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Absence of eye shine and tapetum in the heterogeneous eye of *Anthocharis* butterflies (Pieridae)

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Summary

Insect eyes are composed of spectrally heterogeneous ommatidia, typically with three different types. The ommatidial heterogeneity in butterflies can be identified non-invasively by the colorful eye shine, the reflection from the tapetal mirror located at the proximal end of the ommatidia, which can be observed by epi-illumination microscopy. Since the color of eye shine is determined by the spectral properties of the ommatidia, it has been tentatively related to color vision. In the course of a survey of ommatidial heterogeneity in butterflies, we found that members of the pierid genus *Anthocharis* lack the eye shine. We therefore carried out anatomy of the eye of the yellow tip, *Anthocharis scolymus*, and correlated it with the absence of the tapetum. The butterfly tapetum is a remnant of the ancestral moth tapetum, a trait that has been completely lost in the papilionids and also, as now appears, in the genus *Anthocharis*. Anatomical investigations also revealed that, considering rhabdom shape, peri-rhabdomal pigment clusters and autofluorescence, the ommatidia can be divided in at least two different types, which are randomly distributed in the retina.

Key words: rhabdom shape, ommatidial heterogeneity, yellow tip, orange tip, screening pigment.

Introduction

The Rhopalocera (butterflies) is a recently evolved group in the order Lepidoptera (Grimaldi and Engel, 2005). It contains the day-flying true butterflies (Papilionoidea), the skippers (Hesperioidea), and a newly identified group of the nocturnal Hedyloidea (Scoble, 1986). The eyes of true butterflies are of the apposition type (Nilsson et al., 1988), whereas those of skippers and Hedyloidea are of the superposition type (Yack et al., 2007), which is typical in moths (Nilsson, 1989). The eyes of moths have two characteristic optical structures that probably function to increase the light sensitivity: the tapetal mirror and the corneal nipple array (Miller, 1979). The tapetum is composed of tracheoles at the proximal portion of the rhabdome and reflects unabsorbed light back into the rhabdome, thus providing a second chance for light to be absorbed. The corneal nipple array, known as the 'moth-eye' structure, is a set of protuberances of height about 200 nm, acting as a thin-film antireflection coating. These structures are basically retained in the apposition eyes of true butterflies, but all species in the family Papilionidae lack both (Bernhard et al., 1970; Stavenga et al., 2006).

Recent studies on the cellular organization of butterfly eyes have demonstrated that the ommatidia are spectrally heterogeneous and typically form three classes containing different sets of spectral photoreceptors (Qiu et al., 2002; Arikawa, 2003; Briscoe et al., 2003; Sauman et al., 2005). The three types of ommatidia are randomly distributed in the regularly arranged hexagonal lattice of the compound eye. The ommatidial heterogeneity and random distribution of the different ommatidial types are shared by other insects (Ribi, 1978; White et al., 2003; Spaethe and Briscoe, 2005; Wakakuwa et al., 2005) and are probably related to color vision (Frisch, 1914; Kelber and Henique, 1999; Kelber and Pfaff, 1999; Kinoshita et al., 1999; Kelber et al., 2002; Zaccardi et al., 2006).

The ommatidial heterogeneity of butterfly eyes can be attractively observed by epi-illumination microscopy, because diurnal butterflies exhibit a colorful eye shine due to the tapetum (Miller and Bernard, 1968; Stavenga et al., 2001). The color of the eye shine is determined by three main components: (i) the spectral reflectance of the tapetum, (ii) the absorption spectra of the visual pigments that are concentrated in the rhabdome, and (iii) photostable screening pigments that often exist as clusters of granules in the photoreceptor cell bodies near the rhabdome (Arikawa and Stavenga, 1997; Arikawa, 1999; Qiu et al., 2002; Briscoe and Bernard, 2005; Zaccardi et al., 2006). The colors of the eye shine pattern appeared to be species-dependent (Bernard and Miller, 1970), so we performed a survey of a number of butterfly species to investigate whether there exists a general rule underlying the eye structure and eye shine colors (Stavenga et al., 2001).
In the course of the survey, we recently discovered that the yellow tip Anthocharis scolymus (Pieridae, Lepidoptera) does not exhibit eye shine. This is quite exceptional, because the eye shine has been observed in all inspected species from all butterfly families except the Papilionidae (Miller and Bernard, 1968; Miller, 1979). We therefore investigated the ommatidial anatomy of the yellow tip, and we found that the species does not have a structure resembling the tapetum. The anatomical investigations also revealed that the yellow tip retina is heterogeneous, containing at least two distinct types of ommatidia. The eye of the yellow tip appears to be rather simple compared to other insects.

Materials and methods

Animals

Adults, both males and females, of the yellow tip butterfly, Anthocharis scolymus Butler, were caught in Kanagawa prefecture, Japan.

Eye shine and fluorescence

A butterfly was immobilized with beeswax, with the head gently glued to the thorax, and put on the stage of a fluorescence microscope (BX-60, Olympus, Tokyo, Japan) equipped with an epi-illumination attachment. The possible existence of eye shine was investigated by focusing the microscope at the level of the cornea or the center of the eye. Using white light, in the main, fronto-ventral part of the eyes of pierid butterflies a clear red eye shine is then normally observed (Ribi, 1979; Stavenga et al., 2001; Qiu et al., 2002; Stavenga, 2002a; Stavenga, 2002b). The possible existence of ommatidial fluorescence was also checked under ultraviolet and blue-violet excitation light.

Retinal anatomy

For electron microscopy, compound eyes isolated from the heads were prefixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 mol l⁻¹ sodium cacodylate buffer (CB, pH 7.3) for 30 min at 20–25°C. After a brief wash with 0.1 mol l⁻¹ CB, the eyes were postfixed in 2% osmium tetroxide in 0.1 mol l⁻¹ CB for 2 h at 20–25°C. Embedding in Epon followed dehydration with a graded series of acetone and infiltration with propylene oxide. The tissues were cut into 60–70 nm ultrathin sections, either longitudinally or transversely to the ommatidial long axes. The sections were observed using transmission electron microscopes (JEM 1200EX, JEOL Tokyo; H7650, Hitachi Tokyo). For light microscopy, the prefixed eyes were dehydrated and embedded in Epon without being postfixed with osmium tetroxide. The tissues were cut into 5 µm sections and observed using a fluorescence microscope (BX60, Olympus).

To correlate the ommatidial fluorescence and pigmentation, the fluorescence under 420 nm excitation was first recorded from a large number of ommatidia using a modified epi-illumination optical set-up (Stavenga, 2002b) equipped with an objective lens of large numerical aperture (NA) and long working distance (WD) (Olympus MPLFLN20X, NA=0.45, WD=6.6 mm). The eyes were then processed for light microscopy as described above. When cutting sections, the eyes were carefully aligned so that the ommatidia in the region from which the fluorescence was recorded were cut perpendicular to the ommatidial axes.

Results

Epi-illumination microscopy of the yellow tip eye: absence of eye shine

Observations of butterfly eyes with an epi-illumination microscope always (except for the papilionids) yield an eye shine, which is very characteristic for the species and the inspected eye region (Stavenga et al., 2001). The eyes of both male and female yellow tip Anthocharis scolymus, however, failed to display anything like an eye shine.

The absence of the eye shine in papilionid butterflies can be immediately understood from the absence of tapeta proximally of the rhabdoms. We thus hypothesized that the lack of eye shine of the yellow tip is also due to a non-functional or absent tapetum. To test this hypothesis we investigated the anatomy of the yellow tip retina.

Transmission electron microscopy: absence of a tapetal structure

Transverse sections in the proximal and basal part of an ommatidium are shown in Fig. 1A,B. There are nine photoreceptor cells, R1–R9, of which the rhodomes of the R5–R8 form the proximal part of the rhabdom (Fig. 1A) and the R9 cell body occupies the basal part of the ommatidium immediately distal to the basement membrane (Fig. 1B). Fig. 1C shows a longitudinal section of an ommatidium, in the proximal region of the retina, where normally the tracheoles are folded into a multilayer stack. Such a structure appears to be non-existent in the eye of the yellow tip. A small tracheal cell is nevertheless clearly present, and its cell body exists near the end of the rhabdom. The cell body of the tracheal cell lies around the ommatidium (Fig. 1A), and it seems almost to have crawled under the rhabdom at the base of the ommatidium (Fig. 1B). Each tracheal cell sends four extensions into the retina, very similar to other butterfly eyes (Ribi, 1979; Qiu et al., 2002).

Ommatidial heterogeneity

Histological sections of the yellow tip eye showed clear differences in the rhabdom shape and spatial organization of neighbouring ommatidia in the same eye region (Fig. 2). Sections through the distal part of the retina (Fig. 2A) showed that a subset of the ommatidia featured the four clusters of red pigment around the rhabdom, well-known from another pierid butterfly, Pieris rapae crucivora, located in the distal extensions of the photoreceptor cells R5–R8 (Qiu et al., 2002). The pigment clusters are arranged in a square pattern (Fig. 2A). In proximal sections the complementary subset had clusters of pale-red pigment (Fig. 2C). In the transitional layer (Fig. 2B) both types of ommatidia feature peri-rhabdomial pigmentation. Here the pale-red pigment clusters are trapezoidally arranged (Fig. 2B, broken circle). Note also that the ommatidial type with the pale-red pigment clusters has additional pigmentation in the cell body of R5–R8 in the area remote from the rhabdom (white arrowheads in Fig. 2).

Under 420 nm epi-illumination, a subset of ommatidia emits strong autofluorescence. We cut histological sections through
the eye region where the fluorescence picture of Fig. 3A was taken, and correlated the fluorescence with the ommatidial pigmentation (Fig. 3B). Clearly the fluorescing ommatidia correspond to the ommatidia with trapezoidally arranged pigment clusters. Among the 336 ommatidia we counted, there were 159 (47.3%) non-fluorescing and 177 (52.7%) fluorescing type ommatidia. The fluorescence was evident both in males and females. A similar blue-violet induced fluorescence in *Pieris rapae crucivora* exists only in males (Arikawa et al., 2005).

The shape of the rhabdom appeared to differ between the two ommatidial types. To clarify this further, we cut transverse, ultrathin sections at 10 μm intervals through the entire thickness of the retina in the ventral region of the fronto-lateral eye region. The length of the ommatidia, i.e. the distance between the surface of the cornea and the basement membrane, is about 420 μm. The images in Fig. 4, taken at a depth of 140 μm (A,F), 210 μm (B,G), 260 μm (C,H), 300 μm (D,I) and 340 μm (E,J) from the corneal surface, obviously show ommatidial heterogeneity. Whereas similar anatomical work on the small white *Pieris rapae crucivora* (Qiu et al., 2002), demonstrated the existence of three types of ommatidia, no more than two types of ommatidia can be distinguished in the yellow tip *Anthocharis scolymus*.

In the first ommatidial type, the rhabdom is approximately a circular cylinder. The rhabdom cross-section is round over most of its length (Fig. 4A,B), although it tends towards a square proximally (Fig. 4C,D). In the other ommatidial type, the rhabdom is a much more irregular cylinder (Fig. 4F–J), with a round cross-section only very distally (Fig. 4F), and it has a flat boundary in the middle part of the ommatidium, giving it a characteristic trapezoidal shape in transverse sections (Fig. 4G); the rhabdom tapers off proximally (Fig. 4J).

In the first, round-type ommatidium, the rhabdomeres of the R1–R4 photoreceptors, which form the rhabdom in the distal half of the retina, have curved microvilli (Fig. 4A,B). The cell bodies of R1 and R2 are arranged along the dorso-ventral axis, and those of R3 and R4 are oriented along the fronto-lateral axis (Fig. 4K). The R5–R8 photoreceptors contribute microvilli to the rhabdom in the remaining proximal half of the retina, with the cell bodies arranged diagonally (Fig. 4C–E, Fig. 4I,J; see also Fig. 4K). The R9 is well recognizable at the basal level, immediately distal to the basement membrane (Fig. 1B,C), but it appears that R9 contributes only a minor basal part of the rhabdom, similar to *Pieris*. As for *Pieris*, we define the R1–R4 of *Anthocharis* as the distal photoreceptors, the R5–R8 as the proximal photoreceptors, and the R9 as the basal photoreceptor (Qiu et al., 2002).

The second, trapezoidal-type ommatidium has basically the same arrangement of photoreceptors, but there are striking differences in the structural organization. The rhabdomeres of R1–R4 contribute to the rhabdom over a distinctly longer distance, about the distal two-thirds of the retina, and the rhabdomeres of R5–R8 together form the rhabdom in the proximal one-third (Fig. 4K). The rhabdomere of the R2 (or the R1) photoreceptor in the trapezoidal-type ommatidia has a
This ratio is consistent with the ratio counted among the non-fluorescing (47.3%) and the fluorescing type ommatidia (52.7%).

Discussion

The yellow tip Anthocharis scolymus has a heterogeneous retina with two types of ommatidia, details of which we have identified by light and electron microscopy. Using light microscopic histology, the ommatidial types are characterized by a difference in color and distribution of the perirhabdomeral pigment clusters (Fig. 2). Combined histology and fluorescence microscopy reveals that the trapezoidal ommatidial type emits strong fluorescence under 420 nm excitation light (Fig. 3). Electron microscopy further reveals that the ommatidial types differ in the shape of the rhabdom and the arrangement of the rhabdomeral microvilli (Fig. 4).

The eye heterogeneity of Anthocharis scolymus with two ommatidial types seems rather simple, for other insect species so far studied have three or more types of ommatidia. In another pierid butterfly, Pieris rapae crucivora, three types of ommatidia are evident, even by light microscopy, from three distinct patterns of pigment clusters: trapezoidal, square and rectangular (Qiu et al., 2002). In a papilionid butterfly, Papilio xuthus, histology revealed two ommatidial types, with either red or yellow pigmentation around the rhabdom. The red-pigmented ommatidia in fact form two separate types of ommatidia, as part of the red-pigmented ommatidia fluoresces under ultraviolet epi-illumination (Arikawa and Stavenga, 1997). We have scrutinized the eye of Anthocharis scolymus to determine whether it was possible to distinguish three types of ommatidia, but could identify only two types in the present anatomical study.

We classified the two types of Anthocharis ommatidia as round and trapezoidal, according to the cross-sectional profile of the rhabdom. The main reason for the different shapes of the rhabdom is the enlarged rhabdomere of either photoreceptor R1 or R2 in the trapezoidal-type (Fig. 4). The larger rhabdomere is invariably composed of straight microvilli (Fig. 4G–I). A similar situation has been reported for Pieris, where the enlarged rhabdomere of R1 (or that of R2) also causes a trapezoidal cross-section of the rhabdom and consists of straight microvilli. The trapezoidal ommatidial type of Pieris is called type I, and its fatter rhabdomere has been shown to belong to a blue-sensitive photoreceptor, while the accompanying photoreceptor, R2 (or R1), is ultraviolet sensitive (Qiu and Arikawa, 2003). Presumably, therefore, the fatter R1 (or R2) rhabdomere of the trapezoidal ommatidial type of Anthocharis also belongs to a blue-sensitive photoreceptor, while the more slender R2 (or R1) is an ultraviolet sensitive photoreceptor. The straight microvilli of the enlarged photoreceptor may play a role in polarization detection (Qiu et al., 2002).

We found that the trapezoidal type ommatidia emit fluorescence under 420 nm excitation light. A very similar fluorescence is also observed in the eyes of male Pieris rapae crucivora. In male Pieris, the fluorescing pigment is located in type II ommatidia, whose R1 and R2 photoreceptors both contain a violet-absorbing visual pigment. There the fluorescing pigment acts as a spectral filter, peaking at 420 nm, with the result that the R1 and R2 are double-peaked blue receptors, specific for males (Arikawa et al., 2005). The eyes of Papilio...
**xuthus** contain ommatidia that fluoresce under 360 nm epi-illumination. The fluorescing material, most likely 3-hydroxyretinol, is accumulated in the most distal portion of type II ommatidia, whose R1 and R2 photoreceptors have the same ultraviolet-absorbing visual pigment as that of the ultraviolet receptors in type I ommatidia. Electrophysiological recordings demonstrated that the R1 and R2 of type II ommatidia are violet receptors with an extraordinary narrow spectral sensitivity, due to the filtering effect of 3-hydroxyretinol (Arikawa et al., 1999). We assume that the fluorescing pigment of *Anthocharis* also acts as a 420 nm-peaking spectral filter. If R1 and R2 of the trapezoidal type contain a blue- (e.g. 460 nm) and ultraviolet- (e.g. 360 nm) absorbing visual pigment, similar to *Pieris*, the effect of the fluorescing pigment will be that the sensitivity peaks of R1 and R2 are shifted away from each other, in opposite spectral directions. Experimental evidence for this conclusion will require further electrophysiological analysis.

The rhabdom of *Anthocharis* is tiered, and thus the visual pigments in the distal tier act together as an optical filter in front of the photoreceptors in the proximal tier. For instance, when short-wavelength absorbing visual pigments are concentrated in the distal tier and long-wavelength absorbing visual pigments in the proximal tier, this will result in a reduced absorption and thus a reduced sensitivity of the proximal receptors in the short-wavelength range. In addition to the visual pigments, perirhabdomeral screening pigment clusters affect the light flux in the rhabdom. In the tiered retina of *Papilio* and *Pieris*, the proximal photoreceptors, R5–R8 do not bear microvilli in the distal tier. However, the cell bodies of R5–R8 exist throughout the length of the retina, and they contain dense clusters of pigment granules over most of the length of the distal tier. The pigment clusters thus act as an effective light filter for the proximal receptors themselves. The design principle of a pigment filter in the distal extensions of the proximal photoreceptors is also applied in the retina of *Anthocharis*, although only in the round ommatidial type. In the trapezoidal type the pigment granule clusters are, surprisingly, deposited only in the proximal tier.

The different position of the pigment clusters of the proximal photoreceptors emphasizes the heterogeneity of the ommatidial lattice (Fig. 2). Ommatidial heterogeneity is a widespread characteristic of insect compound eyes. In the honeybee and bumblebee retina, three randomly distributed ommatidial types were distinguished on the basis of the ultraviolet- and blue-sensitive distal photoreceptors (Spaethe and Briscoe, 2005; Wakakuwa et al., 2005). The retina of some nymphaline butterflies is probably organized in a very similar way (Briscoe and Bernard, 2005). Yet, other nymphalids have clearly recruited red photoreceptor screening pigments that act as red filters (Stavenga et al., 2001; Stavenga, 2002a; Stavenga, 2002b; Sauman et al., 2005; Zaccardi et al., 2006), and the same holds for papilionids (Arikawa and Stavenga, 1997; Arikawa, 2003) and lycaenids (Arikawa and Stavenga, 1997; Stavenga, 2002a; Sison-Mangus et al., 2006). Furthermore, detailed molecular biological analyses revealed that in the latter case...
the short-wavelength receptors diversified to such an extent that no less than six ommatidial types exist (Sison-Mangus et al., 2006).

Comparing the retinal organization of the different lepidopteran families reveals an impressive diversification and specialization. Although some exceptional cases are known (Yack et al., 2007), the butterflies are diurnal (Grimaldi and Engel, 2005). The ancestral moth eyes are characterized by so-called ‘moth-eye’ corneal nipple array and tracheolar tapeta, which have been maintained by most butterfly species. Interestingly, both traits have been lost in the papilionoids (Miller, 1979). In a relative of the yellow tip, the European orange tip *Anthocharis cardamines*, the corneal nipple array is well present (Stavenga et al., 2006), but the tapetum has vanished, as concluded from the absence of eye shine (G. D. Bernard and D.G.S., unpublished observations). The American falcate orange tip *Anthocharis midea* also lacks eye shine, and anatomy performed on this species also demonstrated the absence of a tapetum proximal to the rhabdom (G. D. Bernard and W. H. Miller, personal communication). It thus appears that the tapetum is a trait specifically lost by the pierid genus *Anthocharis*. Species in other genera of the tribe Anthochaidini basically share the life style with *Anthocharis*: adults fly only in early spring. The great orange tip *Hebomoia glaucippe*, which belongs to the *Colotis* group, is phylogenetically close to the Anthochaidini (Braby et al., 2006). It has several generations

**Fig. 4.** Transmission electron microscopy of the two ommatidial types, round and trapezoidal, of the yellow tip. (A–E) Transverse sections of an ommatidium with an approximately circular-cylindrical, round rhabdom. (F–J) Transverse sections of an ommatidium having a trapezoidal rhabdom at the middle level of the retina. Bar, 1 μm. (K) Summary diagram of the two ommatidial types. The total length of the ommatidium, including the dioptical apparatus, is about 420 μm. In the round type, the photoreceptors R1–R4 contribute their rhabdomeres over about the distal half of the ommatidium, and the R5–R8 do the same over most of the proximal half. The R9 photoreceptor adds some microvilli at the base of the rhabdom. Clusters of pigment granules exist in the cell bodies of the photoreceptors R5–R8 in most of the distal half of the round type ommatidium, over a distance of about 100–300 μm from the corneal surface, but no pigment clusters exist in the proximal half of the ommatidium. In the trapezoidal-type, the rhabdomeres of R1–R4 form the distal two thirds of the rhabdom, those of R5–R8 form most of the proximal one-third, and the R9 photoreceptor again adds some microvilli at the base of the rhabdom. Clusters of pigment granules exist in the cell bodies of the photoreceptors R5–R8 in virtually only the proximal one-third of the trapezoidal-type ommatidium. The levels of the transverse sections A–J are indicated by arrowheads at the left side of the diagram. Four tracheal extensions (T) invade the retina. BM, basement membrane; D, dorsal, V, ventral; P, posterior, A, anterior.
within a year, however, as is the case for Pieris, and exhibits bright red eye shine (K.A., unpublished observation). A more detailed survey of the Pierinae may reveal whether or not the loss of tapetum is related to the life cycle. The loss of the tapetum in Anthocoris as well as in Papilionidae raises the question whether this inflicts a loss in visual functions. Although the eye shine is a most striking phenomenon, quantitative evaluation of the tapetal reflections indicates in fact only a small contribution of the tapetum to the light sensitivity, at least in a diurnal pierid species Pieris rapae crucivora (Wakakuwa et al., 2004). The tapetum of the nocturnal moths probably raises the sensitivity much more substantially. We conclude that the selective pressure for butterflies to maintain the tapetum is only minor.

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