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The eyes of *Macrosoma* sp. (Lepidoptera: Hedyloidea): A nocturnal butterfly with superposition optics

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Abstract

The visual system of nocturnal Hedyloidea butterflies was investigated for the first time, using light and electron microscopy. This study was undertaken to determine whether hedylids possess the classic superposition eye design characteristic of most moths, or apposition eyes of true butterflies (Papilionoidea), and, to gain insights into the sensory ecology of the Hedyloidea. We show that *Macrosoma heliconiaria* possesses a superposition-type visual mechanism, characterized by long cylindrical crystalline cones, a lack of corneal processes, 8 constricted retinular sense cells, rhabdoms separated from the crystalline cones forming a translucent 'clear zone', and tight networks of trachea that form a tapetum proximal to the retina and which also surround the rhabdoms to form a tracheal sheath. Dark-adapted individuals of *M. heliconiaria*, *M. conifera*, and *M. rubidinarea* exhibited distal retinular pigment migration, forming an eye glow. Correspondingly, light-exposure induced pigment to migrate proximally, causing the eye glow to be replaced by a dark pseudopupil. Other characteristics of the visual system, including relative eye size, facet size, and external morphology of the optic lobes, are mostly 'moth like' and correlate with an active, nocturnal lifestyle. The results are discussed in relation to the evolution of lepidopteran eyes, and the sensory ecology of this poorly understood butterfly superfamily.

Keywords: Vision; Superposition; Hedylidae; Sensory; Evolution; Compound eyes

1. Introduction

Butterflies, according to most current sources, comprise three of 46 superfamilies forming the order Lepidoptera. These include the well-known and cosmopolitan Papilionoidea (true butterflies) and Hesperioidea (skipper butterflies), in addition to a small and little known group from tropical America, the Hedyloidea (Scoble, 1990, 1996; Ackery et al., 1999; Kristensen and Skalski, 1999). There are an estimated 40 species of Hedyloidea (Scoble, 1986), all currently assigned to a single family, Hedylidae, and genus, *Macrosoma*. To date, all have been collected from montane forest in Central and South America, ranging from central Mexico to Southern Peru, and across Bolivia to South-eastern Brasil (Scoble, 1996; Aiello, 1992). Hedylids are of particular interest because they share important morphological traits with both moths (Heterocera) and day-flying butterflies (Rhopalocera), and are thought to represent the long sought after 'missing link' between these two familiar groups. Characterized by several moth-like features, including plumous antennae, largely nocturnal activity, and drab coloration, earlier classification schemes placed hedylids within the Geometroidea moths. More recent morphological and molecular evidence, however, places the hedylids closest to butterflies, although the exact relationship among the three superfamilies remains unresolved (de Jong et al., 1996; Weller and Pashley, 1995; Scoble, 1996; Wahlberg et al., 2005) (Fig. 1). Despite their prominent purported status as the 'living ancestors' of modern butterflies, surprisingly little is known of the anatomy, life history and behaviour of any Hedyloidea species (Aiello, 1992).

This study describes the morphology of the hedylid visual system, a topic of interest for two primary reasons. First, it

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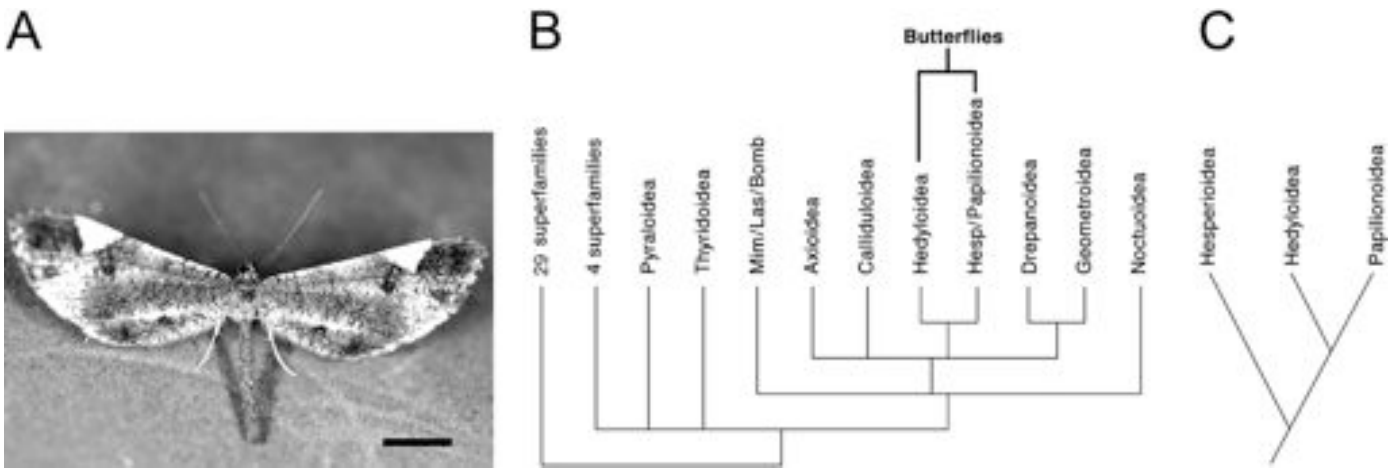

Fig. 1. A. Dorsal view of an adult male *Macrosoma heliconiaria* in its typical resting position, with wings tilted upward. Scale bar: 5 mm. B. Phylogeny of the Lepidoptera adapted from Kristensen and Skalski (1999) depicting the relationship between the three butterfly superfamilies (Hedyloidea, Papilionoidea, and Hesperoidea) and moths. C. An alternative phylogenetic relationship proposed for the three butterfly superfamilies, adapted from Scoble (1986).

was of interest to establish whether hedylid eyes were of the apposition type, typical of papilionoid butterflies, or of the refracting superposition type, typical of most moths (see Warrant et al., 2003 for review). Second, an understanding of the visual system was expected to lend insight into the sensory ecology of this poorly understood group of butterflies.

Butterflies are typically day active with a well developed visual sense. True butterflies (Papilionoidea) characteristically have apposition eyes (Warrant et al., 2003), a design suited for use in bright light. In apposition eyes, each ommatidium is sheathed along its entire length by a sleeve of dark light-absorbing screening pigment that prevents light reaching the light-sensitive structures (the rhabdom) from all but the single corneal lens of the same ommatidium. Even though the tiny corneal lens severely limits photon catch, spatial resolution in apposition eyes is often high. Several morphological features, including a corneal process and a long and narrow rhabdom, are characteristic of the apposition design (Fig. 2). Most arthropod apposition eyes are ''focal'': light focused by the curved outer surface of the corneal lens is imaged on the distal tip of the rhabdom. A second lens interposed between the corneal lens and the rhabdom – the crystalline cone – has little optical effect. In another apposition design, so far identified only within the papilionoid butterflies (the ''afocal'' design: Nilsson et al., 1984; Warrant et al., 2003), the crystalline cone has evolved powerful gradients of refractive index in a stalk-like region that connects to the rhabdom. This stalk efficiently transfers light to the rhabdom, improving both resolution and sensitivity (van Hateren and Nilsson, 1987).

Most moths in contrast, are primarily nocturnally active and their eyes are typically of the refracting superposition type, where light rays reach the rhabdom from hundreds (and in extreme cases thousands) of corneal facet lenses. In contrast to apposition eyes, the rhabdoms of superposition eyes are displaced proximally from the crystalline cones, and in the dark-adapted state the screening pigments are withdrawn both proximally and distally to form a wide optically homogeneous ''clear zone'' between the retina and the lenses. The crystalline cones have powerful gradients of refractive index that allow light to be collected from a large circular ''superposition aperture'' of corneal lenses (or facets) to be focused on a single rhabdom in the retina. This superposition of light arriving from hundreds of facets greatly improves sensitivity but spatial resolution can (but not always) be sacrificed as a result. In light adapted conditions, the screening pigments of many superposition eyes migrate proximally into the clear zone to isolate the ommatidia from one another, effectively converting the eye to an apposition design of lower sensitivity and higher resolution. Superposition eyes are characterized by a number of anatomical features, including thin corneal lenses, long cylindrical cones, constricted retinular cells resulting in rhabdoms that lie deep within the eye separated from the crystalline cones by a translucent 'clear zone', microvillar structural alterations, and sometimes a tracheal tapetum in the proximal region of the ommatidium that reflects incoming light (Yagi and Koyama, 1963; Warrant et al., 2003).

Although the superposition and apposition designs have traditionally been recognized as an important morphological distinction between moths and butterflies, many Lepidoptera do not conform to this rule. For example, skipper butterflies (Hesperioidea) possess a modified superposition focusing system despite their diurnal activity. Some diurnal moths, (e.g. some Agaristidae; Sphingidae) possess modified superposition systems, while others (e.g. Zygaenidae, Syntomidae, Sesiidae) have apposition eyes (Ehnbom, 1948; Yagi and Koyama, 1963; Horridge et al., 1972, 1977; Warrant et al., 1999).

Given the assumptions that hedylids are primarily nocturnal, and taxonomically situated between the other butterflies and moths (Kristensen and Skalski, 1999; Fig. 1B), we predict that hedylids, like most moths, possess superposition eyes. If on the other hand, hedylids are found to possess apposition eyes, this could support the hypothesis that hedylids are closer to the Papilionoidea (Fig. 1C; Scoble, 1986).

Other features of the lepidopteran visual system, including optic lobe morphology, external eye dimensions, and the size

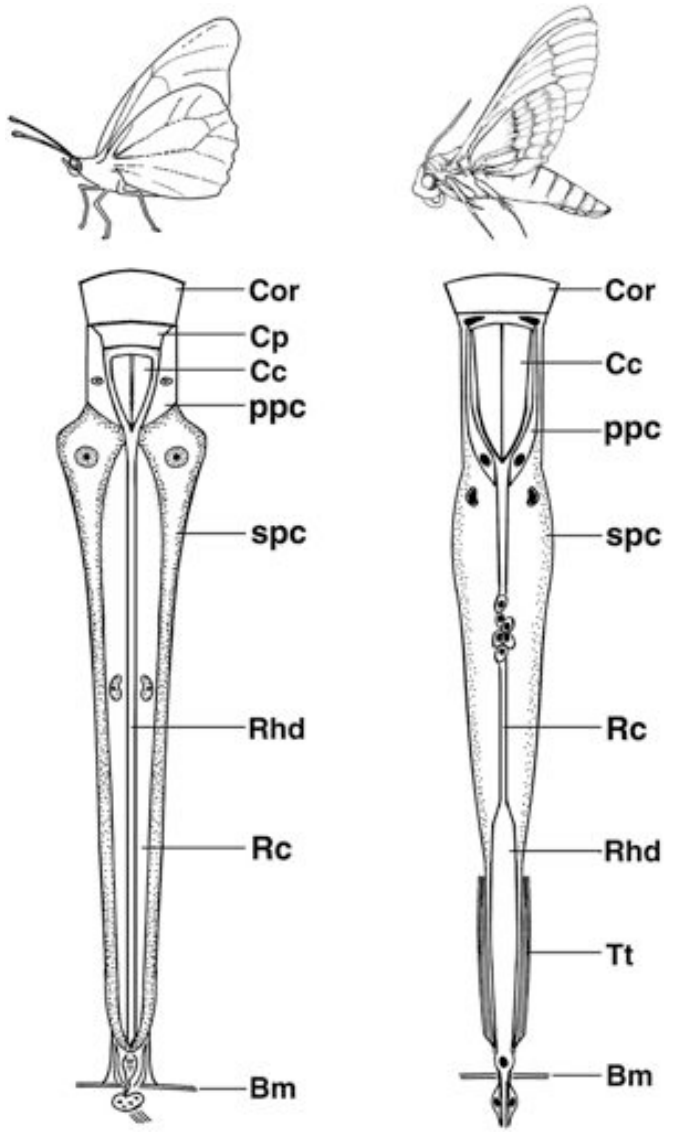

Fig. 2. Diagrammatic representation of a single ommatidium from an apposition eye (left) characteristic of diurnal butterflies, and a superposition eye (right) characteristic of many nocturnal moths. Bm, basement membrane; Cc, crystalline cone; Cor, cornea; Cp, corneal process; ppc, primary pigment cell; spc, secondary pigment cell; Rhd, rhabdom; Rc, retinular cell; Tt, tracheal tapetum. Ommatidia redrawn from Yagi and Koyama (1963).

and topography of individual facets, have been used to infer taxonomic relationships, and behavioural characteristics of different species (e.g. Ehnbom, 1948; Yagi and Koyama, 1963). Thus, our study of the hedylid visual system could offer insights into their sensory ecology, as well as potentially providing further morphological evidence to support their relationship to other Lepidoptera.

In this study, light and electron microscopical investigations were undertaken to characterize the visual system of hedylids for the first time, by focusing primarily on one species, *Macrosoma heliconiaria*. Our results are discussed within the context of the sensory ecology of hedylid butterflies and the evolution of lepidopteran visual systems.

2. Methods

2.1. Animals

All Hedyloidea specimens used in this study, including 20 *Macrosoma heliconiaria* (16 males and 4 females), 2 male *M. conifera*, and 2 *M. rubidinarea* (1 male and 1 female) were collected at mercury vapour and ultraviolet light traps in neotropical lowland rainforest on Barro Colorado Island, Panama, during the months of May, and September to November, between 1998 and 2000. Because *M. heliconiaria* was the most widely collected species, we chose this for the histological work. Species were identified and sexed according to characteristics described in Scoble (1986, 1990). *Cercyonis pegala* (Nymphalidae) and *Catocola subnata* (Noctuidae), used for comparisons of brain morphology, were collected near Ottawa, Ontario, Canada. Live specimens were injected and preserved with one of the following: Bouin's alcoholic fixative (Pantin, 1946), C & C fixative (Chauthani and Callahan, 1966), 70% ETOH, or 2.4% glutaraldehyde in Sorensen's phosphate buffer, and stored at 5 °C until being examined in our laboratories in Canada or Sweden.

2.2. Scanning electron microscopy

The eyes of specimens that had been stored in 70% ETOH were air dried, fixed to aluminum stubs with carbon paint, sputter-coated with gold-palladium, and examined with a JOEL JSM-6400 scanning electron microscope. The lengths and density of interommatidial hairs were measured from the central eye regions of 3 male *M. heliconiaria*.

2.3. Eye shape and size

General eye shape was characterized by the width-to-length ratio (Yagi and Koyama, 1963). Width (L1) was measured as the distance from the anterior to posterior edge of the eye, and length (L2) as the distance from the dorsal to ventral edge of the eye along a line parallel to the posterior margin of the eye (Fig. 3A).

Eye size, relative to other Lepidoptera, was assessed by plotting the square root of the eye surface area against wing expansion (Yagi and Koyama, 1963). Eye surface area was measured from pieces of flattened cornea using the corneal spread method (e.g. Yagi and Koyama, 1963; Lund et al., 2001; Jander and Jander, 2002) as follows: the head from a fixed specimen was removed and either immersed directly in distilled water (previously fixed in C&C) and pinned to a petri dish lined with Sylgard® (Dow Corning Corp., Midland, MI, USA). The eyes were removed, and the inner tissue carefully peeled away from the corneal surface. To flatten the cornea on a microscope slide, a series of cuts were made from the edge of the eye toward the center. The cornea was mounted in a drop of glycerol, cover-slipped, and viewed with a Zeiss Axioplan 2 microscope, and Zeiss AxioCam HRm digital camera. Digitized images of the entire cornea for 6 specimens (5 male, 1 female) were obtained by photographing sections at 10× magnification,

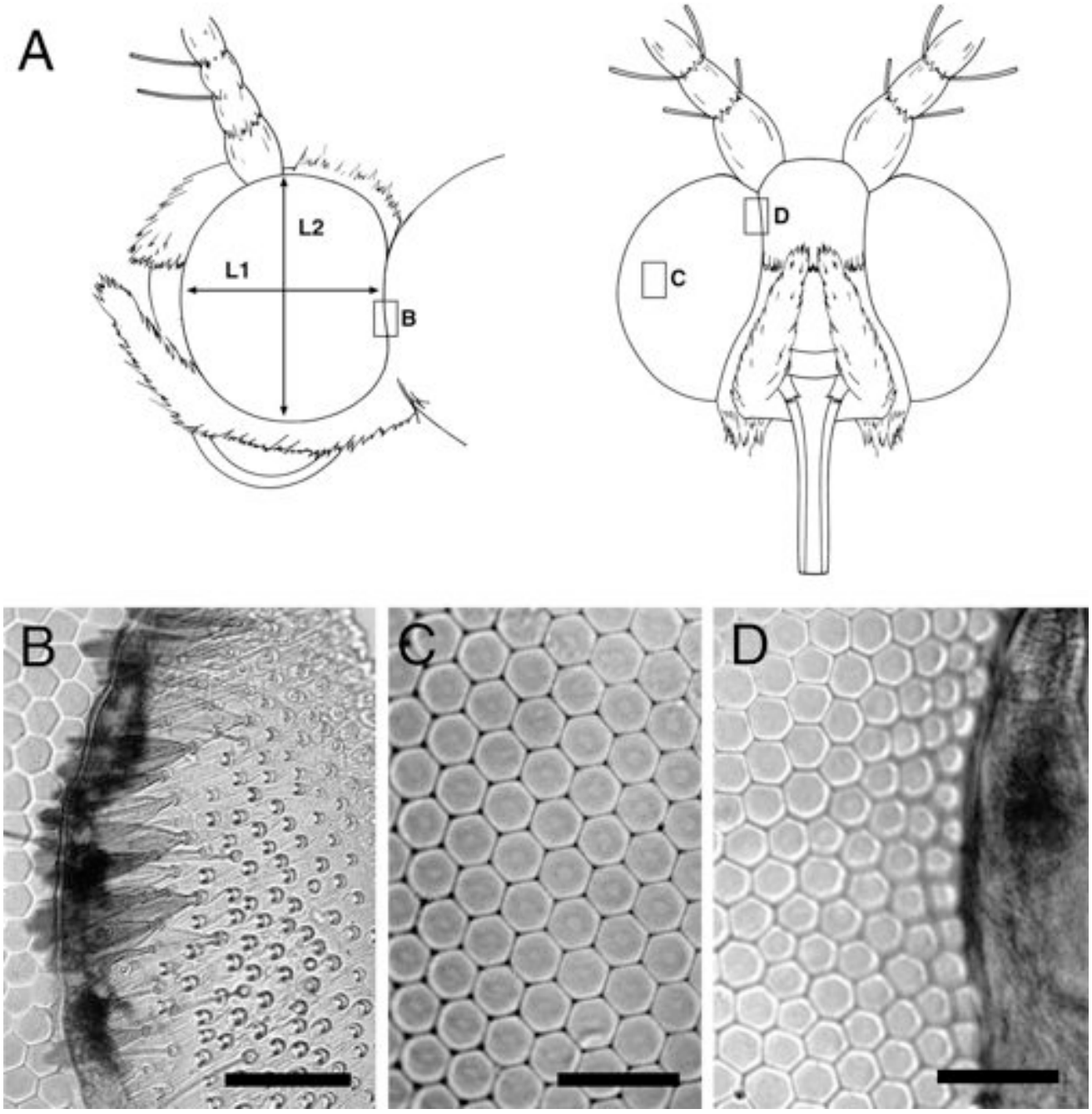

Fig. 3. External features of the hedylid eye. A. Left lateral and frontal views of a male hedylid head showing general eye shape, location of the eye measurements (L1, L2) and the general regions where photographs were taken at the paraocular region (B), central eye region (C) and eye edge (D). Scale bar in B = 60 μm; C = 50 μm, D = 50 μm.

and surface areas estimated by tracing around the perimeter using an outline tool and Zeiss Axiovision software. For each corneal spread, two measurements of the surface area were averaged to control for error in tracing the image.

2.4. Facet size and number

The number of facets for each eye, used to predict average interommatidial angles [(23,818/number of facets)$^{1/2}$ (Land, 1997)], was estimated by manual counts of the corneal spreads (described above). Average facet diameters were calculated from corneal spreads, by measuring the length of 10 facets and dividing by 10 (Lund et al., 2001). For each eye, 2 measurements were made, one each for the center and the perimeter of the eye, and these two numbers were averaged. The areas measured were chosen based on there being 10 consecutive facets in a row with no tears or wrinkles in the tissue.

2.5. Histology

The eyes of 6 male *M. heliconiaria* were examined -5 for light microscopy, and 1 for electron microscopy. All eyes

except one used for light microscopy were fixed while in a light adapted state. For light microscopy, heads were removed from specimens that had been injected and stored in either Bouin's alcoholic fixative (Pantin, 1946) or 2.4% glutaraldehyde in Sorensen's phosphate buffer. Those fixed in Bouin's were rinsed in 70% alcohol saturated with lithium chloride to remove the picric acid, dehydrated in an ethanol series and embedded in Spurr's epoxy resin. Those fixed in glutaraldehyde were rinsed in buffer, dehydrated in ethanol, and embedded in Spurr's. Semi-thin sections (5 μm) were stained with 1% toluidine blue in sodium borate (Pillsbury, 1980), and mounted on microscope slides in Permount.

For electron microscopy, tissue was fixed in 2.4% glutaraldehyde and 2% paraformaldehyde in phosphate buffer (pH 7.2–7.5). Eyes were removed from the head and fixed for 2–3 h at 4 °C before being osmicated (2% OsO_4 in distilled H_2O) for one hour. The heads were subsequently dehydrated in an ethanol series, transferred to propylene oxide and embedded in Epoxy resin (FLUKA). Ultra-thin (50–70 nm) sections were made on a Reichert Ultracut microtome using a Diatome diamond knife. Ultra-thin sections were mounted on Formvar coated slot grids, stained with 6% uranyl acetate (25 min) and 3% lead citrate (10 min), and photographed in a Hitachi H-7100FA transmission electron microscope.

2.6. Gross anatomy of the brain

Brains were dissected out of specimens preserved in C&C fixative, and transferred to distilled water in petri dishes lined with Sylgard®. Janus Green B (Yack, 1993) was periodically applied to enhance the contrast of nerve roots. Sketches were drawn with the aid of a drawing tube.

3. Results

3.1. External eye characteristics

3.1.1. Form and size

The compound eyes of all hedylids examined in this study are ellipsoid to roundish in shape (Fig. 3), with characteristic dorso-medial grooves to accommodate the antennal bases (Fig. 4B). Yagi and Koyama (1963) have characterized the overall shape of different lepidopteran eyes by their width to length ratios (Fig. 3), which for male and female *M. heliconiaria* are 0.85 ± 0.05 ($n = 14$) and 0.81 ± 0.04 ($n = 2$), respectively, and 0.91 ($n = 1$), and 0.94 ($n = 1$) in a female *M. rubidinaria* and male *M. conifera*, respectively.

Eye surface areas for 5 male and 1 female *M. heliconiaria* were 1.79 ± 0.15 and 1.51 mm^2 respectively, while mean wing expansions for males and females were 34.3 ± 0.10 mm ($n = 10$) and 35.6 ± 0.07 mm ($n = 3$) respectively. The relative eye size (assessed by the square root of eye surface area:wing expansion ratio) was 0.52 (male) and 0.42 (female).

3.1.2. Facets

The number of facets in male and female *M. heliconiaria* were 4719 ± 603 ($n = 5$) and 4447 ($n = 1$), respectively. Near the center of the eye the facets are significantly larger (19.1 ± 0.6 μm) and more regularly spaced than those along the edge (17.4 ± 0.6 μm) (Student's t test, $p = 0.01$) (Fig. 3). In the paraocular area (defined as the crescent shaped region located behind the eye [Yagi and Koyama, 1963]), facets are separated from the edge by a chitinous ring (Fig. 3B). Under SEM short interfacetal hairs, approximately 6 μm long, are seen to occur at a density of 4–6 to every 100 facets (Fig. 4C). High magnification of the corneal surfaces reveals a microstructure of hexagonally arranged rows of corneal nipples, each 0.2 μm in diameter (Fig. 4D). If we assume the eye to be spherical and to contain facets of equal size, which the eyes of hedylids coarsely approximate, then the following expression can be used to estimate the average interommatidial angle $\Delta\phi$ of the eye in degrees (Land, 1997):

$$\Delta\phi = \sqrt{\frac{23,818}{n}}, \quad (1)$$

where n is the number of facets in the eye. For male hedylids, $n = 4719$, and $\Delta\phi$=2.2°.

3.1.3. External manifestation of pigment migration

Illumination of dark-adapted individuals of all species examined induced a glowing spot (eye glow) on the eye surface. Continuous illumination of the eye caused a progressive decrease in the size of the glowing area. In the light-adapted state the eye glow converted to a circular dark pseudopupil of approximately the same area (Fig. 4A). In the lateral eye of males this pseudopupil had a width of 413 ± 35 μm and a length of 498 ± 47 μm ($n = 5$).

3.2. Histology

The following describes the histological features of the male *M. heliconiaria* eye according to the position along the length of the ommatidium: (1) dioptric apparatus or light-gathering components; (2) primary sense (retinula) cells; (3) pigment cells; and (4) proximal cells.

3.2.1. Dioptric apparatus

The convex corneal lenses are 19.9 μm ± 1.0 wide and 10.9 ± 0.8 μm thick ($n = 24$ measured from 4 males) near the center of the eye. There is no observable corneal process and thus, immediately proximal to the corneal lens are 4 eucone crystalline cone cells (Fig. 5) which form the crystalline cone, which is 54.2 ± 2.6 μm in length. The cytoplasm of the cone cells is free of organelles, except for 2 nuclei located between the cones and the corneal lens.

3.2.2. Retinula cells

There are 8 retinular cells within each ommatidium, occurring in 3 tiers, of 4 distal, 3 medial, and 1 basal cells. The distal and medial cells each form thread-like structures that extend from the proximal tips of the crystalline cones to the proximally located rhabdom. In the dark-adapted state, the screening pigments migrate distally, creating a 'clear zone' between the

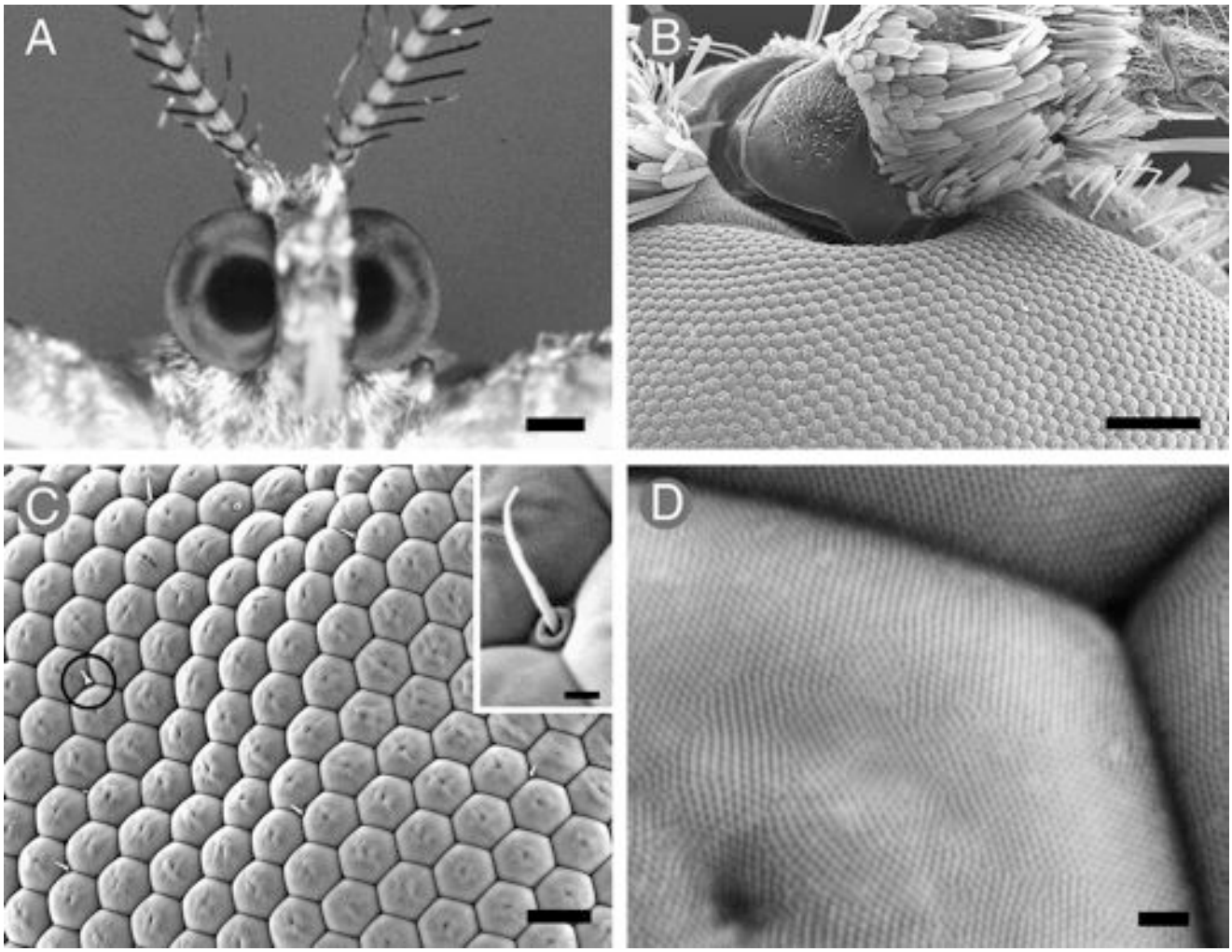

Fig. 4. External features of the eye in *M. heliconiaria* (all specimens male) A. Light micrograph of the eyes in their light adapted state, with pseudopupils. B. Scanning electron micrograph of the eye showing the indentation at the base of the antenna. C. Ommatidial facets interspersed with short interfacetal 'hairs', of which one is circled. Inset depicts an enlarged interfacetal 'hair'. D. Close up of a facet surface showing the characteristic corneal 'nipples' on the facet surface. Scale bar in A = 300 μm, B = 100 μm, C = 20 μm, Inset in C = 2 μm, D = 1 μm.

dioptric apparatus and the rhabdom. The distal and medial retinular cells each internally contribute a portion of the photoreceptive rhabdom. These rhabdom portions, or, 'rhabdomeres', are arranged in a lobed pattern and contain many tightly packed microvilli (Fig. 5D). The microvilli are arranged parallel to one another at the outer edges of rhabdomeres. However, as they progress inward towards the rhabdom center, they shift to a radial arrangement. The rhabdom is 111. 7 ± 19.7 μm long ($n = 16$ measured from 3 male specimens), and at its widest point is approximately 8 μm in diameter.

3.2.3. Pigment cells

Characteristic of other superposition eyes, hedylids have three kinds of pigment cells: iris (= primary), retinular (= secondary), and basal cells, each containing pigment granules that migrate according to the levels of ambient light in their surroundings. Two primary pigment cells surround the crystalline cones in each ommatidium, and these narrow cells contain granular pigment particles. The small cellular proximity in which the iris pigment granules are contained makes it difficult to accurately observe the changes in pigment granule position according to the ambient light conditions. However, careful observation of one preparation fixed under dark-adapted conditions revealed iris pigment granules located in the distal region of the primary pigment cells, leaving the proximal ends of the crystalline cones exposed. In light adapted eyes, these pigment granules migrate proximally to cover the proximal apex of the crystalline cones. Six secondary pigment cells surround all 7 distal and medial retinular cells within the ommatidium. These pigment cells span the 'clear zone' of the eye from the corneal layer to the basement membrane. The pigments within the retinular cells are very dense and granular in appearance. In the cut eye of a fresh specimen, these pigments appeared reddish/brown in colour. The most distinctive feature of these cells is their large-scale longitudinal migration according to the levels of ambient light. In the dark-adapted state, the pigment granules are concentrated in the distal region of the ommatidia and surround the crystalline

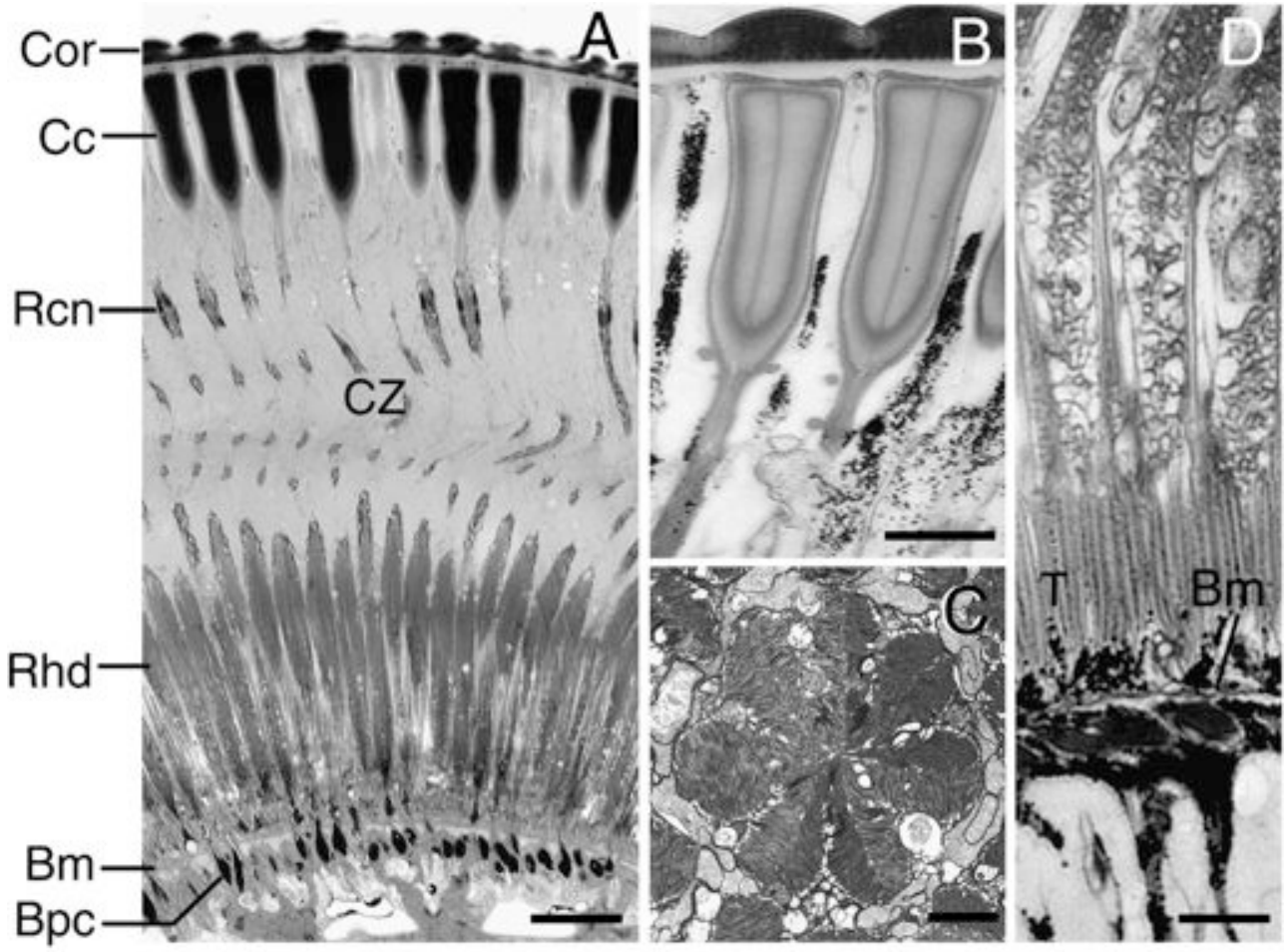

Fig. 5. Histological features of the compound eye in *M. heliconiaria*. A. Longitudinal section through the whole eye showing general organization of the dioptric apparatus with respect to the rhabdoms. B. Crystalline cones in longitudinal section, showing crystalline cone cells, and pigment between crystalline cones. C. Transverse transmission electron micrograph through the proximal region of the eye, showing the flower-shaped rhabdom. D. Longitudinal section through the proximal region of the eye, showing the tracheal bush just distal to the basement membrane. Bm, basement membrane; Bpc, basement pigment cell; Cc, crystalline cone; Cor, cornea; CZ, clear zone; Rhd, rhabdom; Rcn, nuclei of the retinular cells; T: tracheal bush. Scale bar for A = 35 μm; B = 20 μm; C = 2 μm; D = 10 μm.

cones. As the levels of ambient light increase, the pigment granules move proximally until they reach the level of the rhabdom. One basal pigment cell is located on the proximal side of the basal membrane in each ommatidium. No discernable pigment migration in response to changes in illumination was observed.

3.2.4. Proximal cells

Distal to the basement membrane, the trachea branch into many fine tracheoles, forming a 'tapetum'. The tracheal bush ensheaths the proximal 60 to 70% of the rhabdom. One basal retinular cell is located proximal to the tapetum, adjacent to the basement membrane (Fig. 5).

3.2.5. Gross anatomy of the brain

The external structure of the brain was examined to look at the relative sizes of the optic and antennal lobes compared to those of nocturnal moths and diurnal butterflies (Fig. 6). The general features of the brain in *M. heliconiaria* include well developed antennal lobes, moderately developed and somewhat rounded optic lobes, and a short frontal connective.

4. Discussion

The results of this study lead us to conclude that the visual system of Hedyloidea butterflies is more characteristic of nocturnal moths than of true butterflies. They possess morphological features of superposition eyes, and optic lobes more characteristic of moths than true butterflies. Our results, although restricted to a few hedylid species, provide insights into the habits of this unknown group, and their relationship to their moth and butterfly relatives.

4.1. Hedylids have superposition eyes

Based on the morphology of the eyes and visual system, and the dynamics of pigment migration, we conclude that hedylids possess refracting superposition eyes, a design more typical of nocturnal moths than of diurnal butterflies. We

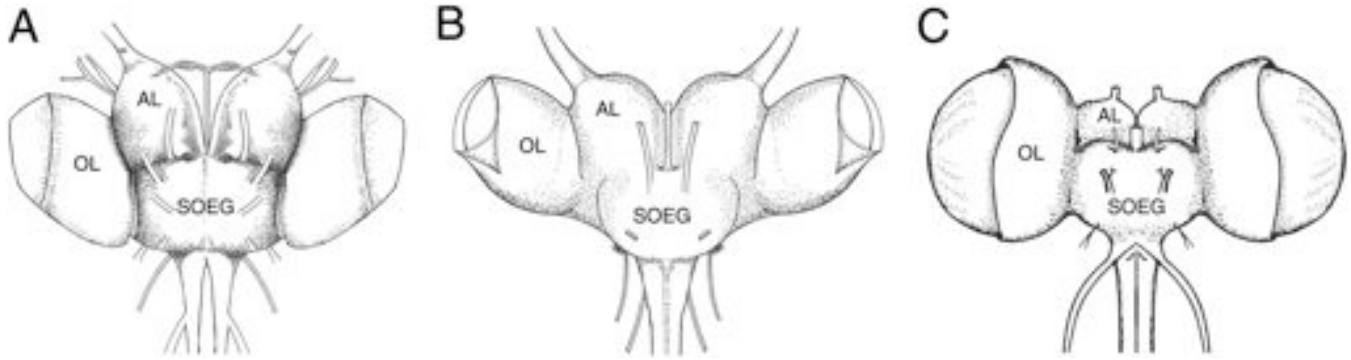

Fig. 6. Ventral views of the brain in: (A) a nocturnal butterfly, *M. heliconiaria* (Hedyloidea); (B) a nocturnal moth, *Catocola subnata* (Noctuoidea); and (C) a diurnal butterfly, *Cercyonis pegala* (Papilionoidea), showing differences in external morphology of the optic lobes (OL), and antennal lobes (AL). SOEG: suboesophageal ganglion.

will now discuss the features of the hedylid visual system that lead us to this conclusion.

4.1.1. External morphology

A variety of external morphological features of lepidopteran eyes have been ascribed either taxonomic or functional importance (see Yagi and Koyama, 1963; Warrant et al., 2003). Here we focus on the general shape and size of the eye, and the size and number of facets.

The general shape of the eye in different taxonomic groups has been characterized by a width:length ratio (Yagi and Koyama, 1963) with most species falling within the range of 0.75 to 0.95. Exceptions include the Lycaenidae, which have distinctively narrow and ovoid eyes, with ratios around 0.56, and some hesperiids and nocturnal sphingids, that have nearly spherical eyes, with ratios around 1. The latter two groups have superposition eyes, and a ratio of 1 implies that the eyes are spherical, which is a usual condition for the optical function of this eye design (Exner, 1891; Warrant et al., 1999, 2003). However exceptions to this rule do exist – the diurnal hawkmoth *Macroglossum stellatarum* has highly aspherical superposition eyes that break the classical rules and endow the eye with remarkable adaptations for life in bright light (Warrant et al., 1999). The average values of width:length ratio obtained for the 3 hedylid species examined in this study range from 0.85 to 0.94, implying that the eyes of hedylids are slightly ellipsoid.

The relative eye size of different Lepidoptera is generally indicative of lifestyle, with large eyes tending to be associated with species active during low light conditions. The wing expansion:square root of eye surface area values obtained for *M. heliconiaria* are indicative of eyes that are relatively large, and most characteristic of actively foraging, nocturnal taxa, such as sphingids and noctuids (Yagi and Koyama, 1963).

Another feature found in hedylids that is a characteristic of nocturnal Lepidoptera is dense rows of prominent corneal nipples covering the corneal surface. These structures, seen when viewed at high magnification, are thought to reduce image glare and perhaps camouflage the organism. If the nipples are large enough, they can even reduce reflection from the corneal surface by up to 4%, thus improving the transmission of light to the retina and boosting sensitivity. Thus, corneal nipples have often been thought to be a characteristic of nocturnal moths, where their function is to increase sensitivity. Indeed, most diurnal butterflies generally have reduced nipples (most Hesperiidae, Lycaenidae and Pieridae), or lack them altogether (Papilionidae). Curiously however the majority of diurnal Nymphalids have very pronounced nipples (Bernhard et al., 1970; Warrant et al., 2003; Stavenga et al., 2006), so their presence on the eye surface is not a guaranteed indicator of a nocturnal existence, nor of being more moth-like than butterfly-like.

4.1.2. Tapetum and eye glow

The presence of a well-formed tracheal tapetum is typical (but not a requirement) of the superposition design. In superposition eyes, the tapetum is formed by the division of the tracheal into a network of many fine tracheoles at the level of the basement membrane. This network sometimes extends distally to ensheath each rhabdom either partially or completely to the rhabdom's distal tips, a feature that has important consequences for both resolution and sensitivity (Warrant and McIntyre, 1991). *M. heliconiaria* has a tapetum typical of those found in superposition eyes, with tracheal sheaths that extend distally to surround the proximal most 60 to 70% of the rhabdoms' lengths. Tracheal tapeta, although not as extensive, are also found in some apposition eyes where they have roles in respiration (e.g. dragonflies) or spectral filtering (e.g. butterflies: Stavenga, 2002).

The appearance of a bright "eye glow" in a dark-adapted superposition eye – clearly visible in *M. heliconiaria* – is caused by light reflected from the tapetum. Incoming light, having escaped absorption during its first passage through the layer of rhabdoms, is given a second chance of absorption before leaving the eye. The presence of a tapetum thus boosts sensitivity by effectively doubling the length of the rhabdoms and the unabsorbed light that leaves the eye forms the bright eye glow. The aperture of facets that comprises the glow – known as the "superposition aperture" (Warrant and McIntyre, 1993) – is the same aperture of facets that initially

collected the light for a single rhabdom in the retina. The eye glow is thus equivalent to the pupil of a superposition eye, and its size is an indicator of the eye's sensitivity.

As mentioned above, the tapetum of *M. heliconiaria* forms sheaths that surround the proximal-most 60 to 70% of each rhabdom. The extent of such tapetal sheathing in a given superposition eye is a good indicator of the quality and/or focal position of the retinal image (Warrant and McIntyre, 1991). When rhabdoms are fully sheathed along their lengths, light rays at all angles of incidence within the focused cone of light are exclusively captured by total internal reflection within the rhabdoms upon which they land. Full sheathing only makes sense if the image is sharply focused at the distal rhabdom tips – if it isn't, then light intended for the target rhabdom will never reach it, being fully absorbed by neighbouring rhabdoms instead, thus degrading resolution. Full sheathing is found in diurnal lepidopteran superposition eyes and in nocturnal hawkmoths (Land, 1984; Warrant et al., 1999), all of which have sharp, diffraction-limited images formed at the rhabdom tips. In *M. heliconiaria* the sheathing is partial, implying that the image quality is lower than in diurnal lepidopteran superposition eyes and/or that the plane of focus is not at the rhabdom tips, but is more proximal. A naked tapetum, without rhabdom sheathing, can significantly degrade spatial resolution due to the reflection of oblique rays (Warrant and McIntyre, 1991, 1993).

4.1.3. Pigment cells and light adaptation

M. heliconiaria exhibits the "dual" pigment migration mechanism (Warrant and McIntyre, 1996): pigment granules within both the primary and secondary pigment cells migrate proximally in light-adapted conditions to reduce the light flux at the retina and distally in dark-adapted conditions to increase it. Upon light adaptation, the proximal migration of pigment reduces the eye glow and thus the superposition aperture. When fully light adapted, the eye glow is replaced by a dark pseudopupil of approximately the same shape and area (Bannister and White, 1987).

4.1.4. Dioptric apparatus and retinula cells

The crystalline cones and retinula cells in *M. heliconiaria* have certain morphological features that are characteristic of typical superposition eyes in Lepidoptera. Firstly, the crystalline cones are bullet shaped, and lack the long tapering shape and corneal process typical of crystalline cones from apposition eyes. The bullet-shaped cone has a strong internal gradient of refractive index (radially from axis to edge) that is crucial to the telescopic optics that underlies the refracting superposition design (Caveney and McIntyre, 1981; McIntyre and Caveney, 1985). Secondly, the retinula cells are of the restricted form, meaning that the rhabdom is located in the proximal region of the ommatidium, and connects to the dioptric apparatus by a thin strand. In the dark adapted state, with the screening pigments distally contracted, a translucent 'clear zone' is seen between the rhabdom and dioptric apparatus, a characteristic feature of superposition eyes and a necessary requirement of superposition optics. A third feature of the *M. heliconiaria* eye that is characteristic of the superposition eye design is a wide rhabdom capable of capturing a large amount of light. Diurnal butterflies typically have very narrow – and much less sensitive – rhabdoms, often only 1–2 μm wide. Since these diameters approach the wavelength of light (ca. 0.5 μm), the rhabdom functions as a waveguide and propagates light in the form of waveguide modes (see Warrant and McIntyre, 1993, for a review). The 8 μm wide rhabdom of *M. heliconiaria* instead functions as a light guide (since rhabdom width greatly exceeds the wavelength of incident light). A fourth feature is the flower shaped rhabdom, so far only found in superposition eyes. The exact meaning of this shape is as yet unknown.

4.1.5. Optic lobe morphology

Variation in external brain morphology has been noted for different groups of Lepidoptera, and has been used to support the monophyly of Hesperoidea with Papilionoidea (Ackery et al., 1999; Kristensen, 1976). Ehnbom (1948) categorized the brains of Lepidoptera into groups, largely based on the relative sizes of the optic and olfactory (= antennal) lobes. Type I, typical of diurnal butterflies, is characterized by enlarged and rounded optic lobes that occupy the largest part of the brain volume, owing to the importance of the visual system in diurnal butterflies, and small antennal lobes (Fig. 6C). In the Type II, or 'Macrofrenate type' (characteristic of most nocturnal macro moths) each optic lobe is divided into 2 distinct regions, and is elongated laterally (Fig. 6B), and the antennal lobes are well developed compared to those of the butterflies, owing to the importance of pheromone perception for mate finding in moths. Nocturnal moths also rely more heavily on olfaction than vision to locate fragrant flowers at night (Balkenius et al., 2006). Based on external brain morphology alone, *M. heliconiaria* appears most characteristic of nocturnal moths, with moderately sized, bilobed optic lobes (Type II), and well developed olfactory lobes (Fig. 6A). However, histological comparisons of brain structures are necessary to make more specific comparisons of brain functionality.

4.2. The resolution and sensitivity of the hedylid superposition eye

How well resolved and how sensitive are the eyes of hedylids compared to the eyes of other Lepidoptera? Two easily determined anatomical measures of the resolution are the interommatidial angle $\Delta\phi$ and the rhabdom's angular acceptance $\Delta\rho_r$.

The interommatidial angle specifies the density of packing of ommatidia in the eye, and thus indicates the eye's potential spatial resolving power. A smaller interommatidial angle indicates a denser ommatidial packing and the potential for higher resolution. We have already estimated $\Delta\phi$ to be about 2.2° using Eq. (1). This value is typical of reasonably well resolved superposition eyes, such as those of dung beetles (McIntyre and Caveney, 1998), but is considerably larger than the nocturnal hawkmoth *Deilephila elpenor*, which has $\Delta\phi = 1.0°$ (Warrant, unpublished data).

The diameter of the rhabdom's receptive field is approximated by its angular acceptance $\Delta\rho_r$. Smaller values of $\Delta\rho_r$

indicate a potentially better spatial resolution. $\Delta\rho_r$ is given (in radians) by the ratio of the rhabdom diameter (d) to the focal length (f), where f in a classical superposition eye is given by the radius of the distal retinal surface (Exner, 1891). In *M. heliconiaria*, $d = 8$ μm and $f = 260$ μm ($n = 14$, from 2 specimens), leading to $\Delta\rho_r = 1.8°$. This value is a little larger than found in the well-resolved superposition eyes of dung beetles (1.5° in *Onitis aygulus*: McIntyre and Caveney, 1998), and hawkmoths (1.5° in *Deilephila elpenor*: Warrant, unpublished), but is considerably smaller than the value found in the Mediterranean flour moth *Ephestia kühniella* (2.7°: Cleary et al., 1977).

The size of the rhabdom's receptive field also influences the optical sensitivity S of the eye (Kirschfeld, 1974; Land, 1981; Warrant and Nilsson, 1998):

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

where A is the diameter of the superposition pupil (456 μm, determined by averaging the short and long diameters of the slightly elliptical dark pseudopupil in 5 specimens), k is the absorption coefficient of the rhabdom (taken as 0.0067 μm^{-1}: see Warrant and Nilsson, 1998) and l is the length of the rhabdom (112 μm), although the presence of a tapetum in hedylids effectively doubles the absorption length of the rhabdom, i.e. l can be taken as 224 μm. The optical sensitivity is a measure of how well a photoreceptor is able to capture light from an extended source of given intensity. In hedylids, with $\Delta\rho_r$ given in radians, $S = 47.9$ μm^2 sr, a value comparable to nocturnal moths with superposition eyes (Warrant and Nilsson, 1998), such as *Deilephila* (69 μm^2 sr) and *Ephestia* (38 μm^2 sr). The apposition eyes of diurnal butterflies, on the other hand, typically have optical sensitivities about 100 times lower (Frederiksen and Warrant, unpublished). Thus, the superposition eyes of hedylids are typical of those found in nocturnal moths. With a high sensitivity to light and the potential for good spatial resolution, their eyes are well suited to nocturnal life.

4.3. Eye design and the evolution of butterflies

There is good reason to suggest that the ancestor of all present-day lepidopterans was a day-active moth with focal apposition eyes. With the exception of a single family (Agathiphagidae: the nocturnal kauri moths), the most primitive extant moth families are day active and have, or are likely to have, apposition eyes (Warrant et al., 2003). These families include the Micropterigidae, the Heterobathmiidae, the Acanthopteroctetidae and the Eriocraniidae (Fig. 7). The kauri moths have superposition eyes, and these probably evolved via afocal apposition optics during a gradual transition to an increasingly nocturnal lifestyle. Two other primitive moth groups – the Lophocoronidae (Australian archaic sun moths) and the Neopseustidae (archaic bell moths), both sister groups to the Neolepidoptera – probably evolved in a similar manner. Both groups are predominantly nocturnal, with the Lophocoronidae almost certainly possessing superposition eyes (Niels Kristensen, unpublished data). The Neolepidoptera include several moth families and the Ditrysia, the group containing most modern moths and butterflies. The Ditrysia are dominated by nocturnal moths with superposition eyes, although apposition eyes are possessed by the true butterflies (Papilionoidea) and can be found in many groups of moths (e.g. selected species of Geometroidea and Drepanoidea (sister groups to the rhopaloceran butterflies), the Hyblaeidae, the Zygaenoidea and the Sesioidea) (Warrant et al., 2003).

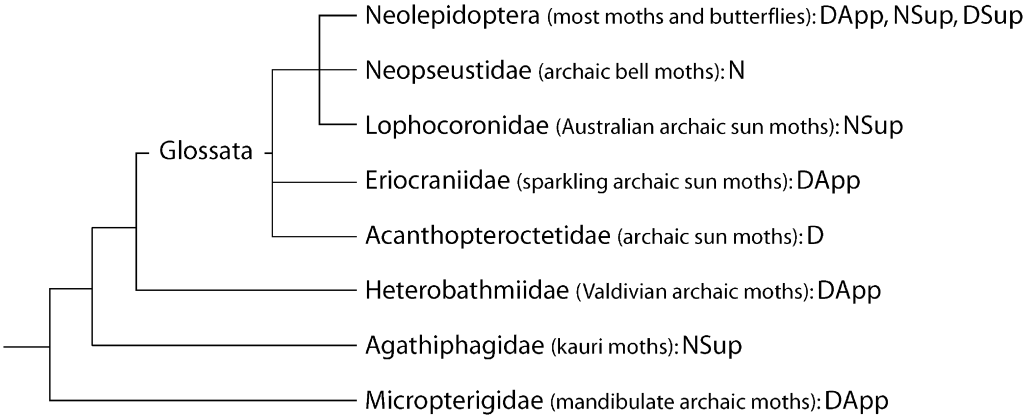

Fig. 7. Phylogeny of the Lepidoptera adapted from Kristensen and Skalski (1999) depicting the relationship between the Neolepidoptera and primitive groups. Eye design (if known) and activity is also indicated. This phylogeny suggests that the ancestor of Lepidopterans was a diurnal moth with apposition eyes. N = nocturnal, D = diurnal, *App* = apposition eye, *Sup* = superposition eye. Explanatory notes. The activities listed for the primitive Lepidopteran groups shown in this figure are derived from the best available knowledge and are intended to show the dominant activity known for the group. Even though the Micropterigidae, Heterobathmiidae, Eriocraniidae and Acanthopteroctetidae are predominantly diurnal, in warmer climates some micropterigid and eriocraniid species are thought to continue their activity into the night (if light trap data can be trusted to indicate nocturnal behaviour). Some acanthopteroctetids are drably coloured and are thus thought to be nocturnal, but in general acanthopteroctetids are caught extremely rarely at light traps. Some exceptions can also be found among the predominantly nocturnal groups. For instance, within the Neopseustidae, the South American *Synempora* has been seen flying actively during the day, although it is also attracted to UV light at night. Such dual diurnal-nocturnal behaviour can also be found within the Neolepidoptera. The authors are deeply indebted to Niels Kristensen (University of Copenhagen) and David Wagner (University of Connecticut) for their help in constructing this figure.

An evolutionary link between focal apposition eyes and refracting superposition eyes is provided by the afocal apposition eye design (Nilsson et al., 1988). Because this design is superior to the focal design in terms of both resolution and sensitivity (van Hateren and Nilsson, 1987), it provides an evolutionary improvement in visual performance. According to the current hypothesis (Nilsson et al., 1988), the afocal optics and gradients of refractive index found in the afocal apposition design probably allowed the evolution of refracting superposition eyes, although an evolution in the reverse direction (i.e. refracting superposition to afocal apposition) is also possible. For instance, refracting superposition optics may have evolved via afocal optics from the focal apposition eyes of a diurnal ancestor that became more nocturnal due to an increased diurnal competition or predatory pressure. As we mentioned above, this almost certainly happened during the early evolution of the Lepidoptera, and probably occurred independently on many further occasions during later evolution throughout the lepidopteran phylogenetic tree.

How then do the Hedylidae fit into this evolutionary scheme? The Rhopalocera is the lineage that contains all butterflies: the Hedyloidea (nocturnal refracting superposition eyes), the Hesperoidea (diurnal refracting superposition eyes) and the Papilionoidea (the few species so far investigated all have afocal apposition eyes). What eye design did the ancestor to all rhopalocerans have? Our discussion above indicates that any of the three eyes designs – focal apposition, afocal apposition and refracting superposition – are all possible. We can say with certainty that if *any* papilionoid (true butterfly) has focal apposition eyes, then the rhopaloceran ancestor also had this eye design – focal apposition eyes, due to their inferior performance, would never evolve from an afocal design.

Since the rhopaloceran ancestor could have possessed any of the three possible eyes designs, it is difficult on the basis of eye design alone to untangle the evolutionary relationships between the Hedylidae, the Hesperiidae and the Papilionoidea. In terms of visual performance, one may not expect a transition from a nocturnal to a diurnal lifestyle to result in an evolution from refracting superposition optics to afocal apposition optics, since diurnal superposition eyes have the potential to significantly out-perform afocal apposition eyes. Indeed, highly optimized diurnal superposition eyes, like those found in day-active hawkmoths, have the potential to code more information than any other compound eye design, including the afocal design (Warrant et al., 1999). However, an evolution from refracting superposition optics to afocal apposition optics is certainly possible, especially if the ancestral superposition eye had poor spatial resolution and a mutation giving a more ''apposition-like'' eye gave the descendent better spatial resolution that increased its fitness.

In isolation, these visual performance arguments suggest that the Hesperoidea may have arisen from the Hedyloidea (or vice versa), assuming the two groups did not evolve independently from the rhopaloceran ancestor. Alternatively, the better performing superposition eyes of hesperoids may have evolved from an ancestor with afocal apposition eyes. As discussed above, the same performance argument also suggests that the afocal apposition eyes of the Papilionoidea are not likely to have evolved from the Hedyloidea (or any other ancestor with superposition eyes), although this evolution could easily have occurred. Indeed, recent molecular phylogenetic evidence (Wahlberg et al., 2005) supports the hypothesis that hedylids are the closest relatives to the butterflies (see Fig. 1C), although whether they are the 'living ancestors' of modern butterflies is more difficult to say. The ancestor of both groups (and indeed also of the hesperoids: Fig. 1C) could have been a diurnal moth with focal or afocal apposition eyes. One other intriguing possibility is that the Hedyloidea are not the ancestors of butterflies at all, but derived from the Papilionoidea. Interestingly, several species of Nymphalidae that fly in low light conditions, also possess a complex tympanal ear that is homologous to that of the Hedyloidea (Yack, unpublished; Yack and Fullard, 2000). It is possible that the Hedyloidea moved back into the night from a papilionoid ancestor. Indeed, further work on the Hedyloidea should prove to be interesting in unfolding the evolutionary history of butterflies.

In conclusion, despite their importance as purported evolutionary links between butterflies and moths, surprisingly little is known about the Hedyloidea. Indeed, as expressed by Aiello (1992), our general knowledge of hedylids is 'meager at every level'. If hedylids do represent the 'living ancestors' of butterflies, studies on their life history and sensory ecology should reveal insights into how primitive butterflies may have lived. The present study provides some insights into the visual ecology of the Hedyloidea. Most significantly, they possess many characteristics of the superposition eye design, which arguably adapts these insects to a nocturnal lifestyle. Our results corroborate other reports of nocturnal activity in the Hedyloidea, including observations that most individuals have been collected at blacklights (Scoble, 1986) and that they possess high frequency tympanal organs that evoke bat avoidance flight maneuvers (Yack and Fullard, 2000). In addition, the size of the optic and antennal lobes of hedylids indicates that they are more moth-like in that they are dependent more on olfaction than vision. The present study provides a preliminary description of the visual system of the Hedyloidea, particularly for one species, *M. heliconiaria*. The information should contribute to a better understanding of this little known group, and its relationship to other butterflies and moths.

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