during the evolution of adaptations that optimise them for a particular light environment (Cronin et al., 1994; Cheroske et al., 2006). Life in dim light, for instance, is particularly challenging to the visual system (Warrant, 2004). The light intensity on a moonless night is over a hundred million times lower than on a bright sunny day (Warrant and McIntyre, 1992). To deal with this extreme range of intensities several eye designs, of various sensitivities, have evolved. This is clearly seen in the compound eyes of insects. Two basic eye designs are found: superposition eyes and apposition eyes. There are several subtypes and variations in these two designs (Nilsson, 1990), but the type of eye that is found in a typical day-active insect is the focal apposition eye. Each rhabdom receives light from a single facet lens, and the apposition eye is thus best suited to insects active in bright conditions. Crepuscular (dusk- and dawn-active) and nocturnal insects, such as moths, many beetles and even some butterflies [Hedyloidea (Yack et al., 2007)], typically have superposition eyes that have evolved to capture as much of the available light as possible. This is achieved by increasing the effective aperture and allowing light from large numbers of corneal facet lenses to be focused onto one rhabdom.

There are, however, interesting exceptions. Diurnal superposition eyes have been reported from several groups of lepidoptera [e.g. Sphingidae (Exner, 1891; Warrant et al., 1999) and Hesperiidae (Swihart, 1969; Horridge et al., 1972)] and beetles [e.g. dung beetles (McIntyre and Caveney, 1985)], and there are nocturnal and crepuscular insects that have retained apposition eyes as they evolved a life in dimmer and dimmer light (Warrant et al., 2004; Kelber et al., 2006).

However, irrespective of eye design, there are adaptations that tune visual systems to specific light intensity windows. For instance, the superposition eye of the diurnal hummingbird hawkmoth *Macroglossum stellatarum* is highly resolved (Warrant et al., 1999) and has a considerably smaller superposition aperture (composed of fewer corneal facet lenses) than found in the nocturnal elephant hawkmoth *Deilephila elpenor* (Kelber et al., 2002). In nocturnal insects with apposition eyes, adaptations for increased sensitivity can likewise be found. The nocturnal halictid bee *Megalopta genalis*, for instance, is an insect with apposition eyes that is active at intensities about ten times dimmer than starlight (Warrant et al., 2004; Kelber et al., 2006). In order to achieve this, *M. genalis* has enlarged facets and rhabdoms, as well as compromised spatial and temporal resolution, all of which favour increased sensitivity (Warrant et al., 2004; Greiner et al., 2004).

There are also many crepuscular insects with apposition eyes. Do these insects, active at intermediate light intensities, also possess important adaptations that improve visual sensitivity in dim light,

but on a smaller scale? If so, do these adaptations reveal anything about the evolutionary transition from a diurnal to a nocturnal lifestyle?

To explore these questions we have examined the anatomical, optical and physiological parameters that determine visual sensitivity in two similarly sized and closely related (Wahlberg et al., 2003; Freitas and Brown, 2004) species of nymphalid butterflies from the neotropical rainforests of Central America – the crepuscular owl butterfly *Caligo memnon* and the diurnal blue morpho *Morpho peleides*. Like all papilionoid butterflies, both species possess afocal apposition eyes (Nilsson et al., 1984; van Hateren and Nilsson, 1987; Nilsson et al., 1988), a design best suited for bright light and in many respects intermediate between the focal apposition design and the superposition design: as in superposition eyes, their lens systems possess graded refractive-index elements (Nilsson et al., 1988). The interesting difference between the two species is that *C. memnon* is active at dawn and sometimes at dusk (Malo and Willis, 1961; Srygley, 1994), when the luminance is 2–4 orders of magnitude dimmer than daylight, while *M. peleides* is active only during the day (Young, 1982; DeVries, 1987).

MATERIALS AND METHODS

Pupae of *Caligo memnon* C. & R. Felder 1866 and *Morpho peleides* Kollar 1850 were purchased from London Pupae Supplies, London, and Stratford Butterfly Farm, Stratford. The pupae were kept hanging in boxes and eclosed within a week. We held the adult butterflies in a cage with free access to food (mashed banana mixed with water) and water. The butterfly cage had a diurnal light cycle of 12 h:12 h light:darkness. We only used male butterflies in the experiments in order to avoid assumptions about the visual physiology being affected by sex-specific visual adaptations related to reproductive biology.

Electrophysiology

In preparation for electrophysiology we removed the wings of the butterfly and inserted it in a tube made of a plastic pipette tip with the small end sliced off to accommodate the head of the butterfly. Only the head of the animal was allowed to protrude through the hole. The animal was fixed to this tube with a tiny amount of 50:50 mixture of bee's wax and violin resin melted onto the mouthparts (proboscis and labial palps) and the dorsal and ventral sides of the head as well as the antennae. The tube containing the animal was attached to a holder on a magnet stand with the aid of dental wax. The indifferent electrode, consisting of a thin silver wire, was inserted through a hole made between the eyes and fixed in position with the same wax mixture used to fix the animal to the plastic tube. A small, approximately ten-facets-wide, triangular hole was cut in the ventral portion of the eye and sealed with Vaseline. After fixation and dissection, we mounted the magnet stand in the centre of the electrophysiology apparatus. The animal was placed with its anterior end facing upwards and the angular position of the animal was noted carefully. Finally the electrode was inserted through the hole in the eye.

The electrophysiology apparatus contained one stimulating section and one recording section, all controlled by a Macintosh computer and LabVIEW 2.2.1 software (National Instruments, Austin, TX, USA). White stimulus light was produced by a Nikon XPS-100 xenon arc lamp. The light was directed through a series of filter wheels and a shutter (UniBlitz T132 shutterdriver/timer; Rochester, NY, USA) before reaching the animal through a quartz light guide. Neutral density filters controlled the intensity of the stimulus and interference filters controlled the wavelength of the stimulating light. The shutter regulated the stimulus light pulse length. The end of the light guide was held in a goniometer arm that allowed the stimulating light to be placed at any position in the visual field of the animal. The stimulus could thus be moved in known angular steps throughout the visual field of the eye. When recording from a photoreceptor we could therefore note its exact position, in terms of latitude and longitude, on an imaginary sphere around the animal. The stimulating end of the light guide had a diameter of 100 μm, and was positioned 115 mm away from the centre of the goniometer arm, making the stimulus a point source subtending a width of 0.050°. Surrounding the point source was a light-adapting device consisting of a set of 15 white LEDs (EL333UWC, Everlight Electronics, Taipei, Taiwan) illuminating a circular plastic diffuser disc with a 40 mm diameter.

For recording we used glass (borosilicate) microelectrodes filled with 2 mol l^{-1} potassium acetate (200–300 MΩ resistance *in vivo*). The electrode was inserted into the hole in the ventral part of the eye using a Märzhäuser PM10 (Wetzlar-Steindorf, Germany) piezo-driven micromanipulator. The electrical responses were amplified on a Biologic VF180 (Claix, France) microelectrode amplifier. Mains noise was eliminated using a HumBug, from Quest Scientific (North Vancouver, BC, Canada). The amplified signal was low-pass filtered at 400 Hz and digitised into a Macintosh computer using LabVIEW 2.2.1 software (National Instruments).

All electrophysiological experiments were performed in a laboratory at a temperature range of 23–25°C. Dark adaptation was performed by switching off all the lights in the room (resulting in a light intensity of 3.5×10^{-4} cd m^{-2}). For light adaptation we used a background illumination of 200 cd m^{-2}. Light and dark adaptation were maintained for at least 30 min prior to recording, and often longer.

Penetration of a photoreceptor cell was indicated by a drop in the baseline of 40–60 mV and depolarising responses to a flashlight. Once a photoreceptor was penetrated, we moved the goniometer arm so that the point source was positioned on the visual axis of the cell. This was indicated by the direction from which the maximum electrical response was generated. Following this we recorded the V–log$I$ curve, the spectral sensitivity, the impulse response and the angular sensitivity of the cell. The sampling frequency was 2.5 kHz in all experiments.

The V–log$I$ curve was plotted from the cell's responses to a series of 40 ms long pulses of white light of increasing intensity, with the point source aligned with the cell's optical axis. The spectral sensitivity was recorded from the cell by stimulation with a series of 40 ms light pulses at different wavelengths. The interference filters used in this experiment had peak transmissions separated by 50 nm and ranged from 350 nm to 700 nm. The band-pass of the interference filters was 40 nm. Because of the broad band-pass of the interference filters the spectral sensitivity function could not be measured exactly. Nevertheless, it gave a good estimation of the wavelength range where the cell was maximally sensitive. Although we occasionally penetrated cells that were maximally sensitive in the blue and UV range of the spectrum, the vast majority of the cells were 'green-sensitive' with a sensitivity peak at around 550 nm. Only these cells were used for further experiments, since these are considered to be the part of the pathway for contrast and luminance vision in insects (Osorio and Vorobyev, 2005). Following the measurement of spectral sensitivity, the impulse response was recorded. The shutter was set to deliver 2 ms light pulses and the neutral density filters were adjusted to give a dim stimulus that resulted in a 2–3 mV depolarising response in the cell. Responses from 100 pulses were recorded and averaged from each cell. Lastly,

we recorded the angular sensitivity function of the cell. The shutter was reset to deliver 40 ms flashes and the neutral density filters were adjusted to an intensity that resulted in the cell giving a depolarising electrical response of 50–75% of maximum. The point source was displaced from the cell's optical axis outside the visual field. During the recording, the stimulus was moved in known angular steps across the visual field. At each step one stimulus flash was delivered and the electrical response of the cell was recorded. When recording from *C. memnon* we used 0.5° steps and 0.25° steps from *M. peleides*. The responses were converted to equivalent intensities through the *V*–log*I* curve and the sensitivities at each angular step were calculated.

In total, 33 cells from nine individuals of *C. memnon* and 28 cells from six individuals of *M. peleides* were used for electrophysiology. Of these, only cells with a spectral sensitivity maximum of about 550 nm, and a maximum response of at least 30 mV, were used for analysis of the impulse response and angular sensitivity function. We also rejected all cells that did not have symmetrical angular sensitivity functions, since an asymmetrical angular sensitivity function may indicate damaged optics or an artificial double cell recording. The final number of cells used for analysis was 11 (9 dark-adapted recordings and 6 light-adapted recordings) from *C. memnon* and 12 (10 dark-adapted recordings and 6 light-adapted recordings) from *M. peleides*.

Maps of interommatidial angles

We made two eye maps of males of each species using standard methods (Land and Eckert, 1985; Rutowski and Warrant, 2002). Briefly, the animal was mounted in a plastic tube in the same way as in the preparation for electrophysiology. The left eye was dusted with chalk dust particles that were used as landmarks in the analysis. We placed and centered the preparation in a goniometer that in turn was placed under a microscope adapted for orthodromic illumination: an axial light source illuminates the insect eye with white light that is reflected by the mirror-like tapetum below the retina. This reflection is seen as a 'bright pseudopupil', a brightly lit spot on the eye surface facing upwards into the microscope, light that is not absorbed by the photopigments.

A magnified image of the eye, with pseudopupil and landmarks, was taken at the central front of the butterfly's visual field (latitude=0°, longitude=0°). We changed the angular position of the goniometer in 10° steps of latitude and longitude and took a new image at each. The procedure was repeated until the limits of the goniometer were reached (latitudes 80°, –80° and longitudes 80°, –80°). From the images the facet diameters were measured to produce an additional map with isolines of facet diameters. The local interommatidial angle was calculated by counting the number of facet rows (in *x*, *y* and *z*-axis) that the pseudopupil moves between the angular steps. This data was used to make a map with isolines of interommatidial angle.

Histology

Living animals were decapitated and the eyes were removed from the head and put into fixative for 24 h. We used a fixative made of 90 ml ethanol (80%), 5 ml concentrated acetic acid and 5 ml formalin (40%). Following fixation, the eyes were dehydrated in an ethanol series (70% 2×20 min, 96% 2×20 min, 100% 2×30 min) and then put in a series of acetone mixed with Epon (Poly/Bed® 812, Polysciences, Eppelheim, Germany) plastic (acetone 2×30 min, 2:1 acetone/Epon 1 h, 1:1 acetone/Epon over night, 1:2 acetone/Epon over day, and pure Epon over night). The eyes were embedded in new Epon and polymerised for 48 h at 60°C. We cut 2 μm thick sections for light microscopy using a LKB Bromma 11800 pyramitome (Bromma, Sweden) with a glass knife. From the sections we measured the rhabdom length in the frontal visual field from two eyes in each species using five sections from each eye.

In preparation for transmission electron microscopy, the animals were dissected in the same way as for light microscopy. The eyes were fixed in 2% paraformaldehyde, 2.5% glutaraldehyde and 2% sucrose in a 0.15 mol l^{-1} sodium cacodylate buffer, pH 7.2, for 12 h. After the fixation the eyes were rinsed in 0.15 mol l^{-1} sodium cacodylate buffer and postfixed in 1% osmium tetroxide (in the same buffer as above) and then rinsed again. The dehydration and embedding process was the same as for the light microscopy preparation. Thin sections (0.05 μm) were cut using a Leica Ultracut UCT ultratome (Wetzlar, Germany) with a diamond knife and mounted on grids for transmission electron microscopy. The sections were stained with uranyl acetate (3%, 30 min) and lead citrate (1%, 4 min). The microscope used was a Jeol JEM-1230 (Tokyo, Japan). Photographs of the rhabdoms of both species were taken and the rhabdom diameter measured.

For scanning electron microscopy we used air-dried specimens that were mounted on stubs and sputter coated with gold/palladium (Polaron SC7640 sputter coater; Quorum Technologies Ltd, Ringmer, E. Sussex, UK) at 1.2 kV, 11 mA and 0.03 mbar (3 Pa). The microscope used was a Jeol JSM-5600 LV scanning electron microscope. Photographs of the surface of the eyes of both species were taken for eye-size comparison.

RESULTS

Morphology of the eyes

C. memnon has exceptionally large facets in the frontal visual field (maximal *D*=48 μm, Fig. 1A) while *M. peleides* has more moderate facet diameters (maximal *D*=34 μm, Fig. 1C). Even though the two species are approximately the same size, this difference is essentially due to the much larger total eye size of the former (Fig. 2). An enlarged eye is beneficial not only for increased sensitivity due to enlarged apertures, but also for increased spatial acuity, since it allows smaller interommatidial angles (Fig. 1B,D), as will be described in detail in the next section.

C. memnon has rhabdom lengths of 451±65 μm in the part of the retina viewing the frontal visual field. In *M. peleides*, rhabdoms at the corresponding position have lengths of 430±48 μm. The rhabdom lengths of both species increase from the posterior to the anterior, and from the ventral to the dorsal, parts of the eye.

Rhabdom diameters are larger in *C. memnon* (3.9±0.2 μm, Fig. 3A) than in *M. peleides* (2.0±0.1 μm, Fig. 3B). Wide rhabdom diameters can potentially increase sensitivity by increasing the solid angle ($\pi d^2/4f^2$ steradians) of visual space that is viewed by the receptor (where *d* is the rhabdom diameter, and *f* is the focal length). A larger solid angle contributes to a higher sensitivity but worsens spatial resolution (Kirschfeld, 1974; Land, 1981; Warrant, 2004).

Spatial resolution

The spatial resolution of a compound eye is ultimately dependent on the rhabdom acceptance function, the focused image quality, and the sampling density (Land, 1981). The angular sensitivity function (ASF) accounts for the two first of these parameters and its angular width at 50%, the acceptance angle $\Delta\rho$, is a convenient measure to quantify spatial resolution.

C. memnon has slightly broader ASFs than *M. peleides* (Table 1). In both species the smallest acceptance angles (Fig. 4) were found near the equator of the visual field, 10° to 20° lateral of anterior.

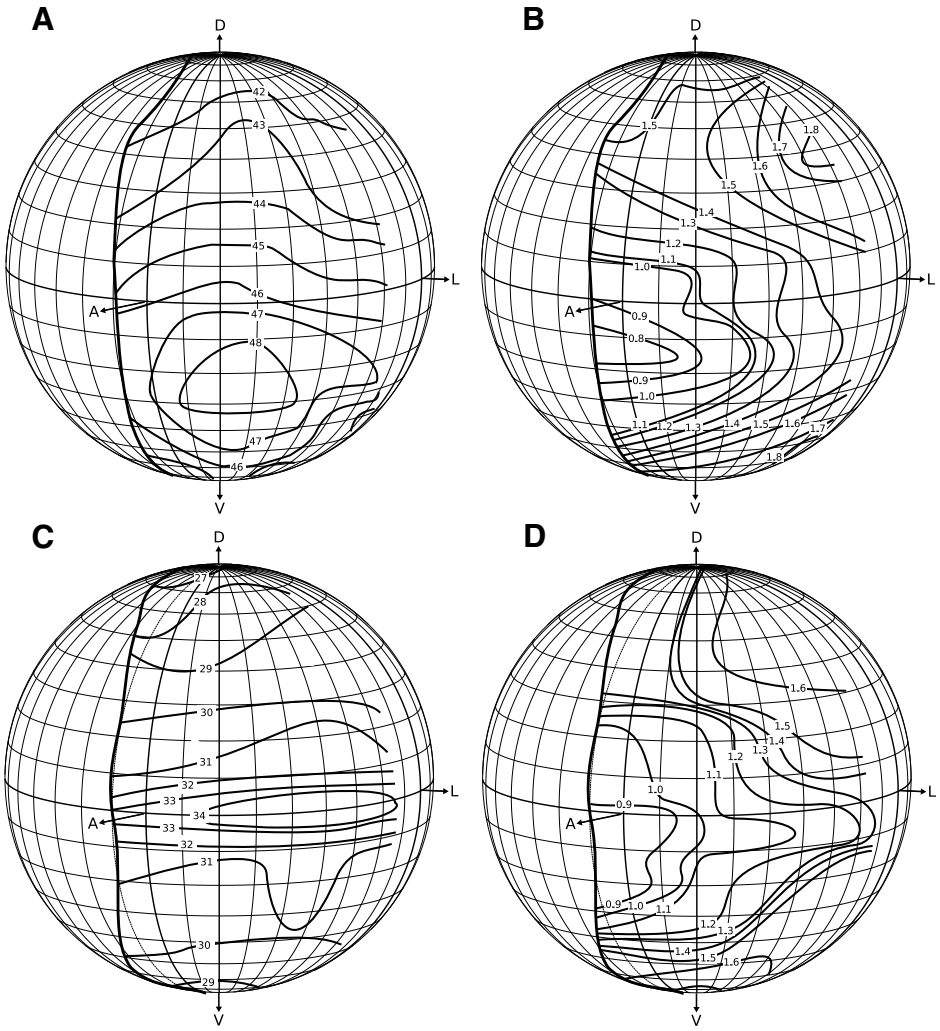

Fig. 1. Maps of the frontal visual fields of the left eyes of male *Caligo memnon* (A,B) and male *Morpho peleides* (C,D) with isolines representing facet diameter *D*, in μm (A,C), and interommatidial angle Δφ, in degrees (B,D). The globes represent the three-dimensional space around the butterfly (with lines of latitude and longitudes in 10° steps), with the eye located at the globe's centre. The arrows indicate the orientation of the butterfly: A, anterior; D, dorsal; V, ventral; L, lateral. The boundary of the eye's receptive field is also indicated by the dorso-ventrally oriented contour in each panel.

This is also an area of small interommatidial angles and large facet diameters (Fig. 1).

So far we have mentioned the parameters that determine the spatial resolution and sensitivity of a single rhabdom. To get the full picture, the visual sampling density of the eye, defined by the interommatidial angle Δφ, the angular spacing between two neighbouring ommatidia, must also be considered. The interommatidial angle is defined by the ratio of the facet diameter *D*, to the radius of curvature *R* of the eye [*D*/*R* rad (Land, 1981)].

Both species have interommatidial angles that are in the same size range (about 1–2°, depending on position in the eye), but the distribution pattern in the visual field is rather different (Fig. 1).

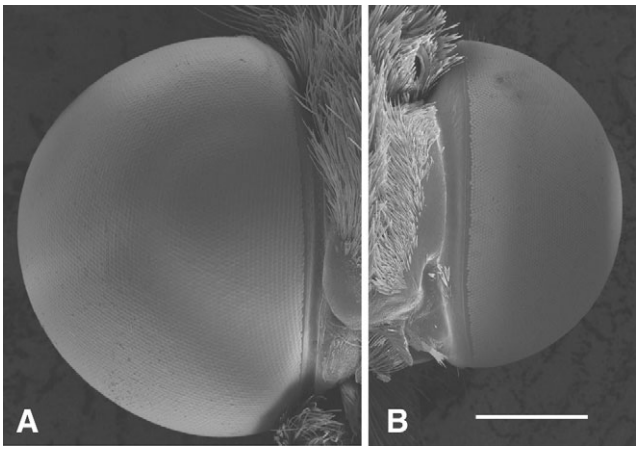

Fig. 2. Scanning electron micrographs of the eyes of a male *Caligo memnon* (A) and a male *Morpho peleides* (B). The local eye radius of *C. memnon* is almost twice as large as that of *M. peleides*. Scale bar for A and B, 1 mm.

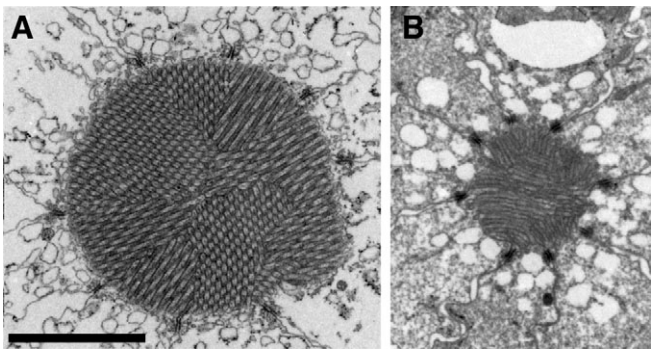

Fig. 3. Transmission electron micrographs showing distal transverse sections through the rhabdoms of male *Caligo memnon* (A) and male *Morpho peleides* (B). Scale bar for A and B, 2 μm.

Table 1. Spatial and temporal resolution of the photoreceptors of *Caligo memnon* and *Morpho peleides* in the light-adapted and dark-adapted states

Species	LA/DA	Δρ (degrees)	τ_p (ms)	Δ*t* (ms)	*N*
Caligo memnon	LA	1.10±0.24	17.8±0.8	9.5±0.6	6
	DA	2.06±0.30	29.4±4.6	18.2±3.4	9
Morpho peleides	LA	0.96±0.08	16.5±0.6	8.2±0.8	6
	DA	1.63±0.25	27.1±2.1	15.4±2.5	10

LA, light-adapted state; DA, dark-adapted state, as represented by acceptance angle (Δρ) from the angular sensitivity function and two parameters from the impulse response: τ_p, the time-to-peak and Δ*t*, the integration time.
Values are means ± s.d., based on *N* cells, as indicated in the table. See text for details.

The visual field of *C. memnon* has a frontal acute zone 10–30° ventral of the equator. *M. peleides*, on the other hand, has a visual streak along the equator of the visual field where the largest facets are also found. Both species have the smallest interommatidial angles in the anterior part of the eye, about 10–20° ventral of the equator.

Temporal resolution

The impulse response is the cell's response to a very short and dim flash of light that elicits an electrical response similar to that of one photon (a photon bump). The half-width, or integration time Δ*t*, of the impulse response is a good measure of the temporal resolution of an eye (Pinter, 1972; Howard et al., 1984).

Both species show considerably lower temporal resolution in the dark-adapted state than in the light-adapted state, both in terms of time-to-peak τ_p (the time from the stimulus onset to the maximal response amplitude) and Δ*t* (Table 1). Moreover, *C. memnon* has longer integration times and times-to-peak than *M. peleides* in the dark-adapted state. In the light-adapted state, the difference is minor (Fig. 5, Table 1).

By taking the squared absolute value of the Fourier transform (Fig. 5C,D) of the impulse response (Fig. 5A,B) we obtain its power spectrum and can determine the temporal frequencies that can be perceived by the animal's visual system at a particular state of light adaptation. The shorter time course of the light-adapted impulse response translates into a wider range of perceivable frequencies compared to the smaller range possible in the dark-adapted state. The corner frequency f_c of the power spectrum [the frequency at which the power has fallen off to 50% of its maximum (Howard et al., 1984)] is useful for comparing power spectra.

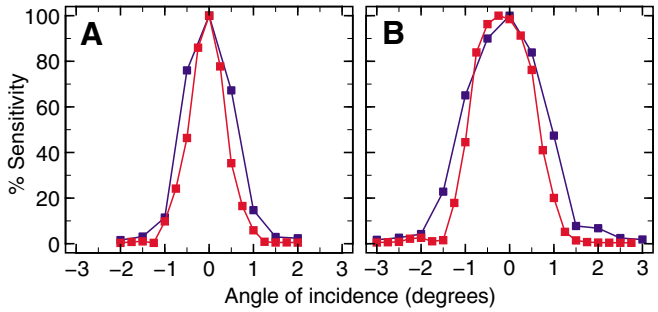

Fig. 4. Typical angular sensitivity functions recorded from single cells in a male *Caligo memnon* (blue) and male *Morpho peleides* (*red*) in (A) light-adapted (LA) and (B) dark-adapted (DA) conditions. The acceptance angles of the angular sensitivity functions for these cells are slightly larger in *C. memnon* ($\Delta\rho_{LA}$=1.4°, $\Delta\rho_{DA}$=2.1°) than in *M. peleides* ($\Delta\rho_{LA}$=0.9°, $\Delta\rho_{DA}$=1.7°).

The power falls off at lower frequencies in *C. memnon* ($f_{c,LA}$=30.7±2.3 Hz, $f_{c,DA}$=18.9±7.0 Hz) than in *M. peleides* ($f_{c,LA}$=37.0±4.0 Hz, $f_{c,DA}$=18.2±5.3 Hz). This is due to the wide and skewed dark-adapted impulse response (Fig. 5) in *C. memnon*. The difference between the species is, however, small.

Sensitivity

Several optical and physiological parameters contribute to the sensitivity of an eye. In order to obtain a more complete picture we calculated the sensitivity *S* (μm²sr) of the eyes in *C. memnon* and *M. peleides* (Kirshfeld, 1974; Land, 1981; Warrant and Nilsson, 1998):

$$S = \left(\frac{\pi}{4}\right)^2 D^2\Delta\rho^2\left(\frac{kl}{2.3+kl}\right), \quad (1)$$

where the new parameters are the absorbance coefficient of the rhabdom, *k*, and the rhabdom length, *l*. The factor (*d*/*f*) from the equation's original formulation has been replaced by Δρ (Warrant, 1999), an approximation that is relevant for apposition eyes (Stavenga, 2004a; Stavenga, 2004b). The parameters used in the calculation, and their values, are summarised in Table 2.

The calculations show that *C. memnon* (*S*=1.3 μm²sr) is about 3.3 times as sensitive as *M. peleides* (*S*=0.4 μm²sr). If we also account for the 1.2 times longer integration time (Table 1) in *Caligo*'s photoreceptors, we end up with a sensitivity difference of about four times between the species.

DISCUSSION

Due to the rapidly changing light environment at dusk and dawn, crepuscular insects experience a wide range of intensities. There are thus likely to be opposing selective pressures working on the visual system that on the one hand tend to increase the optical sensitivity of the eye in order to capture enough photons to produce a reliable image, while on the other hand attempt to maintain a reasonable acuity (Warrant and McIntyre, 1992). The crepuscular *C. memnon* has evolved large eyes with large facets but has retained a reasonably high spatial and temporal resolution. Nonetheless, its vision is 3–4× more sensitive than that in the diurnal *M. peleides*. The major visual adaptations contributing to this sensitivity difference are the enlarged facets (2× increase), the larger acceptance angles (1.7× increase) and the longer integration time (1.2× increase). The effect of the somewhat longer rhabdom lengths found in *C. memnon* is negligible. Interestingly, we observe that all visual properties that contribute to sensitivity in an apposition eye, and thus adapt it for a crepuscular or even a nocturnal lifestyle, do not seem to contribute equally and probably do not change at the same evolutionary pace.

Morphology, optics and spatial resolution

Perhaps the most evident adaptation for crepuscular vision in *C. memnon* is its enlarged facets. The optical sensitivity increases with the lens diameter squared, D^2 [the lens area: $\pi D^2/4$ (Kirschfeld, 1974; Land, 1981)]. If we compare the facet diameters of *C. memnon* with those of *M. peleides*, in the eye region of highest resolution, we find that the facet diameters alone account for a doubling of the optical sensitivity ($48^2/34^2$=2). It is clear that it is the large eye size of *C. memnon* (Fig. 2) that allows sensitive vision

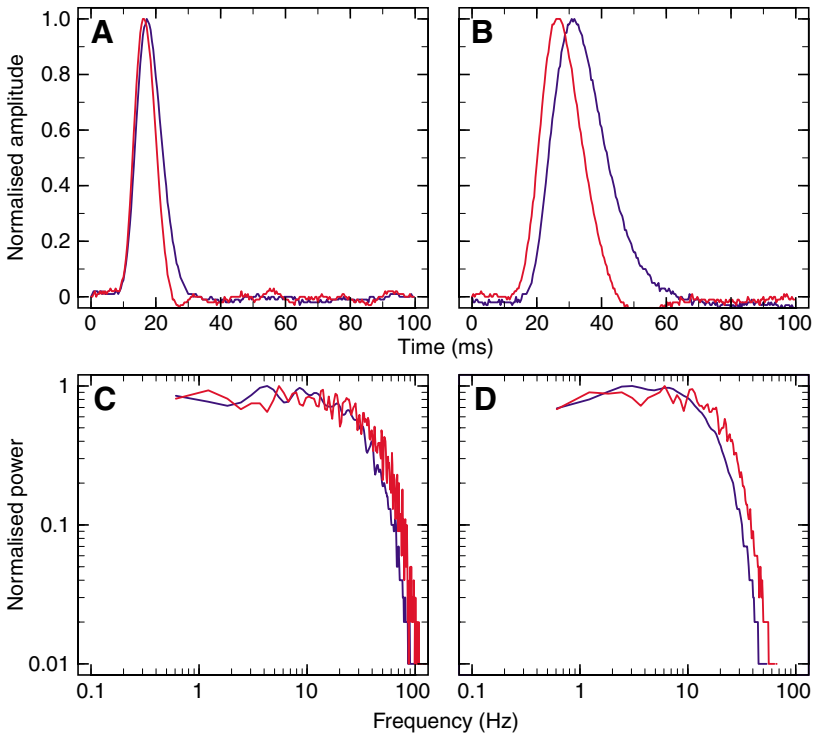

Fig. 5. Typical impulse responses (A,B) with corresponding calculated power spectra (C,D) recorded from single cells in a male *Caligo memnon* (blue) and a male *Morpho peleides* (red) in (A,C) light-adapted (LA) and (B,D) dark-adapted (DA) conditions. The integration time and time-to-peak of these recordings are: *C. memnon* Δt_{LA}=9 ms, Δt_{DA}=18 ms, $\tau_{p,LA}$=17 ms, $\tau_{p,DA}$=31 ms, and *M. peleides* Δt_{LA}=8 ms, Δt_{DA}=15 ms, $\tau_{p,LA}$=16 ms, $\tau_{p,DA}$=26 ms. The corresponding corner frequencies are: *C. memnon* $f_{c,LA}$=33 Hz, $f_{c,DA}$=16 Hz and *M. peleides* $f_{c,LA}$=42 Hz, $f_{c,DA}$=23 Hz.

whilst maintaining reasonably high visual acuity (Figs 1, 4). If we use our data to calculate a local radius of curvature ($R=D/\Delta\phi$) in the eye region of highest spatial resolution, we find that *C. memnon* (R=3.4 mm) has a local radius of curvature that is 1.8× greater than that in *M. peleides* (R=1.9 mm). Since the interommatidial angle is very similar in the two butterfly species, the doubled sensitivity due to enlarged facets in *C. memnon* is mostly due to its very large eyes (Fig. 2). Because an enlargement in eye size and aperture size increases sensitivity while maintaining high acuity, we believe that this is likely to have happened early in the evolution towards increased sensitivity in crepuscular insects. Such enlargements of eye and aperture size have also been reported from other crepuscular and nocturnal insects with apposition eyes (Warrant et al., 2004) as well as from insects with superposition eyes (McIntyre and Caveney, 1998). Moreover, they have also been reported from nocturnal vertebrates such as birds (Brooke et al., 1999; Thomas et al., 2006; Hall and Ross, 2007) and primates (Kay and Kirk, 2000; Kirk, 2004), suggesting that a relative enlargement of eye size is a common evolutionary strategy in animals adapted for reliable vision in dim light. The size of the eye cannot, of course, increase indefinitely but is eventually limited by developmental constraints and opposing selection forces, for instance, limited energy resources (Laughlin et al., 1998). Thus, at some point in the evolution of nocturnality, eye enlargement will cease; acuity is then likely to be sacrificed in order to achieve higher sensitivity. This sacrifice in acuity in the service of higher sensitivity can result from the evolution of one or more of the following three morphological strategies: (1) further increases in facet diameter (that will decrease the sampling density), (2) increases in rhabdom diameter (that will widen the acceptance angle) or (3) spatial summation of signals from groups of several photoreceptors.

Interestingly, different strategies or combinations of different strategies have been reported from different species. The nocturnal bee *Megalopta genalis*, for instance, uses a combination of all of them (Warrant et al., 2004). The nocturnal wasp *Apoica pallens* has large eyes but with many ommatidia, all with small corneal facet lenses. Their rhabdoms, however, are very wide and this, together with their large numbers of small facets, implies the likelihood of spatial summation (Greiner, 2005). In *Caligo memnon*, the eyes, facets and rhabdoms are all larger than in *Morpho peleides*, suggesting that this scaling-up of eye size should lead to improvements in sensitivity without compromising spatial resolution. As we discuss below, this is indeed the case, but further slight improvements in sensitivity have also occurred *via* modest increases in acceptance angle.

C. memnon has a rhabdom diameter, d, about twice as wide as that in *M. peleides* (Fig. 3). Wide rhabdoms increase sensitivity by

Table 2. Parameters used to calculate the optical sensitivity of *Caligo memnon* and *Morpho peleides*

Symbol	Parameter	Unit	*Caligo memnon*	*Morpho peleides*
$\Delta\rho$	Acceptance angle[a]	rad	0.036	0.028
D	Facet diameter	μm	48	34
l	Rhabdom length[b]	μm	902	860
k	Absorption coefficient[c]	μm^{-1}	0.0067	0.0067
S	Optical sensitivity	$\mu m^2 sr$	1.3	0.4

The eye of *C. memnon* has an optical sensitivity S^* (from Eqn 1) that is 3.3 times as high as that in the eye of *M. peleides*.

[a]The factor (d/f) in the original formulation of the sensitivity equation has been replaced by $\Delta\rho$ in our calculations.

[b]We used double rhabdom lengths in the calculations to account for the presence of a *tapetum lucidum*.

[c]k measured by Bruno et al. (Bruno et al., 1977).

increasing the acceptance angle [approximated by *d*/*f* (Stavenga, 2004a; Stavenga, 2004b)]. The mismatch between the small difference in electrophysiologically measured acceptance angles (Fig. 4) and the large difference in rhabdom diameters can be explained by a large local radius of curvature, *R*, in the eye of *C. memnon* (Fig. 2) and its correspondingly longer focal length, *f*. However, the focal length is very difficult to measure optically because of the complex graded refractive index lens system of afocal apposition eyes. Electrophysiologically measured acceptance angles are therefore a much more reliable measure of the eye's resolution and sensitivity than what can be predicted from the ratio of the rhabdom diameter and focal length.

We found somewhat wider acceptance angles in *C. memnon* than in *M. peleides*, indicating more sensitive eyes in the former (Fig. 4). The difference, however, is not as great as one might expect. The dark-adapted apposition eyes of the nocturnal bee *Megalopta genalis*, for instance, have much wider acceptance angles: $\Delta\rho_{DA}$=5.6° (Warrant et al., 2004). This species is active at intensities several orders of magnitude dimmer than those in which *C. memnon* is active, and its much wider acceptance angles (due to much wider rhabdoms) reflect its need for greater visual sensitivity. Perhaps a more relevant comparison is with other lepidopteran apposition eyes. The acceptance angles of both species investigated here are similar to, or smaller than, those of other day active butterflies (Land, 1990). We must therefore conclude that in terms of acceptance angles alone, improvements in sensitivity are modest in *C. memnon* (1.7 times), suggesting that it has been important for this species to maintain high acuity.

A similar pattern is seen in the maps of interommatidial angles (Fig. 1): compared to other butterflies neither of the species have exceptionally large interommatidial angles (Stavenga et al., 2001). These, moreover, should follow the acceptance angles in order to provide an optimal sampling ($\Delta\rho/\Delta\phi$=2) of the image (Snyder, 1977; Snyder et al., 1977; Land, 1997). We calculated the sampling ratio ($\Delta\rho/\Delta\phi$) for *C. memnon* and *M. peleides* in the frontal visual field and found that in the diurnal *M. peleides* ($\Delta\rho_{LA}/\Delta\phi$=1.1 and $\Delta\rho_{DA}/\Delta\phi$=1.8) the light adapted value is very close to the average for insects [$\Delta\rho_{LA}/\Delta\phi$=1.07 (Land, 1997)]. *C. memnon*, on the other hand, has larger sampling ratios ($\Delta\rho_{LA}/\Delta\phi$=1.4 and $\Delta\rho_{DA}/\Delta\phi$=2.6). The oversampling in the dark-adapted eye of *C. memnon* will increase photon capture and produce a brighter image. The trade-off between sensitivity and spatial resolution becomes evident once again: acuity must be sacrificed to achieve a greater sensitivity.

Thus, if during evolution the angular sensitivity functions widen more quickly than the ommatidial sampling density coarsens, the image will be oversampled and thus brighter. An already oversampled image will suffer little from a degradation in acuity caused by an eventual spatial summation at a later stage of processing such as the lamina (Warrant et al., 2004; Greiner et al., 2005): spatial resolution cannot be further coarsened by an equally wide summation of photoreceptor signals, but sensitivity and visual reliability, on the other hand, can be greatly improved. We have no evidence as yet, however, of spatial summation in the lamina of *C. memnon*.

As we have discussed in the previous paragraphs, *C. memnon* achieves most of its sensitivity through its enlarged eyes and corneal facet lenses. Visual acuity is obviously important to *Caligo*, but without sufficient sensitivity the eyes will not capture enough light to exploit this acuity (Warrant and McIntyre, 1992). What might the relatively high acuity be used for? During reproduction, male *Caligo* gather at 'hot spots' for lekking. Although chemical cues have been suggested to be important in the courtship display (Wasserthal and Wasserthal, 1977), vision could also play an important role, in particular for landmark detection when navigating to the lekking site (Srygley and Penz, 1999). Vision might also be important for detecting conspecifics during perching behaviour. There are, however, no previous studies that definitely link reproductive behaviour in *Caligo* to its visual system. *Morpho*, on the other hand, has a more mobile reproductive behaviour and does not aggregate on hot spots for lekking (Young and Muyshondt, 1973). Colour and contrast cues seem to be important for the courtship display in *Morpho* (Young, 1971), and this can be part of the explanation as to why this species has high acuity. However, these differences in reproductive behaviour between the two species cannot on their own satisfactorily explain the differences in their visual systems.

Temporal resolution

Compared to *M. peleides*, does *C. memnon* have adaptations for crepuscular vision in terms of temporal resolution?

Photoreceptors of nocturnal and crepuscular animals have been shown to have slower impulse responses with longer integration times (Δt) than those of diurnal animals with the same type of eye (e.g. Warrant et al., 2004). There is also a trade-off between photoreceptor sensitivity and temporal resolution (Warrant and McIntyre, 1992). Low temporal resolution – from impulse responses with long integration times – means a compromised bandwidth in the frequency response and a lower corner frequency, properties that result in increased motion blur at high angular velocities. High frequency noise is attenuated, however, and reliability is improved, at lower frequencies (Laughlin, 1996).

Although the dark-adapted impulse response of *C. memnon* is slower than that of *M. peleides* (Table 1; Fig. 5), it is not by any means slow compared to other insects. A similar pattern is seen in the power spectra of the two species: power falls off at slightly lower frequencies in *C. memnon*, indicating a somewhat more pronounced low-pass filtering of the visual signal and a greater reliability at lower frequencies. However, it is very likely that other factors apart from light intensity alone have had a large impact on the evolution of light-response dynamics in the two butterfly species studied here (Howard et al., 1984; Laughlin and Weckström, 1993).

Sensitivity

Apart from the much enlarged facet diameters found in *C. memnon*, the individual optical, anatomical and physiological properties of vision that influence visual sensitivity do not seem to contribute much on their own. Nevertheless, their combined effects make the eyes of *C. memnon* about four times as sensitive as those of *M. peleides*. Supporting this, Järemo Jonson et al. (Järemo Jonson et al., 1998) found that the pupil mechanism in the closely related *C. eurolochus* closes at about one log unit dimmer intensities compared to diurnal butterflies and thus indicates a more sensitive eye in the former.

This difference in sensitivity can at first thought appear to be minor, considering that there is a difference in ambient intensity of 2–4 orders of magnitude between the activity peaks of the two species. We do not know, however, if either of the species regularly experiences microhabitats of higher or dimmer light intensity than what is considered to be their 'normal' intensity window. Nor do we yet know whether *C. memnon* increases sensitivity further by employing neural summation, a strategy likely to be used by nocturnal bees (Warrant et al., 2004).

If we compare the calculated sensitivities of the butterflies to other diurnal and nocturnal insects, we find that *M. peleides* (S=0.4 $\mu m^2 sr$) has a sensitivity similar to the honeybee *Apis mellifera* [S=0.1 $\mu m^2 sr$ (Greiner et al., 2004)]. *C. memnon*'s

sensitivity (S=1.3 $\mu m^2 sr$) is not as great as that of the nocturnal bee *Megalopta genalis* [S=2.7 $\mu m^2 sr$ (Greiner et al., 2004)] or the elephant hawkmoth *Deilephila elpenor* [superposition eye, S=69 $\mu m^2 sr$ (Warrant, 2004)], but it is well above that of the honeybee. This shows that adaptations that improve sensitivity can be found not only in nocturnal apposition eyes, but also on a smaller scale in crepuscular apposition eyes.

Concluding remarks

We conclude that the visual systems of crepuscular insects have evolved adaptations that improve visual reliability in dim light. The most important adaptations found in the species we studied are the enlarged facets allowed by a greater total eye size. Our data strongly suggest that the visual systems of insects are sufficiently flexible to evolve to be matched and optimised for a particular intensity window, a conclusion also recently drawn for closely related ants (Greiner et al., 2007).

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