Structural and functional changes of insect eyes in cases of miniaturization

by

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Statutory declaration

I, Stefan Fischer, hereby declare that this thesis is my own work and effort and that it has not been submitted anywhere for any award. Where information has been derived from other sources, this has been indicated in the thesis.

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List of original publications

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Abstract

In spite of the numerous studies on insect compound eyes within the last 100 years, the number of investigations specifically dealing with the eyes of species of small body size is near to negligible. Given that the greater proportion of insects is made up of small and very small species and that small size is known to possess a considerable potential for reductions, structural simplifications, and morphological novelties (Hanken and Wake 1993), the fact that so few of them had their eyes studied is certainly astonishing. Moreover, conclusions by Warrant and McIntyre (1993) and Meyer-Rochow and Gál (2004), based on theoretical optical examinations, suggested that an unlimited eye size reduction was not possible; a suggestion that begged the question as to how the tiniest insects might deal with these restrictions. First indications of specific structural and functional changes in small eyes came from a study of small scarabid beetles (Gokan and Meyer-Rochow 2000) and a tiny lepidopteran species (Honkanen and Meyer-Rochow 2009). To demonstrate that optical and structural modifications were widespread, a detailed study of eye miniaturizations involving additional species and a quest to determine limiting factors governing such modifications seemed timely and interesting.

The main goals of the present work were therefore A) to test through the description of ultrastructural and functional design features of compound eyes of different species of tiny lepidopterans, the prediction of Meyer-Rochow and Gál (2004) that the possession of a superposition type of eye was of no benefit to small eyes B) to describe possible adaptations occurring in these eye of reduced size and C) to carve out through a comparative analysis of eyes of different size, the impacting factors that set the limits for the miniaturization of compound eyes generally. In order to answer the last question, it was deemed helpful not to restrict the study to Lepidoptera alone, but to include a tiny hymenopteran species with apposition optics in the investigation as well.

The present work, nevertheless, focuses on small Lepidoptera and revealed that the conclusions reached by Meyer-Rochow and Gál (2004) based on their theoretical approach holds true and adaptations in small eyes are manifest in form of an intermediate eye type. This eye type combines features of apposition and superposition optics and is present in nearly all of the investigated small
lepidopteran species. While structurally the eyes possess the general anatomy of a superposition eye, specific modifications include a distal rhabdom found in place of the standard clear zone, a close connection between the dioptric apparatus and the broad, proximal rhabdom, the latter frequently shaped like a bottle with a distally placed neck.

Based on the comparative investigation of compound eyes of species of different body sizes, it was possible to demonstrate a positive correlation between eye size (and eye radius) with body size. With decreasing eye size the diameter of the distal rhabdom increases presumably for sensitivity reason; limits are given especially by the size of the nuclei of the retinula cells. As the interommatidial angles increase (a consequence of the relation between similar sized facets and the decrease in the eye radius), this decreases the probability for superposition, which efficiency depends on a large number of participating facets. Given that sensitivity is reduced, a nocturnal lifestyle cannot be maintained with the small optics, and the investigated species had to shift to a more diurnal lifestyle. Connected with this is a change to functional apposition optics as the location of screening pigments in the light-adapted eye prevent light from being channelled to neighbouring ommatidia. This functional change from superposition to functional apposition optics due to miniaturization reveals that the borders of the categories of both functional types become less and less distinct. This aspect of an unclear categorization of eye types, and the intermediate eye type in particular, are further discussed in relation to the evolution of the different eye types and specifically the question of which kind of eye most likely the original eye type within the Lepidoptera could have been.

The quest into factors impacting on the construction of the smallest possible functional superposition eye in the dark-adapted state lays bare the necessity of additional investigations on the optics of such eyes. The smallest functional size of the dioptric apparatus will play an important role in miniaturization, a factor that can not be tested with morphological investigations alone (as in this study) and specialized optical investigations are needed to validate the functional conclusions. Adaptations recognizable in the investigated eyes as a consequence of miniaturization are discussed on a functional basis, as is the aspect of limiting factors operating on the smallest possible optics generally. Especially for the latter aspect information available from the small compound
eye of the tiny hymenopteran *Trichogramma evanescens*, several times smaller than the smallest investigated lepidopteran, has been useful.

The present work, in addition to confirming that even the smallest eyes are formed by the same cellular components as the larger eyes, revealed the existence of a new type of basal matrix present in the Lepidoptera. Furthermore the ultrastructural make-up of the ommatidia was generally similar amongst the smaller species, but different from the larger lepidopterans in relation to the numbers of retinula cells. The presence of 8 retinula cells, found in the basal groups, has to be regarded as the original state of the Lepidoptera. The literature on retinula cell numbers present in lepidopteran eyes is reviewed and discussed in relation to the original state and functional aspects.
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Chapter 1: General introduction

Sensory perception plays an important role in the lives of insects as it provides the opportunity to gain information about the environment. This is needed for many activities during the life of these animals, like finding a mate, searching for prey or food resources, control over location in space and detection of enemies. Besides mechanoreceptors and chemoreceptors, photoreceptors, especially the highly evolved compound eyes are of importance in performing the aforementioned activities. The importance of visual information is further revealed by the fact that other than cave species and species with subterranean lifestyles virtually no eyeless species can be found in environments where at least some light is present (Meyer-Rochow and Nilsson 1999).

The study of compound eyes of insects has a long tradition and many features of insect eyes were already described more than 100 years ago (Exner 1891). At that time the differences between apposition eyes (the typical eyes of daytime-active butterflies, wasps and bees) and superposition eyes (eyes that are characteristics of nocturnal and crepuscular moths and beetles) had been distinguished. Since Exner’s days extensive studies have been carried out on the function of these eye types (see Goldsmith 1989 for review of major events in the study of arthropod vision). After years of uncertainty about Exner’s superposition theory, its validity was finally proved (Kunze 1969, 1971; Kunze and Hausen 1971). In the 1980s Autrum (1981) summarized what was known at that time about dark-light adaptational changes in the insect eye’s structural organization. A so-called afocal apposition type of butterfly eyes was discovered by Nilsson et al. in 1984 and described in more detail a few years later (Nilsson et al. 1988). Theoretical limitations for optics operating in compound eyes were described for apposition (Barlow 1952, Kirschfeld 1976, Snyder 1977), as well as for superposition eyes (Warrant & McIntyre 1990, 1993; Meyer-Rochow & Gál 2004). However, with few exceptions most of the investigations that yielded the above-mentioned results were based on species of medium to large body size, irrespective of the fact that a large percentage, or perhaps even the majority of insects, are of small to very small size (e.g. Chown and Gaston 2010, Fig.3).
This neglect of small species is astonishing as small size is known to possess a potential for reductions, structural simplification and morphological novelties (Hanken and Wake 1993) due to the fact that, for example, an unlimited miniaturization of cells and the neural system is not possible (Beutel et al. 2005; Polilov and Beutel 2010). So far eyes have not been in the focus of work about miniaturization and only a single article about eye size in miniaturized vertebrates was published with regard to the lens eyes of salamanders (Linke et al. 1986).

Irrespective of the lack of morphological investigations, there is theoretical knowledge about optical parameters impacting eye size (and vice versa), making an unlimited reduction in eye size without a change in functionality quite unlikely. In this context it is especially the superposition type of eye that is of interest (Meyer-Rochow and Gál 2004), and this is the reason why the focus of this work has been on a group of insects, for which this eye type is the rule: the nocturnal lepidopterans (“moths”). Theoretical aspects of vision with this kind of eye shall be described after a comprehensive introduction of the major structural design features of compound eye generally in the following chapters. The introduction is concluded by a chapter summarizing the available information through the literature on lepidopteran eye-designs to reveal why this group is of special interest.

1.1 General anatomy of compound eyes

Within the hexapods, including the insects, the typical eye type of the adults is the compound eye (Land and Nilsson 2002), composed of a certain number of similar, repetitive units, the ommatidia. Closely packed, the ommatidia form an array, which is visible from the outside as a cluster of typically hexagonal facets (Fig.1). The number of ommatidia in an eye ranges from only a few in some ant species (Chapman 1998) to up to several tens of thousands in larger eyes like those of dragonflies (Warrant 2001). Within the Lepidoptera facet numbers range from 203 in a nepticulid species of the genus *Stigmella* to an estimated number of 27,000 found in a sphingid (Yagi and Koyama 1963).

Each ommatidium can be divided into two functional units: the dioptric apparatus made up of cornea and crystalline cone, channelling and focusing
the light onto the receptive unit: the retinula, which is made up of retinula (= photoreceptive) cells that give rise to the rhabdom with its light perceiving, visual pigment containing membrane specializations (Fig.2).

Figure 1. The compound eye of *Argyresthia goedartella* (Linnaeus, 1758): a) ventral view on the head and the left compound eye b) close-up of single facets and two interfacetal hairs.

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Figure 2. General anatomy of an ommatidium, representing the apposition type of eye. The transparent cornea consists of a number of horizontally stacked layers and in some taxonomic groups the outermost layer is covered by corneal protuberances, the so-called corneal nipples (Bernhard and Miller 1962, Bernhard et al. 1970, Gemne 1971). The crystalline cone, subjacent located to
the cornea, is made up of four cone cells (often referred to as Semper cells in the older literature), each of them developing a cone projection that reaches down to the basal matrix to anchor and stabilize the dioptic apparatus and the ommatidium. This pattern is a highly conserved feature within insect and crustacean taxa, although the composition of the basal matrix differs in other respects, i.e. cellular components (Odselius and Elofsson 1981). The crystalline cone is enveloped by two types of pigment cells: primary pigment cells (usually two cells) restricted to the longitudinal extension of the crystalline cone and secondary pigment cells (or intermediate pigment cells, usually counting six), situated in-between two neighbouring ommatidia, adjoining and accompanying the ommatidium down to the basal matrix. Both pigment cell types contain ommochrome granules of screening pigments, which are used for adaptations to different light intensities, due to their ability to migrate intracellularly (Autrum 1981, Meyer-Rochow 1999). The retinula is composed of usually eight and sometimes slightly more photoreceptors, the retinula cells. Each retinula cell contributes to the light-sensitive rhabdom a rhabdomere, consisting of numerous microvilli, housing the visual pigments in their membranes (Schwemer 1989). The rhabdom itself can be of the fused type (all rhabdomeres fuse and build a single rhabdom) or of the open type, in which the rhabdomeres remain separated over their entire lengths. At the proximal end the basal matrix separates the eye from the optical neuropil. Axons of each retinula cell pass through the basal matrix to project to their respective lamina or medulla terminations, depending on the cell’s position in the ommatidium (Ribi 1981; Meinertzhagen 1991; Morante and Desplan 2008).

### 1.2 Different types of compound eyes

During the last few decades, several different types of compound eye were described (see Land and Nilsson 2002 for review), which can be classified according to their non-identical light-guiding abilities or the way how signal processing of the visual information takes place in them. However, basically the two major types of eye (defined by Exner 1891) are still valid and distinguished: apposition and superposition eyes. The first type is primarily present in diurnally active species, like butterflies (Yagi and Koyama 1963, Kolb 1977, Kolb 1978), ants (Menzel 1972, Greiner et al. 2007) bees and wasps (e.g. Perrelet 1970,
Wachmann et al. 1973), whereas superposition eyes are normally developed in nocturnal species like moths (Yagi and Koyama 1963), neuroptera, caddis-flies and some carabid and scarabaeid beetles (Ehnbohm 1948, Nilsson 1989). Some exceptions are known for both (a) apposition eyes, which can also be present in certain nocturnal Hymenoptera (Menzi 1986, Frederiksen and Warrant 2008, Greiner et al. 2004, 2007), and (b) superposition eyes, which have been described from some species of day-active lepidopterans (Yagi 1953, Horridge et al. 1972; Warrant et al. 1999, Lau et al. 2007). In the following the focus will be on the structural and functional differences between apposition and superposition eye types present in insects.

Figure 3. Schematic drawing of two different types of compound eyes: a) apposition and b) superposition (after Nilsson 1989). a. In apposition eyes each ommatidium is functioning separately from each other. Light entering the eye not on-axis is absorbed by pigment granules surrounding the crystalline cone. Retinula cell pigment granules show a radial migration within dark-light adaptation (light-adapted state shown schematically on the left, dark-adapted state on the right). b. In superposition eyes light of several neighbouring facets is channelled to a central rhabdom in the dark-adapted state (right). In the light adapted-state (left) proximal-directed longitudinal pigment migrations of the secondary pigment cells prevent the superposition process for many neighbouring facets. C: cornea, CC: crystalline cone, RCP: retinula cell pigment, RH: rhabdom, TR: tracheole.

Focal and afocal apposition optics

The focal apposition eye represents the typical apposition eye, named focal as the light is focused on the apex of the rhabdom, which is directly connected to the dioptic apparatus. In apposition optics each ommatidium is separated from its neighbouring ommatidia by the dense sheath of pigment bearing primary and secondary pigment cells (Fig. 3a). In this way stray light is eliminated and the aperture for the incoming light is thus represented by the facet diameter of the
ommatidium. Afocal apposition eyes that can be found in butterfly species (except skipper butterflies: Horridge et al. 1972, Nilsson et al. 1988) are more complex with regard to the dioptric apparatus than simple focal apposition eyes. The crystalline cone reveals an elongated shape, exhibiting a cone stalk. Unlike focal apposition optics, the focal plane of the afocal eye does not lie on the apex of the rhabdom, but within the tip of the cone, due to the particular refractive index distribution in the crystalline cone (Nilsson et al. 1988). Because of that, a parallel beam of light leaves the cone, as is also the case in superposition optics (Land and Nilsson 2002). Due to this similarity, this type of eye is seen to be a link between apposition and superposition eyes (Nilsson et al. 1988, Nilsson 1989). Afocal apposition optics, compared with focal apposition eyes, are approximately 10% more efficient in terms of light gain, as the light is channelled towards the rhabdom in a different way (Nilsson et al. 1988). It was therefore assumed that the focal apposition type did not evolve from the afocal type as this would result in a loss of efficiency.

Refractive superposition optics

In contrast to the described apposition optics, a pigment-free area, the so-called “clear zone” separates the dioptric apparatus from the rhabdom in the dark-adapted superposition eye (Exner 1891). This distance, in combination with a gradient of refractive index in the crystalline cones (refractive superposition optics), makes it possible that parallel light rays, entering the eye through several neighbouring facets, can be channelled to a central rhabdom (Fig. 3b). This increases photon catch and thus sensitivity (Exner 1891, Kunze 1979). Consequently, unlike with apposition optics, each ommatidium is not working separate and independently from its neighbours. The number of participating facets in the superposition process can range from only few facets to up to several hundreds and the degree of enhancing sensitivity is dependent on the number of participating facets.

In order to avoid cross light from light rays entering the rhabdom at a steep angle, each rhabdom is surrounded by a reflective structure, the tracheal tapetum (a system of air-containing blind ending tubes). The tracheal tapetum not only avoids cross talk, but also increases sensitivity as light that has not been absorbed in the rhabdom before, can be absorbed after being reflected at
the tracheal tapetum owing to the lower refractive index (i.e., air) of the latter. The tracheal tapetum is typical (but not a prerequisite) for superposition optics and is missing in the apposition type in this form. Butterflies with apposition optics possess proximally situated tapeta below the rhabdom (Ribi 1979), except for the papilionids, where a tapetum is missing (Stavenga and Arikawa 2006, Wakakuwa et al. 2007).

1.3 Dark-light adaptations

Pigment migrations within different cell types allow the eye to adapt to different light levels by adjusting the amount of light entering the rhabdom (for reviews see Autrum 1981, Meyer-Rochow 1999). This is performed not only for efficiency reasons, but also to protect the eyes from UV-A damage under intense light levels (Meyer-Rochow 1994, 2001; Mishra and Meyer-Rochow 2007). Pigment migration mechanisms operate in different cells and do vary between apposition and superposition optics (Walcott 1975).

In apposition optics the primary and secondary pigment cells deal as “pupil mechanism”, reducing the aperture for the incoming light under high light-intensities. Additional mechanisms for adaptations to different light levels involve radial pigment migrations within the retinula cells (Ribi 1978). In light-adapted eyes these pigment granules envelop the rhabdom closely, whereas they are positioned at a greater distance peripherally displaced in the dark-adapted eyes (Fig. 3a). Due to the wave-guide nature of the rhabdom (rhabdom diameter < 2 µm) in butterflies (van Hateren 1989), part of the propagating light is travelling outside the rhabdom and is absorbed by the pigment granules. The larger the distance between pigment granules and rhabdom, the more wave-guide modes can propagate (Snyder 1979). This mechanism allows butterflies to adapt to different light levels within short time scales.

Although the pigments of primary pigment cells play a role in dark-light adaptation in superposition optics, it is mainly the longitudinal pigment migration within the secondary pigment cells that prevents light to pass from one neighbouring ommatidium to another in the light-adapted eye (Fig.3b). Thus different from the dark-adapted state, the ommatidia are largely shielded from each other as in an apposition eye. Due to this, each ommatidium perceives mainly light that entered through its own dioptic apparatus.
1.4 Optical properties of compound eyes and limiting factors

Sensitivity versus spatial resolution

Sensitivity and spatial resolution are the key features determining the quality of an eye. The eye must be sensitive enough to separate light information from photon noise under given light illumination levels (comparable to noise in digital photo cameras under low light conditions) and simultaneously possess enough spatial resolving power to determine the direction of incident light (Warrant and McIntyre, 1993). Unfortunately these two functional features of an eye cannot be increased together in an eye of given size as they are counter-dependent on each other. An eye of a given size, as will be presented in the following, always has to compromise between resolution and sensitivity.

It is mainly the diameter of the aperture through which light is entering the eye (D) and the focal length (f) that are controlling sensitivity in terms of light available for the photoreceptor. The ratio between these factors is known as the F-number of the eye (Warrant and McIntyre 1993) and can be described as:

\[ F = \frac{f}{D} \]

As in photographic cameras, lenses with large apertures in relation to their focal length can form the brightest image. This relationship can also be applied to insect optics: eyes with small F-numbers are potentially more sensitive to light than eyes with larger F-numbers (= smaller apertures). The formula reveals the importance and impact of the aperture to sensitivity. In order to increase sensitivity it would be of interest to increase the facet diameter (= aperture), which will simultaneously decrease the number of facets in an eye of given size. At this point the negative impact on resolution becomes obvious, as the number of ommatidia in an eye is generally a good indicator of spatial resolution (Warrant 2001): a higher number and denser packing of ommatidia in an eye of given size, increases spatial resolution but decreases sensitivity. Thus due to the interconnection between sensitivity and resolution a compromise, fitting best to the needs of a species and to the environment, has to be found for an eye of given size. A useful parameter here is the interommatidial angle (\( \Delta \phi \)), defining the spacing of ommatidia (Snyder et al. 1977) in relation to the eye radius (R) and facet diameter (D) of the eye (\( \Delta \phi = 180 \frac{D}{\pi R} \)). Figure 4a presents a
schematic drawing of hypothetical eye designs, visualizing the dependency of the parameters facet diameter, eye radius and interommatidial angle.

On the basis of the interommatidial angle ($\Delta \phi$) and the aperture (D), the resolving power of an eye can be described by the eye-parameter ($\rho$) as $\rho = D \Delta \phi$ (Snyder et al. 1977). In general diurnal insects active under bright light conditions (and thus not light limited) maximise spatial resolution by combining relatively small facet diameters and small interommatidial angles and thus possess small eye parameters; night active species by comparison show higher values (Snyder et al. 1977). The equation furthermore reveals that the combination of a narrow interommatidial angle with a large facet diameter can result in greater resolving power and sensitivity. But this is only possible by maximising the size of the eye, which is limited by the size of the head. Indeed a simultaneous increase in sensitivity and spatial resolution was shown for apposition optics of butterflies with increasing eye size by Rutowski et al. (2009).

**Small optics and optical limits**

The connection between sensitivity and resolution reveals the difficulty in small optics and the need to balance both parameters. In addition to this, optical parameters also impact on an unlimited reduction of facet diameter.

All optics are affected by the wave nature of light and properties of refractive indices between layers of different optical densities. Due to that, aberration, chromatic aberration and diffraction are affecting also the eyes of insects (Warrant and McIntyre 1993, Land and Nilsson 2002). Whereas aberration and chromatic aberration increase with the size of the lens and are of less concern for small eyes, the reverse applies to diffraction (Land and Nilsson 2002). Irrespective of the design of the optics, diffraction increases the smaller the facet diameter gets, resulting in more and more blurred information gained by the receptor.

The so-called airy disc is a diffraction pattern that is the result of the fact that light passing through the centre of the lens will be delayed, as it has to pass through denser material than nearer to the edges of the lens. Due to that, a wave front of parallel light is curved into a spherical shape afterwards. As all rays are bend to a focal plane, different parts of the wave front will interfere at
the focal plane. Some of the parts will be cancelled (parts that are out of phase), while others will be reinforced (components that are in phase). This leads to a bright spot in the middle (the airy disc) that is surrounded by a darker ring. The size of the airy disc (Barlow 1952) depends on the aperture D and the wavelength $\lambda$:

$$\Theta = \frac{1.22 \lambda}{D}$$

Resulting from this formula, the size of the airy disc will increase with a reduced aperture and it is possible that the airy disc does not only capture the whole rhabdom, but also affect neighbouring receptors. This leads to blurred information in this receptor, as the information was not gained along the axis of its own optical components but involved neighbouring ommatidia. A loss in resolution is the result. A blurred image, like that of the airy disc covering several receptors, can also be produced by a focal misalignment, when the focal plane is not fitting the top of the rhabdom (Warrant and McIntyre 1993, Land and Nilsson 2002).

Barlow (1952) concluded on the basis of diffraction a minimal possible facet diameter of around 10 µm to be present in insect eyes. This feature is therefore of special interest in small eyes as it should limit resolution. Down to a certain eye radius, resolution might be maintained at the cost of sensitivity loss by reducing the facet diameter down to 10 µm. Below that limit, eye size reduction will only be possible by reducing the number of ommatidia and thus reducing spatial resolution.

The limited facet diameter in combination with the interommatidial angle can also be used (as in the following) to reveal the problematic nature of the superposition process and to visualize the problematic consequence of a decreasing width of the clear zone.

Given two hypothetical eyes of different eye radius (R) possessing both the same facet diameter D, it is obvious that due to the smaller eye radius the interommatidial angle has to increase (compare Fig. 4a). This correlation can also be shown by the mathematical correlation interommatidial angle = $D/R$ (in radians). As according to Barlow (1952) the minimal facet diameter should be around 10 µm, the interommatidial angle has to increase in small eyes, as the facet diameter cannot be reduced any further.
Figure 4. Schematic drawings of hypothetical compound eyes. a. The figure illustrates the dependence of facet diameter (D) and eye radius (R) on the interommatidial angle (Δφ). In both cases the facet diameter is the same but the eye radius on the right is only half of the length compared to the left side. b. Two eye-halves of an eye with the same eye-radius and facet diameter reveal the dependence of the exit angle needed from the dioptic apparatus (dotted lines) to be able to channel light to the median rhabdom in order to maintain superposition. On the left side the exit angle is smaller, allowing more facets to take part in the superposition process, but the rhabdom is rather short. On the right side the rhabdom is clearly larger, but the smaller clear-zone increases the needed exit angle between the dioptic apparatus of neighbouring ommatidia and the central rhabdom. If this angle cannot be fulfilled, less ommatidia can take part in the superposition process.

The large interommatidial angle will lead to a reduction of the number of ommatidia taking part in the superposition process as the exit angle that must be reached by the dioptic apparatus to focus the light on the central rhabdom is inevitably increasing (Fig. 4b). As the ommatidial length is correlated to the eye-radius, the available space for all components is decreasing.

The exit angle can be reduced by a deeper situated rhabdom (= broader clear-zone), but not without diminishing the extension of the latter and thus a loss in sensitivity. Furthermore, a drastic decrease in length of the dioptic apparatus also seems impossible in connection with the need to maintain a proper function to reach a certain exit angle.

Based on this theoretical background and on anatomical data from 78 investigated species of scarab beetles (Gokan and Meyer-Rochow 2000), for which superposition optics are also the rule, Meyer-Rochow and Gál (2004) calculated a minimal limit for eyes of the refractive superposition type for an eye radius of 250 µm, below which possessing superposition optics was considered not to be beneficial. In the smallest investigated scarab beetles (Gokan and Meyer-Rochow 2000) the clear zone was missing altogether and the rhabdom
was in direct contact with the crystalline cone. This raised the question how small species of Lepidoptera, even smaller than most of the investigated scarab beetles, could cope given this calculated minimal limit.

**Sensitivity formula and receptor diameters**

The impact on aperture and the limiting factor of diffraction in small facets has been described, but the photoreceptor itself has not yet featured directly as part of this consideration. As light perceiving structures, rhabdoms and their dimensions are critical elements in the context of sensitivity, especially in small eyes sensitivity more than any other feature of vision is affecting the quality of photoreception. As long as an eye is not sensitive enough to be able to separate information from noise, high spatial resolution has no benefit at all (Warrant and McIntyre 1993).

In terms of visual perception, the main difference between diurnal and nocturnal activity ranges is the light available at the times of maximal activity. The difference is around 8 log units (Land and Nilsson 2002), the brightest sunshine being about 100 million times brighter than the available light on a moonless night (Warrant 2001). The less available light results in the need of a higher sensitivity in crepuscular and nocturnally active species. The sensitivity in these species is indeed outclassing the sensitivity of diurnally active insects with apposition optics by a factor of 25-30 (diurnal versus nocturnal bees and wasps: Greiner et al. 2004, Greiner 2006) in case of apposition optics and up to factors close to 500-700 (Warrant et al. 2004) in species with large superposition eyes as in the nocturnal dung beetle *Onitis aygulus* or the elephant hawkmoth *Deilephila elpenor*.

In order to determine sensitivity-impacting factors it is worth taking a look at the sensitivity formula (originally formulated by Land 1981) used in the aforementioned calculations, and modified for white light perception by Warrant & Nilsson (1998):

\[
S_w = \left(\frac{\pi}{4}\right)^2 A^2 \left(\frac{d}{f}\right)^2 \left(\frac{k l}{2.3 + k l}\right)
\]

with \(A\): aperture (facet diameter), \(d\): rhabdom diameter, \(f\): focal length, \(k\): absorption coefficient (0.0067 \(\mu m^{-1}\) for invertebrates) and \(l\): rhabdom length.
The formula reveals the sensitivity impacting factors aperture, rhabdom diameter, focal length and length of the rhabdom and emphasizes the importance of each factor.

As discussed briefly in the previous chapters, an increase in aperture will increase sensitivity. Indeed many nocturnal species have larger facets than their diurnal counterparts and in general possess larger eyes (Greiner et al. 2004, 2007; Frederiksen and Warrant 2008). The importance of the factor aperture in this formula becomes even more obvious in the case of superposition optics in which the aperture is increased in relation to the diameter by several facets. This explains the very high sensitivity values for \( S_w \).

The second impacting factor \((d/f)^2\), corresponding to the square of the acceptance angle of the rhabdom \(\Delta\rho\), reveals the portion of the focal length and diameter of the rhabdom. Both, a decrease in focal length and an increase in rhabdom diameter, will result in an increase in sensitivity. Especially the latter is found in apposition optics of nocturnal bees and wasps, allowing these species to gain visual information at lower light intensities (Greiner et al. 2004, Greiner 2006). Yet, the comparison between superposition and apposition (see above) reveals that the increase in rhabdom diameter cannot reach the sensitivities gained by the superposition process. However, an increase in rhabdom diameter is also thinkable in small compound eyes as an adaptation to the very limited, small amount of space, keeping in mind that other space-limiting factors on cellular level might prevent an unlimited increase of the diameter of the rhabdom.

However, the increase of sensitivity gained by a larger acceptance angle \((\Delta\rho = d/f)\) is only possible at the cost of spatial resolution. Resulting from this, a smaller rhabdom diameter can increase spatial resolution, but has drawbacks in terms of sensitivity. Additionally, rhabdoms with a diameter below 2 µm cannot be explained by geometrical optics (total internal reflection) as the rhabdoms act as wave-guides in case the diameter is close to the dimensions of the wavelength (van Hateren 1989, Snyder 1979). As a result part of the light is propagating outside the wave-guide. In case of the presence of pigment granules, surrounding the rhabdom, this proportion of the light is ending up absorbed and therefore lost in terms of detection by the rhabdom.
Summing up, one can conclude that different options exist to adapt an eye to different light levels, but that these adaptations will not be possible without drawbacks in other respects. The complexity of the consequences of any adaptational change reveals that in case of an optimally adapted eye of a certain size and to a certain need, the parameters had to evolve successively and become one by one adjusted to their new requirements. Therefore an investigation into small compound eyes is not only of functional interest, but also to understand how nature deals with the described complexity in face of the limited space to operate under in the tiny eyes.

1.5 Earlier studies on lepidopteran compound eyes

As mentioned before both apposition and superposition optics occur in the Lepidoptera. Dominating are the superposition eyes in the nocturnal lepidopterans ("moths"), whereas afocal apposition optics are restricted to the eyes of some diurnal butterflies (Warrant et al. 2003). However, apposition optics, as yet unknown whether focal or afocal, are also found within more basal diurnal active groups of Lepidopterans like, for instance the Adelidae and Zygenidae (Ehnbohm 1948, Warrant et al. 2003).

In the following a short review of some earlier studies on the lepidopteran compound eye shall be given in order to display the focus and the amount of previous works in relation to taxonomic groups and the body sizes of the investigated species. An additional focus will be placed on annotations found within the mentioned works in relation to uncommon eye designs, which so far have largely been neglected in the recent literature.

Lightmicroscopical studies

Besides some descriptions of lepidopteran compound eyes of single species in the early to mid 20th century (Johnas 1910, Bugnion and Popoff 1914, Nowikoff 1931, Yagi 1953) three major comprehensive reviews including a broader systematically spectrum of lepidopteran compound eyes were published by Ehnbohm (1948), Tuurala (1954) and Yagi and Koyama (1963).

Ehnbohm (1948) focused primarily on the anatomy and histology of the central nervous system, but also discussed "peripheral sense organs connected with it".
He investigated 165 different lepidopteran species, but focused in his description of the compound eyes on single groups of interest. In addition to this he also described apposition eyes for groups (e.g., *Adelidae*, *Zygenidae*, *Sesiidae* and *Syntomidae*) that are not considered related to the true butterflies (Ehnbohm 1948). Furthermore, besides the regular apposition and superposition optics, he determined a third type of eyes, referred to as "superposition type, but of a simpler kind", whose key features were an extended rhabdom, occupying one third to one half of the length of the ommatidium, and nearly touching the apex of the crystalline cone. He differentiated this type of eyes from apposition optics by the shape of the cone, which was more extended in this "simpler superposition kind of eye". Ehnbohm furthermore noted the similarity of this optical type to one investigated caddies-fly (Trichoptera) species, suggesting that this type also occurred in the sister group of the Lepidoptera.

Tuurala (1954) investigated 88 lepidopteran species of different families by light microscopy and classified different subtypes of ommatidia on the basis of their morphological characters, like the construction of the cornea, shape of the crystalline cone, position of the nuclei of the retinula cells, construction of the retinula, the shape of the basal retinula cell and the kinds of cells harbouring pigment granules. In addition to the regular eye types (apposition and superposition) Tuurala described also atypical superposition eyes, found only in day-active species. The main characteristic of the "atypical superposition type", according to Tuurala, was that the rhabdom was in direct contact with the crystalline cone (Tuurala 1954). All eyes had in common that they were found in species of small body size and Tuurala noted that a detailed description of three eyes (of the seven eyes found for this type) was not possible by light microscopical methods due to their small sizes.

The number of investigated lepidopteran species by Ehnbohm and Tuurala was even excelled by Yagi and Koyama (1963). According to their species list in total more than 400 species were included in their investigation. Unlike Ehnbohm and Tuurala, the authors took measurements, allowing them (and readers of their work) to draw functional conclusions from their quantitative data, which were based on morphological descriptions made by light microscopy. The two Japanese investigators present for a large number of species total lengths
of the ommatidia and ratios of the components of the dioptric apparatus and the retinula. Yagi and Koyama furthermore correlated eye size with body size and showed that a positive correlation existed between both parameters. Additionally their work revealed a sexual dimorphism in eye size: generally the eyes of males were larger than those of the females.

Limited by their light microscopical methodologies all of these earlier investigators, like Ehnbohm, Tuurala and Yagi and Koyama, were restricted to histological descriptions and crude measurements. Detailed ultrastructural descriptions of rhabdom diameters, cell numbers of retinula cells and pigment cells are only possible with instruments that allow for higher magnifications combined with improved resolution. For this reason more detailed descriptions could not be carried out until one or two decades later, when electron microscopes became available.

**Ultrastructural descriptions (TEM)**

In relation to ultrastructural descriptions of insect compound eyes generally, the list of descriptions of lepidopteran compound eyes up to date is restricted to a relatively small number of publications (Table.1). Except for *Ectoedemia argyropeza* (Zeller, 1839; see chapter 1.6) and *Tineola bisselliella* (Hummel, 1823), all of the investigated species were of medium or large size (smallest species *Tineola bisselliella*, wingspan 9-16mm (Kuchlein and Bot 2010)). The majority of the descriptions dealt with species with superposition optics (24); much fewer dealt with apposition optics (5). With the sphingids *Macroglossum pyrrhosticta* (Butler, 1875) and *Cephenodes hylas* (Linnaeus, 1771), the noctuid *Phalaenoides tristifica* (Hubner, 1811) and the skipper butterflies (Hesperiidae) also the eyes of some diurnal lepidopterans with superposition optics were described (Horridge et al. 1972, Horridge et al. 1977, Fernandez-Moran 1958).

Taken all the information of both, light microscopically and ultrastructural studies, together and displayed on a systematic tree for which taxa information on compound eyes are known (Fig. 5), it is possible to illustrate the general lack of knowledge concerning the eyes, especially the eyes’ ultrastructural organization of the basal groups. By including information about the smallest known body sizes (expressed as wingspan (WS) or length of the front wing
(FW) in Fig. 5) of the presented taxa, it is possible to illustrate the lack of knowledge about compound eyes in species of small Lepidoptera (= wingspans below 10mm) as only one description of a nepticulid species was published by Honkanen and Meyer-Rochow (2009).

Table 1. Earlier ultrastructural descriptions on lepidopteran compound eyes, listed according to systematic position. The systematic classification is according to Kristensen et al. (2007).

<table>
<thead>
<tr>
<th>family</th>
<th>valid species name</th>
<th>source</th>
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<tbody>
<tr>
<td>Nepticulidae</td>
<td><em>Ectoedemia argyropeza</em></td>
<td>Zeller, 1839</td>
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<td></td>
<td></td>
<td>Honkanen &amp; Meyer-Rochow 2009</td>
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<tr>
<td>Tineidae</td>
<td><em>Tineola bisselliella</em></td>
<td>Hummel, 1823</td>
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<td>Faucheux 1987</td>
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<td>Plutellidae</td>
<td><em>Plutella xylostella</em></td>
<td>Linnaeus, 1758</td>
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<td>Wang &amp; Hsu 1982</td>
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<td>Tortricidae</td>
<td><em>Adoxophyes orana</em></td>
<td>Fischer von Roélerstamm, 1834</td>
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<td>Hämmerle &amp; Kolb 1987</td>
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<tr>
<td>Pyralidae</td>
<td><em>Amyelois transitella</em></td>
<td>Walker, 1863</td>
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<td></td>
<td><em>Ephesia kuehniella</em></td>
<td>Zeller, 1879</td>
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<td>Horridge &amp; Giddings 1971</td>
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<td></td>
<td><em>Galleria mellonella</em></td>
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<td></td>
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<td>Stone &amp; Koopowitz 1976</td>
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<td>Crambidae</td>
<td><em>Acentria ephemereella</em></td>
<td>Denis &amp; Schiffermüller, 1775</td>
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<td>Lau et al. 2007</td>
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<td>Hedylidae</td>
<td><em>Macrosoma sp.</em></td>
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<td>Bombycidae</td>
<td><em>Bombyx mori</em></td>
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<td></td>
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<td>Eguchi 1962</td>
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<td>Saturniidae</td>
<td><em>Antheraea polyphemus</em></td>
<td>Cramer, 1776</td>
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<td>Anton-Érxleben &amp; Langer 1988</td>
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<tr>
<td>Sphingidae</td>
<td><em>Cechenena lineosa</em></td>
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<td>Eguchi 1982</td>
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<td><em>Cephonodes hylas</em></td>
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<td><em>Deilephila elpenor</em></td>
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<td><em>Macroglossum pyrrhosticta</em></td>
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<td>Eguchi 1982</td>
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<td>Hesperiidae</td>
<td><em>Epargyreus sp.</em></td>
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<td></td>
<td><em>Hesperilla ornata</em></td>
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<td><em>Parnara guttata</em></td>
<td>Bremer &amp; Grey, 1852</td>
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<td>Shimohigashi &amp; Tominaga 1986</td>
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<td><em>Trapezites symmomus</em></td>
<td>Hübner, 1823</td>
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<td></td>
<td>Horridge et al. 1972</td>
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<tr>
<td>Papilionidae</td>
<td><em>Papilio aegus</em></td>
<td>Donovan, 1805</td>
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<td>Pieridae</td>
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<td>Kolb 1985</td>
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<td><em>Danaus plexippus</em></td>
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<td><em>Pararge aegeria</em></td>
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<td></td>
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<td></td>
<td><em>Orgyia antiqua</em></td>
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<td></td>
<td></td>
<td>Lau &amp; Meyer-Rochow 2007</td>
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<td></td>
<td><em>Phalaenoides tristifica</em></td>
<td>Hubner, 1811</td>
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<td></td>
<td>Horridge et al. 1977</td>
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<td></td>
<td><em>Spodoptera exempta</em></td>
<td>Walker, 1856</td>
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<td>Meinecke 1981</td>
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In addition, Figure 5 demonstrates that although information and suggestions about the life style and optical type for the most basal moth groups have been reported (see Yack et al. 2007), detailed combined histological and
ultrastructural descriptions of the compound eyes of these species are still lacking.

Figure 5. Cladogram of lepidopteran superfamilies and superfamily assemblages modified after Kristensen et al. (2007). Markings indicate the presence of light microscopical (+) and ultrastructural information (number of studied species) on the compound eyes of specimens of this taxon. Furthermore the smallest known front wing lengths (FW) or wingspans (WS) in millimetre are presented as representation of small body sizes found in these taxa. Values, as far as available, are taken from Kristensen (1998), Davis and Gentili (2003), Kuchlein and Bot (2010) and Scoble (1990).
1.6 Rationale and objective of the study

As elaborated in the introduction, a multitude of optical factors are theoretically affecting an unlimited size decrease of the compound eye and the results found in scarab beetles as well as the theoretically calculated minimal limit (Meyer-Rochow and Gál 2004) allows one to suspect that size-related adaptations should also be present in other taxa possessing superposition optics.

Besides parameters impacting on the optics, also parameters that depend on the cellular level (cell sizes and numbers, cell positions and shapes) might additionally influence eye size reductions. First of all, the question arises how far retinula cells can be reduced in size, as enough space must be available to store the nucleus and other essential cell organelles in them. Screening pigment granules, as one example, are used for adjusting the amount of light entering the eye and the photoreceptor and for that reason cannot be dismissed. Mitochondria, as another, representing the powerhouses of the cell need to be present in sufficient density. A comparison of eyes of different lepidopterans of different body- and eye sizes reveals that the pigment granules possess similar diameters in a range of 0.4-0.7 µm irrespective of body- and eye-size (Meinecke 1981, Kolb 1985, Hämmerle and Kolb 1987, Lau et al. 2007, Lau and Meyer-Rochow 2007, Meyer-Rochow and Lau 2008; Honkanen and Meyer-Rochow 2009). If the pigment granules cannot be reduced in size, their total number should decrease in order to adapt to the reduced available space. The same holds true for mitochondria. But how far a size reduction of these organelles is possible without impacting critically on the dark-light adaptational process and cell survival remains an unanswered question. It seems therefore that also at the cellular level factors are operating that prevent unlimited size reductions and, therefore, exert some control on the functional designs of small compound eyes.

The descriptions of “atypical superposition eyes“ (Tuurala 1954) and “superposition eyes of a simpler kind” (Ehnbohm 1948) suggested that certain adaptations were to be found in small lepidopteran eyes. The first evidence for this, which included also ultrastructural methods, came from the study by Honkanen and Meyer-Rochow (2009), so far the only investigation on the functional anatomy and ultrastructure of the compound eye of a tiny nepticulid,
namely *Ectoedemia argyropeza* (Zeller, 1839), a parthenogenetic moth with a wingspan of 5-6 mm. A modified superposition eye was found, with characteristics of an apposition eye, since the clear zone, seen as an essential character for a superposition eye (Land 1981), was missing. The rhabdom in this small moth was hourglass-shaped and separated into a proximal and a distal part by a narrow desmosomal connection. This was a new finding for the Lepidoptera in particular and also a rather unusual rhabdom shape for insects in general.

A similar design with a separation of a dorsal and proximal rhabdom was known from the sphingid moth *Cechenena lineosa* (Walker, 1856), but in the latter species only two retinula cells were responsible for the rhabdom at the distal end (Eguchi 1982), contrary to the seven retinula cells which are participating in *Ectoedemia argyropeza*. These results on *Ectoedemia argyropeza* provided first impressions of changes in the eyes of miniaturized species of Lepidoptera and it therefore seemed of considerable interest to compare these findings with eyes of additional species of similar size or even smaller built.

The main goals of the present work are thus A) to test the theoretical approach of Meyer-Rochow and Gál (2004) by describing ultrastructural and functional designs of eyes of different species of tiny lepidopterans B) to describe possible functional adaptations manifest in these eyes and C) to carve out impacting factors, limiting the miniaturization of eyes by a comparative investigation of eyes of different sizes. The focus is on eyes of lepidopteran species (chapter 2.2; 2.3; 2.4), but as a comparison especially for question C) one of the smallest known species with apposition compound eyes, a small parasitic wasp (Hymenoptera) is included in the investigation (chapter 2.1).
Chapter 2: Results

2.1 How small can small be: The compound eye of the parasitoid wasp *Trichogramma evanescens* (Westwood, 1833)(Hymenoptera, Hexapoda), an insect of 0.3- to 0.4-mm total body size.
How small can small be: The compound eye of the parasitoid wasp *Trichogramma evanescens* (Westwood, 1833) (Hymenoptera, Hexapoda), an insect of 0.3- to 0.4-mm total body size

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Abstract

With a body length of only 0.3–0.4 mm, the parasitoid wasp *Trichogramma evanescens* (Westwood) is one of the smallest insects known. Yet, despite its diminutive size, it possesses compound eyes that are of oval shapes, measuring across their long axes in dorsoventral direction 63.39 and 71.11 µm in males and females, respectively. The corresponding facet diameters are 5.90 µm for males and 6.39 µm for females. Owing to the small radii of curvature of the eyes in males (34.59 µm) and females (42.82 µm), individual ommatidia are short with respective lengths of 24.29 and 34.97 µm. The eyes are of the apposition kind, and each ommatidium possesses four cone cells of the eucon type and a centrally fused rhabdom, which throughout its length is formed by no more than eight retinula cells. A ninth cell occupies the place of the eighth retinula cell in the distal third of the rhabdom. The cone is shielded by two primary and six secondary pigment cells, all with no apparent extensions to the basement membrane, unlike the case in larger hymenopterans. The regular and dense packing of the rhabdoms reflects an effective use of space. Calculations on the optics of the eyes of *Trichogramma* suggest that the eyes need not be diffraction limited, provided they use mostly shorter wavelengths, that is, UV light. Publications on the visual behavior of these wasps confirm *Trichogramma*'s sensitivity to UV radiation. On the basis of our findings, some general functional conclusions for very small compound eyes are formulated.

Keywords: Vision, Photoreceptor, Diffraction, Retina, Miniaturization

Introduction

The design of compound eyes and the balance between different optical parameters have been discussed and reviewed in several studies (Snyder, 1979; Land, 1981; Warrant & McIntyre, 1993; Land & Nilsson, 2002). It is known that for an eye of a given size, sensitivity and spatial resolution are in conflict with each other, and a distinction between superposition eyes (useful under dim light conditions and characterized by a wide pigment-free clear zone between dioptric and light-perceiving structures) and apposition eyes (with no such clear zone and typical of diurnally active species) has been made ever since Exner (1891) described these two eye types. In the apposition eye, large facets, for example, can lead to an increase of the eye’s overall sensitivity but will decrease spatial resolution if the interommatidial angle is also large; smaller facets and the resultant smaller interommatidial angles, on the other hand, would favor higher resolution but then allow less light to enter each facet and, thus, lead to a reduction in sensitivity.

For butterflies, Rutowski et al. (2009) were able to show that an increase in the size of the eye was accompanied by an increase in sensitivity and resolution. The large radius of the eye allowed for a simultaneous decrease of the interommatidial angle (with the result of improved resolution) and large facet diameters (leading to increased sensitivity). Yet, how small insects with body sizes below 1 mm cope with the demands for optimal sensitivity and acuity in face of the extremely limited space for the eyes on the head are questions that to date have not been examined.

One consequence of reducing the size of the head in an insect is that the space for the eyes decreases as well and the question arises how far the size reduction of an eye and its components can be pushed before the reduction impacts on the function of the eye. As smaller facets are increasingly limited by diffraction (Barlow, 1952), one would expect a certain minimal facet size to exist that cannot be undercut. As the eye approaches this lower limit in size, it should be accompanied by a reduction in the total number of facets. However, one also needs to consider that the dioptric apparatus must have a certain size to focus (or at least channel) the impinging light on the underlying rhabdom and that the microvillar membrane fringes of the latter have to be sufficiently voluminous to house an adequate amount
of visual pigment necessary to intercept incoming photons. How and to what extent in very small eyes neighboring facets are insulated from each other by screening pigment sleeves and how thin and curved a cornea can be made in order to serve equally well as a dioptic and a protective structure are further questions. Thus, in order to understand more fully the general features and functional limitations of compound eyes, we felt a look at the optical components, anatomical organization, and ultrastructure of very small eyes was needed. In this first paper on miniaturized compound eyes, we focus on the eye of the minute wasp Trichogramma evanescens Westwood (1833).

With a body size of only 0.3–0.4 mm, the parasitoid T. evanescens is one of the smallest insects known to possess compound eyes. Because of its use as a biological pest control agent against crop-impacting moths and butterflies, commercial rearing has made the species easily obtainable, and information on its biology and life cycle has been available for several decades (Schulze, 1926; Salt, 1937; Quednau, 1958; Romeis et al., 2005). Since the females of this wasp react to the pheromones of a variety of lepidopteran species, special attention was given to chemosensory features of Trichogramma (e.g., Lewis et al., 1971; Noldus & van Lenteren, 1985). In contrast, not much is known about this species’ visual capacity and the role that vision plays in its life. Altogether missing is information on the anatomy and ultrastructure of its tiny eye.

Quednau (1956) assumed that visual cues could be used by T. evanescens for host detection in the near field, but so far, this has not been confirmed. On the other hand, the functionality of the eyes of T. evanescens was demonstrated by Quednau (1958), who showed that the species reacted positively phototactically and increased its activity during illumination, and by Brower and Cline (1984), who reported a stronger attraction to black (i.e., UV-A) than to white light. Romeis et al. (1998), furthermore, described a selective response to colored sticky traps that differed for males and females of various representatives of the genus Trichogramma. This suggests that there could be sexual differences in the optical devices of males and females.

Materials and methods

Transmission and scanning electron microscopy

T. evanescens wasps were purchased from a commercial distributor (Biologische Beratung, Berlin, Germany) and raised under natural day/night rhythm. Specimens, shielded from direct sunlight, were obtained from parasitized lepidopteran eggs that had been glued to the head of specimen to be examined and split in half on top pieces of cardboard. Following determination of the sexes, the heads of specimens to be examined were decapitated and split in half during daytime hours under room light conditions before being fixed for 1 day in a modified Karnovsky’s (1965) fixative solution, containing 2% paraformaldehyde and 2.5% glutaraldehyde, buffered with 0.1 M cacodylate (pH 7.4).

After a 2-h period of postfixation in 2% OsO₄ solution, also buffered with 0.1 M cacodylate (pH 7.4), the samples were rinsed 3 times in the same buffer. The samples were dehydrated in a graded series of ethanol before being passed through different acetone/Epon mixtures (3:1, 1:1, 1:3, pure Epon). Pure Epon-812 resin was used for final embedding and the samples were then polymerized at a temperature of 60°C for 3 days. For light microscopy (LM), semithin sections were cut on an ultramicrotome (RMC, Boeckeler Instruments Inc., Tucson, AZ) with a glass knife and stained with 0.5% aqueous solution of toluidine blue on a hot plate for 30 s. Ultrathin sections were cut with a diamond knife and picked up on formvar-coated copper slot grids. The ultrathin sections were stained with lead citrate and 2% aqueous uranyl acetate for 20 min each (modified after Reynolds, 1963). The sections were mainly examined under Zeiss EM 900 and Jeol JEM-1011 transmission electron microscopes (TEM), operated at 80 KV. The TEM was calibrated with a calibration grid (Plano S104) before any measurements commenced. For scanning electron microscopy (SEM), osmium-fixed heads were dehydrated in a graded series of ethanol and then subjected to critical-point drying (EMITECH 850, Emitech Ltd., Ashford, UK). The dried samples were coated with a layer of approximately 15-nm gold in a sputter coater (Quorum Q150T S, Quorum Technologies Ltd., East Grinstead, UK) and observed under a JEOL JSM-5900 SEM, operated at 20 KV.

Morphometric analyses

SEM was used to determine dorsoventral and anterior–posterior lengths of the eye (i.e., height and width, respectively), total number of ommatidia per eye, facet diameters, and diameters of the ocelli. Longitudinal sections for LM were used for measurements of ommatidial lengths, radii of curvature of eye, and corneal facets as well as interommatidial angles. The radius of curvature of the eye was measured on the broadest anterior–posterior extension (eye width) of the eye, in sections done in dorsoventral direction, by laying a circular measurement tool over the eye surface in the image analysis software. The interommatidial angles were measured at the point of intersection of the normals to the eye surface through the ommatidial axes of central ommatidia. TEM micrographs of cross-sections were used to measure rhadom diameters, shapes, and diameters of pigment granules of primary pigment cells (PPC) and secondary pigment cells (SPC) as well as retinula cells. Measurements of rhadom diameters were taken at the distal tip of the rhadom. Longitudinal sections were used to determine cone length, corneal thickness, and shapes and positions of the pigment granules within the retinula cells.

Five eyes of each sex were used for measurements under the SEM. For examinations involving LM and TEM, five eyes of either sex were used. All measurements were gathered by image analysis software (Image J, Rasband, W.S., U.S. National Institutes of Health, Bethesda, MD) and based on an identical number of objects per specimen to avoid a weighting in the statistics later.

Statistical analyses

In each set of data, normality of distribution was tested with the Shapiro–Wilk normality test. Comparisons between both sexes were performed with Mann–Whitney’s U Test for datasets that were not normally distributed, while the independent t-test was chosen for normally distributed data. The analytic software SPSS was used for all of the statistical tests.

Results

General organization of the eye

All measurements and observations reported in this paper are based on light-adapted specimens fixed under room light conditions during the day. A separate paper is planned that will deal specifically with light/dark adaptational changes in the eye of T. evanescens.

The reddish-colored compound eyes of T. evanescens are of an oval shape (Fig. 1a) and show a significant sexual dimorphism. Females have larger eyes than males; typical of the eye of a female
are an eye height (dorsal–ventral length) of 71.11 μm, an eye width (anterior–posterior length) of 59.09 μm, and contains 128 facets of a relatively uniform diameter of 6.39 μm (Table 1). The eyes of the males are similarly shaped, but of smaller size (eye height of 63.39 μm and eye width of 45.47 μm), and consist of only 106 ommatidia with facet diameters of 5.90 μm. Sementin sections revealed a smaller radius of curvature of the eye in males (34.6 μm) than in females (42.8 μm). The facets are basically of hexagonal shape, but the strong curvature of the facets leads to an impression of somewhat circular outlines (Fig. 1b). In the eyes of both sexes, regularly arranged interfacetal hairs (up to 6–7 μm long and 0.45–0.70 μm thick) are present (Fig. 1c). Besides compound eyes, three ocelli are present, two smaller dorsally placed ones with diameters

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Table 1. Measurements of external and internal features of the compound eyes of male and female Trichogramma evanescens, fixed during the day under room light conditions.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Female</th>
<th>Male</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eye height</td>
<td>μm</td>
<td>71.11±3.52</td>
<td>63.39±3.39</td>
<td>*</td>
</tr>
<tr>
<td>Eye width</td>
<td>μm</td>
<td>59.09±2.76</td>
<td>45.47±1.90</td>
<td>*</td>
</tr>
<tr>
<td>Number of facets</td>
<td></td>
<td>128±6.51</td>
<td>105.8±4.02</td>
<td>*</td>
</tr>
<tr>
<td>Radius curvature eye (outside)</td>
<td>μm</td>
<td>42.82±0.92</td>
<td>34.59±1.91</td>
<td>***</td>
</tr>
<tr>
<td>Radius curvature cornea (inside)</td>
<td>μm</td>
<td>4.59±0.27</td>
<td>3.97±0.33</td>
<td>***</td>
</tr>
<tr>
<td>Radius curvature cornea (outside)</td>
<td>μm</td>
<td>6.39±0.82</td>
<td>6.43±0.85</td>
<td>NS</td>
</tr>
<tr>
<td>Facet diameter</td>
<td>μm</td>
<td>6.39±0.33</td>
<td>5.90±0.41</td>
<td>***</td>
</tr>
<tr>
<td>Length ommatidium</td>
<td>μm</td>
<td>34.97±3.88</td>
<td>24.29±1.58</td>
<td>***</td>
</tr>
<tr>
<td>Interommatidial angle</td>
<td>deg</td>
<td>9.98±0.57</td>
<td>12.31±2.15</td>
<td>***</td>
</tr>
<tr>
<td>Length dioptric apparatus</td>
<td>μm</td>
<td>10.26±0.98</td>
<td>8.61±0.68</td>
<td>***</td>
</tr>
<tr>
<td>Maximum thickness cornea</td>
<td>μm</td>
<td>2.25±0.29</td>
<td>1.62±0.33</td>
<td>***</td>
</tr>
<tr>
<td>Cone length</td>
<td>μm</td>
<td>7.88±0.71</td>
<td>6.93±0.78</td>
<td>NS</td>
</tr>
<tr>
<td>Maximum cone diameter</td>
<td>μm</td>
<td>5.51±0.52</td>
<td>4.58±0.50</td>
<td>***</td>
</tr>
<tr>
<td>Rhabdom diameter</td>
<td>μm</td>
<td>1.67±0.12</td>
<td>1.41±0.17</td>
<td>***</td>
</tr>
<tr>
<td>Diameter of rhabdom microvilli</td>
<td>nm</td>
<td>62.22±4.02</td>
<td>58.28±5.35</td>
<td>***</td>
</tr>
<tr>
<td>Pigment granule diameter (PPC)</td>
<td>μm</td>
<td>0.44±0.04</td>
<td>0.46±0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Pigment granule diameter (SPC)</td>
<td>μm</td>
<td>0.33±0.03</td>
<td>0.34±0.02</td>
<td>NS</td>
</tr>
<tr>
<td>Pigment granule diameter</td>
<td>μm</td>
<td>0.28±0.04</td>
<td>0.26±0.03</td>
<td>***</td>
</tr>
</tbody>
</table>

Data are expressed as means ± s.e.; NS = not significant. *P < 0.05, **P < 0.01, ***P < 0.001 in the independent t-test for normally distributed data and Mann–Whitney U Test for datasets that were not normally distributed.

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Fine structural organization

Dioptric apparatus and primary pigment cells

In both sexes, the cornea is a transparent biconvex lens that consists of horizontally stacked microvilli, typical for cuticle-covered eucharthopods. In the female, the cornea is approximately 2.25 μm thick with an external radius of curvature of 4.63 μm, while in the male, it is 1.62 μm thick with a radius of curvature of 3.97 μm. The inner radius of curvature of the cornea does not vary between the sexes and measures approximately 6.40 μm. Due to the biconvex profile of the facets, the corneal thickness is least in the interommatidial areas. Beneath the corneal lens of each ommatidium, we find two pigmented corneagenous cells, the so-called PPC (Fig. 4a–c). Their moderately osmiophilic cytoplasm contains pigment granules of high electron density that measure approximately 0.46 μm in
The cone is of the eucone type (cone matrix located inside the cone cell body); it has an average length of 7.88 μm and a maximum width of 5.51 μm in the female (corresponding values for males are 6.93 μm and 4.38 μm) and is formed by four cone cells, the so-called Semper cells. The cone matrix is rich in ribosomes and tiny particles of diameters below 2 nm that most likely consist of glycogen. Bundles of microtubuli, stabilizing the cone, are regularly encountered in the peripheral less-condensed cytoplasmic sheath of the cone cells when sectioned longitudinally. Furthermore, a ring of tiny, osmiophilic spherical structures lines the outer border of the cone as seen in cross-sections (Fig. 4b). The nuclei of the cone cells, characterized by high concentration of heterochromatin, can be found in the most distal region of the cone cells just below the cornea (Figs. 3 and 4c).

Proximally, the cone tapers down to the diameter of the distal tip of the rhabdom. In the transition zone between the cone and subjacent rhabdom, the four cone cells separate and each of them sends a long process running proximally between the retinula cells (infraretinular space) and alongside the rhabdom all the way down to the basement membrane, where the processes end in four swollen tips just above the basement membrane (Fig. 4c).

Secondary pigment cells

Six accessory pigment cells, here termed SPC, are present per ommatidium. Unlike the situation in other more typical apposition eyes, the SPC in *Trichogramma evanescens* do not accompany the retinula cells down to the basement membrane and can thereby not be classified as interommatidial pigment cells.

Measuring only 0.33–0.34 μm in diameter, the pigment granules of the SPC are of a significantly smaller size than those of the PPC, but with regard to optical density or chemical composition (judging by their coloration), there seems no difference between the granules of the two cell types. Granule diameters were the same in males and females. The nuclei of the PPC can be found near the cornea, close to the plane of the widest extension of the cone.

Pigment granules with diameters similar to those of the PPC are numerous below the basement membrane. The same holds true for pigment shielding the eye from the rest of the head (Fig. 2a). In both areas, tracheoles can be found that are missing in the eye itself. All pigments exhibit a reddish color in toluidine blue–stained semithin sections.

Retinula cells and rhabdom

The rhabdom, with a length of approximately 25 μm in females and 14 μm in males, extends from the proximal tip of the cone to just above the basement membrane. Longitudinal sections of the eye of *T. evanescens* reveal that the nuclei of the retinula cells are not situated in one plane but make use of almost the entire length of the retinula cells, from the distal end of the rhabdom down to the lower third. No retinula cell nuclei, tracheoles, or basal pigment cells were detected in the region above the basement membrane. Two retinula cells of neighboring ommatidia rarely both have a nucleus at the same level, at any given plane (Fig. 2b). In fact, the limited space is used more effectively by alternating the positions of the nuclei in transverse as well as longitudinal planes. This results in somewhat “wavy” interdigitating outlines of neighboring retinula cells.

Most distally, where the rhabdom has its widest expansion, the nuclei of up to three retinula cells per ommatidium can be found in one horizontal plane, but further proximally, owing to the diminishing radius of the ommatidium, the number of nuclei seen at any given plane decreases. Typical cell organelles of the retinula cells, like mitochondria of the cristae type, are predominantly found close to the cell borders (e.g., Fig. 5c).

The centrally fused columnar rhabdom of *T. evanescens* is formed throughout its entire length by eight retinula cells, all of them contributing rhabdomeres. In agreement with the finding of nine axons per axon bundle, a ninth retinula cell has been identified, which participates in the formation of the rhabdom over two third of its length, replacing the eighth retinula cell.

At the distal end of the rhabdom, the microvilli of the rhabdomeres of two opposite sets of two neighboring retinula cells each (involving four retinula cells) are aligned in the same direction. The microvilli of
the rhabdomeres of the other four retinula cells are also aligned in parallel but at right angle to the microvilli of the aforementioned two sets of rhabdomeres; thus, transverse sections through rhabdoms at this level show microvilli running in just two orthogonal directions (Fig. 5a). These retinula cell duplets are separated by the four proximal projections of the cone cells that accompany the retinula cells down to the basement membrane. A divergent position of the cone cell projections can be found sporadically in some rhabdoms of the males (Fig. 5c). In the distal part, just below the cone, the rhabdom has its greatest diameter, measuring 1.67 μm in the female and 1.41 μm in the male. Apart from the difference in rhabdom diameter, there was also a significant sexual difference with regard to the diameter of the microvilli (62 nm in the female vs. 58 nm in the male).

The rhabdom is surrounded by cisternae of the smooth endoplasmic reticulum (ER). This so-called perirhabdomeric or “palisade” ER becomes enlarged as the eye becomes dark adapted (not shown in this paper). In eyes fixed under room light, the development of perirhabdomeric cisternae is restricted to a pigment-free sleeve of approximately 0.3–0.7 μm width around the rhabdom. Pigment granules of the retinula cells, measuring about 0.28 μm in diameter in the female and 0.26 μm in the male, are most abundant close to the cisternae. Considering the elongated ovoid shape of the pigment granules, the mathematically calculated significance may have been influenced by unavoidable inconsistencies in the measurements. As longitudinal sections show (Fig. 4d and 4e), the pigment granules are oriented predominantly lengthwise along the perirhabdomeric ER cisternae parallel to the ommatidial axis.

Considering the presence of an irregular ninth retinula cell, it is possible to number and identify individual retinula cells (cf., Fig. 3d and 3e). Starting with the ninth cell, the retinula cells are counted from one to eight in the direction of the adjacent duplet partner irrespective of whether it is on the right or on the left (thus in a direction away from the cone cell projection). The ninth cell starts in the upper third of the rhabdom, replacing the eighth cell. The Eighth cell is initially displaced to the periphery and its axon can later be found near the cone cell projection (Figs. 3d, 3e, and 5c). The different alignment of the microvilli of the ninth cell’s rhabdomere, compared with the eighth cell, leads to a situation, in which three neighboring rhabdomeres all have their microvilli oriented in the same direction (Fig. 5d), thus forming a rhabdomeric triplet.
In some rhabdoms of the eyes of females, and even more commonly in the males, an irregular rotation in the orientation of the microvilli of the fourth cell (oppositely placed to the ninth cell) may be seen, but such irregular rhabdoms are not restricted to particular regions of the eye. In situations like these, single cells with identical microvillar orientations separate two opposite rhabdomeres that have their microvilli aligned at right angle to those of the two cells occupying the space between the two triplets (Fig. 5c). Arrangements intermediate between this and the arrangement of four orthogonally arranged cell duplets (see above) can also be found (Fig. 6b).

Further proximally, the retinula cells 4, 3, and 7 are the first to diminish in size and turn into axons. In this lower part of the rhabdom, a dislocation of some retinula cells can be observed, leading to minor shifts in the microvillar alignments (Fig. 6a). The next two retinula cells changing into axons are those placed opposite to each other but with identical microvillar orientations, namely retinula cells 1 and 5; the ninth retinula cell follows. At the very end of the rhabdom, only retinula cells 2 and 6, facing each other, remain. They are the longest retinula cells and contribute to the rhabdom from its most distal to its most proximal end (Fig. 6c).

At the basement membrane, axon bundles, each containing nine fibers, leave the ommatidium through separate holes in the thin and structurally homogenous basement membrane. In each bundle, usually two larger fibers are observed to be enveloped by three smaller fibers on either side with one small fiber in the center of the axon bundle (Figs. 3 and 4f). The nine axonal processes are surrounded by glial cells. Pigment granules and mitochondria were noticed in some axons.

Dark/light adaptational changes

Although we intend to describe in detail structural and ultrastructural responses of the eye of *T. evanescens*, caused by an exposure to light and darkness, in a separate paper, it may suffice to mention here that photomechanical events do occur. We observed displacements of the osmiophilic screening pigment granules along the ommatidial axis. This pigment migration reduces the amount of light reaching the rhabdom upon an exposure to bright light. Additionally, we noticed a progressive formation of perirhabdomeric ER cisternae around the edge of the rhabdom, accompanying an adaptation to lower ambient light levels. Total lengths of the ommatidia or interommatidial angles were apparently not affected by exposures to different environmental brightnesses.

Discussion

General remarks

Compared with the compound eyes (just termed eyes in the following) of other common nonparasitic Hymenoptera, the eyes of *T. evanescens* are rather small. With a dorsoventral height of 60–70 μm and facet numbers between 100 and 135, they are nearly 1/100 the size of the eye of the largest known hymenopteran, the Japanese giant hornet *Vespa mandarina* ssp. *japonica* (whose bean-shaped eyes extend dorsoventrally over 5.8 mm and consist of at least 8000 facets), and approximately 1/40 the size of the eye of the common wasp *Paravespula vulgaris*, in which the eye contains approximately 3000 facets and the dorsoventral eye height is 2.5 mm (Fischer et al., unpublished). As eye size depends on the available space for the eyes on the head, eye size has to diminish with decreasing body size. A relation between eye size and body size is not only apparent during the postembryonic growth of an insect (Meyer-Rochow & Keskinen, 2003; Keskinen & Meyer-Rochow, 2004) but has also been demonstrated for the adults of various insect species of different body size (e.g., Jander & Jander, 2000; Rutowski et al., 2009).

Barlow (1952) carried out measurements on the eyes of 27 different species of Hymenoptera and showed that there was a relationship between the diameters of their ommatidia and the square root of the height of the eye. *T. evanescens* falls below the size of the smallest species that Barlow included in his study and both size of the eye and facet diameters in *T. evanescens* are smaller than any of Barlow’s data; yet they are in line with Barlow’s general conclusion.

Adaptations of the ultrastructural organization to the small size of the eye

The eye of *T. evanescens* displays a compact and well-organized usage of space. With a total length of only 39.97 μm (24.29 in the males), a single ommatidium of *T. evanescens* is considerably shorter than that found in eyes of larger hymenopteran species (*Apis* drone: 480 μm; Perrelet, 1970), *Formica polyctena* 140 μm (Menzel, 1972).

Space available in one ommatidium is not only limited due to ommatidial length but also restricted by the small facet diameter; yet each ommatidium must provide sufficient space for the retinula cells (including the rhabdom) and other cells, their nuclei, pigment granules, mitochondria, and other cell organelles involved in or influenced by photomechanical adaptations (see summary, Eguchi, 1999).

The uniquely modified arrangement of the nuclei of adjacent retinula cells, dividing up available space in a most efficient way, must be understood as a direct effect of miniaturization in the *Trichogramma* eye. This arrangement allows more ommatidia per eye to be developed and results in more densely packed and larger rhabdoms than if retinula cells and their nuclei were conventionally arranged. The smallest amount of space in the retinula cells is obviously present around the most proximal part of the rhabdom, where no nuclei at all occur and the whole of the small available space is occupied by pigment granules and cell organelles aligned in parallel to the ommatidial axis. The kinds of cell organelles present, their dimensions, and their relative abundance, however, are not unlike those seen in the much larger eyes of the honeybee *Apis mellifera* (Perrelet, 1970; Skrzypek & Skrzypek, 1971). As the size of
the pigment granules is not critically smaller than that seen in larger hymenopterans, it appears obvious that, just as predicted by Meyer-Rochow and Reid (1996), there is a minimal limit for the diameter of screening pigment granules. Whether chemical, physical, or developmental factors are involved in setting the size limit of the pigment granules is still unclear.

Given that a minimum size exists for cell organelles, then retinula cells, too, cannot infinitely be reduced in size, for they need to accommodate not only pigment granules but also nuclei, mitochondria, ER, and other essential organelles. Furthermore, considering the virtually ubiquitous ability of organelles to translocate and cells to change shape during light/dark adaptation in

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**Fig. 5.** Transmission electron micrographs of the rhabdom of *Trichogramma evanescens*. (a) Transverse section of the most distal part of the rhabdom, showing a regular arrangement of four duplets, consisting of two neighboring rhabdomeres with the same microvilli orientation. The duplets are separated by the four cone cell projections (CP). In the cisternae of the endoplasmatic reticulum, enveloping the rhabdom, large bulges can be noticed in the intercellular spaces between the retinula cells in the distal part of the rhabdom (arrows). (b) Oblique sections reveal that the pigment-free extensions of the primary pigment cells (PPC) reach into these bulges. (c) Cross-section through the eye of a male. In the upper third, the ninth retinula cell (white arrow) replaces the eighth cell. Due to the irregular position of the proximal processes of the cone cells (CP), one of which can be seen to the left of the rhabdom, this rhabdom is of a different (not the normal) appearance. To the right side, a neighboring rhabdom is visible, with irregularly orientated microvilli of retinula cell R4. At this level, the eighth cell (R8) had already been replaced by the ninth cell (R9) in this rhabdom. Mitochondria of the cristae type (M) are usually present near the borders of all retinula cells. (d) The ninth cell shows a different microvillar orientation from that of the eighth cell, which is dislocated from the rhabdom in this plane.
Fig. 6. Cross-sections through the basal part of the rhabdom of *Trichogramma evanescens* in more proximal section planes. (a) Cross-section through different planes near the lower end of the rhabdom. In some rhabdoms (RH), the microvilli of the rhabdomeres show a different orientation to each other, so that the adjacent rhabdomeres are not arranged at an angle of 90 degrees to each other anymore (arrows). The dislocated eighth cell (R8), as well as the axons (AX), is positioned between the two cone cell projections (CP) of adjacent rhabdoms. (b) The position of the cone cell projections (CP) facilitates the identification of the changed orientation of the microvilli of one rhabdomere each of the two duplicates in some of the rhabdoms. In these rhabdoms also, the fourth retinula cell (R4) exhibits a changed orientation of its microvilli. (c) At the basal end of the rhabdom, only two cells are facing each other (R2 and R6), surrounded by the axons (AX) of the other retinula cells and the cone cell projections (white arrows).
insect photoreceptors, space and conditions need to exist to allow such adjustments to changing ambient light levels to occur.

Contrary to what has been described in the eyes of other Hymenoptera, the SPC in *T. evanescens* do not reach the basement membrane. Only extensions of the PPC, although free of pigments, were found in between the intercellular spaces of adjacent retinula cells. With regard to the limited space between cones of neighboring ommatidia, nearly all that space is filled by the PPC; the space for the SPC is restricted to a small area close to the cornea. It is not possible to decide whether this tight packing of the PPC is the reason why extensions of the SPC into the retina do not occur or if it is the interlocking nature of the retinula cells of neighboring ommatidia. At least, it would seem that the loss of interommatidial (secondary) pigment cells (assumed to be a ground pattern component of mandibulate ommatidia generally: Müller et al., 2003) is a further result of miniaturization.

**Organization of the retinula cells and the rhabdome**

The rhabdome arrangement into four duplets in the distal part of the rhabdome conforms to that of the larger worker honeybee (Skrzipek & Skrzipek, 1971, 1974). The eye of *T. evanescens* also shares the presence of a ninth retinula cell with other hymenopterans (ants: Formica; Menzel & Lange, 1971). *Cataglyphis* (Brunnert & Wehner, 1971), and bees: workers and drones of the honeybee *A. mellifera* (Perrelet, 1970; Grundler, 1974; Skrzipek & Skrzipek, 1974) and halictid bee *Megalopta* (Greiner et al., 2004). What is different, though, is that in the eye of *T. evanescens*, the ninth cell starts earlier, that is, more distally, probably due to the short total length of the rhabdome, than in workers of *A. mellifera* and *Megalopta*. Nevertheless, the retinula cells in *T. evanescens* still show the same order in which the cells are contributing to the rhabdome as in *A. mellifera* (Skrzipek & Skrzipek, 1974). This finding underscores the conservative nature of compound eye organization in pterygote taxa generally (cf., Paulus, 1979) and the Hymenoptera in particular, but it could also suggest that in evolutionary terms, eyes of *T. evanescens* have not diverged much from the original type inherited from the last common ancestor of the Hymenoptera. Our results support Paulus (1979), who suggested that a corona made of nine retinula cells belong to the hymenopteran ommatidial ground pattern.

Irregularities, similar to those that were occasionally seen to involve the organization of the rhabdom in *T. evanescens*, were also reported from other hymenopterans (e.g., Skrzipek & Skrzipek, 1974). In *T. evanescens*, some of the irregular rhabdoms were of similar shapes to those described from the dorsal rim area of the eye of *Megachile rotundata* (Wachmann et al., 1973), but in *T. evanescens*, the presence of the irregular rhabdoms was not limited to any particular eye region. It is thus unlikely that *T. evanescens* possesses regionally differently specialized rhabdoms, and it is more likely that the presence of the relatively large number of irregularly shaped rhabdoms indicates that selective pressure on retaining high visual acuity has not been strong in this tiny eye.

It is conceivable that the decreasing space in the lower part of the rhabdome has an influence on the orientation of the retinula cells. This could explain why the irregular orientation of the fourth cell is found more often in the smaller eyes of the males. The fact that other rhabdomeres also frequently show a change in their microvillar orientation toward the lower end of the rhabdome shortly before they terminate supports this hypothesis. With diameters of 1.4–1.6 μm, the rhabdomes of *T. evanescens* are no more slender than many known from much larger insects. Actually, rhabdom diameters in *T. evanescens* are wider than those of rhabdomeres R7 and R8 (1 μm) in the open rhabdom of the fly (Kirschenfeld & Snyder, 1976). Thus, even in the tiny eye of *T. evanescens*, the minimum possible rhabdome diameter may not yet have been reached.

**Optical features**

Facets in the compound eye of *T. evanescens* are not only much smaller than those of all other hymenopterans examined to date but also to the best of our knowledge, they are smaller than the facets of any other insect compound eye. With regard to the diffraction limit, described by Barlow (1952), one would assume that it is optically counterproductive to have a compound eye with facets as small as those seen in *T. evanescens*. However, if we consider the small eye radius, it would seem that diffraction may not be a problem for the *T. evanescens* eye. The diffraction limit is given by $D\Phi = 0.58 \lambda$ (Snyder, 1977) with facet diameter $D$, interommatidial angle $\Phi$, and the wavelength $\lambda$. According to Snyder (1977), $D\Phi$ (the P-formula) can equally well be expressed by the square of the facet diameter divided by the eye radius ($D^2/R$) since the interommatidial angle $D\Phi$ equals $D/R$. Transposing this formula, it is possible to plot the theoretical minimal facet diameter $D$ for different eye radii for different wavelengths, showing the impact of a small eye radius on the minimum possible facet size for a diffraction limited eye (Fig. 7).

A comparison with the measured interommatidial angles and facet diameters reveals that the eyes of *Trichogramma* are not diffraction limited ($D\Phi > 0.58 \lambda$). Minimal optical limits are therefore set by the minimal functional size of the lens in order to fulfill the requirements of a short focal length, the reduced amount of light entering the dioptric apparatus due to the decrease in facet diameter and rhabdome length as well as diameter. In order to reduce focal length, lens power must increase. And this is achieved by a smaller radius of curvature of the lens. Focusing the light on the tip of the rhabdome would mean that the focal length must be equal to the distance between the cornea and the tip of the rhabdome, resulting in a lens power of around 100,000–120,000 diopters (diopters = $1/f$, in meters). This can be tested against the calculation of the focal length of the lens using the thick lens approximation (Jenkins & White, 1976). As the real refractive indices are not known for the components of the eye of *T. evanescens*, approximations (taken

![Fig. 7. Diffraction limit plotted for different facet diameters, wavelengths, and eye radii. The values for *Trichogramma evanescens* are illustrated for males (o) and females (x).](image-url)
from measurements on the compound eye of *A. mellifera* (Varela & Wittanen, 1970) were used for the lens \( (n_l = 1.452) \) and for the cone \( (n_f = 1.348) \). Taking into account the limitations of the formula, namely that the refractive indices may not be homogeneously distributed in the lens (Varela & Wittanen, 1970; Stavenga et al., 1999), the calculated image focal lengths (Table 2) for both sexes lead to a focal plane behind the tip of the rhabdom: 1.74 \( \mu \)m behind the tip in the females and 1.93 \( \mu \)m in the males. As the distance from the principal point to the front surface of the cornea must be added to the image focal length (see Stavenga, 2003, for optical diagram), the distance to the tip of the rhabdom is slightly longer.

Nevertheless, as approximations were used and the values for the refractive index of the cornea (but not cone) showed a gradient in *A. mellifera* (Varela & Wittanen, 1970), it is likely that the dioptric apparatus is able to focus the light on the tip of the rhabdom. Already, a slightly higher corneal refractive index (e.g., 1.49, the highest value mentioned for *A. mellifera*, and not an uncommon value for the cornea of other insects: Meyer-Rochow, 1975, 1978) would bring the lens even more into focus (image focal point 0.49 \( \mu \)m behind the tip of the rhabdom in females and 0.86 in males).

### The rhabdom

The amount of light that can be absorbed by the rhabdom is defined by its diameter and the total length of the receptor (Warrant & McIntyre, 1993; Warrant & Nilsson, 1998). A decrease in diameter as well as in the total length will cause a decrease in the amount of absorbed light. The amount of absorbed light can be calculated with the sensitivity formula, modified for light perception by Warrant and Nilsson (1998) using a value of 0.0067 \( \mu \)m \(-1\) for the absorption coefficient of the rhabdoms for invertebrates. This gives a value of \( S_m = 0.040 \mu \text{m}^2/\text{sr} \) for the female and 0.026 \( \mu \text{m}^2/\text{sr} \) for the male of *T. evanescens*. According to Stavenga (2003), the formula is weak for eyes with \( F\)-numbers < 2 and rhabdoms that function as waveguides (rhabdom diameter < 2 \( \mu \)m). Since both limitations would apply to the eye of *T. evanescens* (Tables 1 and 2), the results have to be treated with caution. However, they nevertheless show that the amount of absorbed light in comparison with that of larger species, for example, *Apis* (0.1 \( \mu \)m \(^2/\text{sr} \) Greiner et al., 2004) is somewhat smaller.

Light propagation in rhabdoms that act as waveguides cannot be explained by geometrical optics (Snyder, 1979; van Hateren, 1989, Warrant & McIntyre, 1993). Unlike the situation in larger rhabdoms, light is not guided simply by total reflection in the rhabdom; moreover, stable waveguide modes travel on the inside and partly on the outside of the rhabdom as well. How many modes are possible for a certain rhabdom diameter and wavelength can be calculated by the V-parameter (Snyder, 1979); more modes increase the total light intensity as well the receptive angle over which light can be perceived (Stavenga, 2003). Fig. 8 shows the plotted results for \( V \) of the rhabdoms of male and female *T. evanescens* for different wavelengths using a value of 1.36 for the refractive index for the rhabdom and 1.34 for the surrounding. The graph indicates that the second order mode is only possible for wavelengths below \( \lambda = 500 \) nm in the female and below approximately \( \lambda = 400 \) nm in the male. For the female, the third order mode is also possible in the far UV. As part of the light power is traveling also on the outside of the waveguide (amount of that fraction increases with mode order), the fraction of light inside the rhabdom \( (n_r) \) has to be calculated in order to determine the part that is effective for absorption. Stavenga (2003) calculated \( n_r \) for different wavelengths and rhabdom diameters and showed that the light fraction within the rhabdom decreases with increasing wavelength for all modes.

This means for *T. evanescens* that sensitivity increases in the blue, violet, and ultraviolet and that these wavelengths furthermore represent the largest fractions of light power inside the rhabdom. These results fit very well with the findings of Brower and Cline (1984) who showed a stronger preference of *T. evanescens* for black light (=UV-A) rather than white light. We therefore predict that *T. evanescens* has UV-sensitive photoreceptors, which is not exactly surprising as other hymenopterans have also been shown to be UV sensitive (Peitsch et al., 1992).

### What do the findings tell us about vision in *Trichogramma*?

Despite its small size, *T. evanescens* possesses a typical apposition eye, the functionality of which as a photoreceptor was demonstrated as long ago as 1958 by Quednau (1958). The small rhabdom, in connection with the small facet diameter, limits *T. evanescens* to bright light conditions, and its vision seems to be already light limited under room light conditions, given the typical ultrastructural signs of dark-adapted apposition eyes like, for example, the swelling of perirhabdomeric ER cisternae in the photoreceptive cells and positions of screening pigment granules (unpublished observations). Due to the even shorter rhabdoms in the eye of the male, the latter is expected to be especially light insensitive.

### Table 2. Optical parameters calculated on basis of measurements on the eye of *Trichogramma evanescens* and approximations for refractive indices of the dioptric apparatus. Parameters \( f \) and \( f' \) describe the focal length and the image focal length (both in micrometer), respectively. The \( F\)-number gives the ratio of the focal length to the facet diameter and serves as a value for comparing optical properties of different compound eyes. The acceptance angle of the ommatidium (\( A_p \)) is presented in radians and in degree (in brackets).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>( f )</th>
<th>( f' )</th>
<th>( F)-number</th>
<th>( A_p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>8.9</td>
<td>12.0</td>
<td>1.39</td>
<td>0.19 (10.88)</td>
</tr>
<tr>
<td>Male</td>
<td>7.8</td>
<td>10.5</td>
<td>1.32</td>
<td>0.18 (10.31)</td>
</tr>
</tbody>
</table>

### Fig. 8. V-Parameter plotted against the wavelength for the rhabdom diameter of the female (1.67 \( \mu \)m) and the male (1.41 \( \mu \)m) of *Trichogramma evanescens*. Dashed lines indicate the minimal limits for the second \( (V = 2.4) \) and third \( (V = 3.8) \) order mode for the waveguide.
Differences in the structural organization of the eyes can often be linked to different life styles or visual requirements (Land, 1989; Meyer-Rochow & Nilsson, 1999; Greener et al., 2004; Kelber et al., 2006). On the other hand, the size of the eye can also be simply the consequence of the limited space for it in a given insect head.

Behavioral studies of *T. evanescens* males and females have shown that males show a lower activity than females (Romeis et al., 1998) around a smaller radius of the hatching site, where most of the copulations take place. Females instead are more active and disperse over larger areas searching for hosts to deposit their eggs in (Martel & Bovin, 2004). Therefore, the presence of smaller eyes in the males could be linked to the lower activity and a lesser need of vision of the males.

Except for a few solitary species, Hymenoptera have trichromatic vision with peaks at wavelengths of 340, 430, and 535 nm (Menzel, 1971; Menzel & Blakers, 1976; Meyer-Rochow, 1981). Some species, furthermore, appear to possess a fourth peak in the red (>600 nm: Peitsch et al., 1992). With regard to the calculations of the *V*-parameters of the rhabdoms of male and female *T. evanescens*, it seems feasible that the first two orders of modes can be used by the females in connection with the peak near 430 nm, while the third mode would involve the UV. The males instead would only benefit from the second order mode in the UV. This suggests sexually different spectral sensitivities in the mid range of the wave spectrum, although we must not hastily conclude that the *V*-parameters alone explain the different attractiveness of differently colored sticky traps for females (white > clear > green) and males (yellow > green > white) as reported by Romeis et al. (1998) for *Trichogramma* spp.

The *V*-parameter shows that peak sensitivity in the UV is a property shared by both sexes but that the females are even more sensitive to light generally than the males. The use of UV in insects can help them find food as in nectar-seeking species (Manning, 1956), be involved in partner selection (Meyer-Rochow, 1991), or be of use in navigational tasks (Möller, 2002). For the small eye of *T. evanescens*, it is difficult to relate a specific function to its presumed ability to perceive UV, keeping in mind the small amount of light that can be absorbed in the short rhabdom. Since UV can indicate open space better than any other wavelength (Mazokhin-Porshnyakov, 1969), it could possibly lead *T. evanescens* into open areas, when entrapped by foliage. Many insects appear to use UV in this way. Quite common among Hymenoptera is also the use of UV radiation in connection with the e-vector (Duelli & Wehner, 1973; Wehner, 1989, Horvath & Varju, 2004), but preliminary behavioral tests with *T. evanescens* by us did not reveal any polarization sensitivity (unpublished observations).

**General functional conclusions relevant for small compound eyes**

As insects evolved smaller and smaller species, increasing less space had to remain in the head capsule for essential structures like the musculature to operate mouthparts and antennae and the brain to integrate sensory inputs. Small insects frequently do not feed and have degenerate mouthparts. A reduction in eye size had to be one consequence of smaller heads. However, giving up photoreception completely (in contrast to feeding) has not been an option. A total disappearance of eyes is only known from some (and then not always minute) cavernicolous species (Meyer-Rochow & Nilsson, 1999), which demonstrates that maintaining a compound eye makes sense even in the smallest members of insects, provided sufficient light is available to enter the facets and excite the visual pigments in the rhabdom.

To have small eyes means first and foremost a reduction of the diameter of the eye, one consequence of which a reduction of the total length of an ommatidium is. A shorter ommatidium by necessity will have a rhabdom of reduced length and this in turn is likely to result in a decrease in absolute sensitivity because a small rhabdom houses fewer visual pigment molecules in its microvillar membranes. The effects outlined above are independent of the size of the diameter of the facet lens, but a larger facet diameter does, of course, allow more light to enter the facet. Yet, facet diameters can only become enlarged when interommatidial angles concomitantly increase, and this leads to a decrease in the resolving power of the eye.

Furthermore, an isometric relationship between cone and total ommatidium length is deleterious; yet, a reduction of the total length of the ommatidium will affect somehow the total length of the cone (reduction of the length between the lens and the tip of the rhabdom (=f)). As long as the lens still focuses the light onto the tip of the rhabdom, a decrease in the F-number (f/βD) is the consequence. Generally, small F-numbers refer to highly photosensitive eyes (or optical systems), and larger F-numbers are typical of eyes (or optical systems) with reduced photosensitivity. In larger compound eyes, small F-numbers result from an increase in facet diameters, but small F-numbers can equally well be achieved given similarly sized f and D values (≈1). In order to achieve a short focal length, the curvature of the lens has to increase. A maximum limit for the curvature is set by the facet diameter, as the radius of curvature of the lens (r) should not go below D = 2 × r, as otherwise only a part of and not the whole facet would be covered.

Further size restrictions of a facet are set by the diffraction limit and the need of the underlying ommatidium to accommodate all the essential retinal cell types and their organelles. As Fig. 7 shows, the evolution of smaller eye radii is possible with smaller facet diameters without reaching the diffraction limit. But decreasing facet diameters will result in increasingly smaller interommatidial angles and, thus, less and less space for retinula cells and other ommatidial cell types, including the wealth of essential organelles dispersed therein. As the extent to which rhabdom diameter can be reduced is limited in respect to its light gathering function, sufficient place for the retinula cell nuclei will remain only in the upper somewhat wider third of the ommatidium. Thinner rhabdoms, with shorter microvilli to house the visual pigment, will be able to absorb fewer photons than larger rhabdoms, and there has to be a limit below which the gain for the insect obtained from photoreception no longer outweighs the cost to maintain photoreceptive cell specializations.

Thus, the minimum eye size seems to depend on the rhabdom length and diameter in combination with eye radius and facet diameter. To avoid crossing the limit (given through the interaction of these eye characteristics), one should expect a reduction in the number of facets, in which the latter can either be of the minimum possible dimensions or may have larger diameters. In *T. evanescens*, facet sizes appear to have reached their functionally possible minimum size limit, but does this hold true for the rhabdoms as well? Even if somewhat narrower rhabdoms do exist in the eyes of some larger insect species, such rhabdoms are considerably longer than the slightly wider ones seen in the eye of *T. evanescens*. Considering it is photon absorption, based on the amount of visual pigment molecules incorporated into each rhabdomere, which limits rhabdom size, the rhabdom dimensions encountered in the eye of *T. evanescens* most probably also represent the functional limit of this structure. What is worthy of note, however, is that the diameters of the microvilli in the rhabdoms of *T. evanescens* are no different from those making up the rhabdoms of larger species. As with the diameters of the screening pigment granules, the exact reason for this uniformity across vastly differently sized species of insects remains unknown.
The miniature compound eye of Trichogramma evanescens

Perspective

As there are other and even smaller Hymenoptera besides Trichogramma that still possess compound eyes, like, for example, the chalcid species Chloë micropterum (Gibson & Huber, 2000), further investigations on insects with small compound eyes are needed to test whether the theoretical minimal size limits and conclusions on compound eye miniaturizations advanced in this paper are generally valid. As in this paper approximations were used for the refractive index gradients, measurements of the real indices by interference microscopy would be helpful in order to carry out more precise calculations. However, how realistic it is to expect such measurements to be obtained on the minute dioptric structures of the eye of T. evanescens is anyone’s guess at this moment. It would seem easier to carry out behavioral tests to determine absolute and spectral sensitivities as well as flicker fusion frequencies. It would also be interesting to determine whether temporal summation events occur in the eye of T. evanescens to compensate for its short rhadom length.

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References


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2.2 Neither apposition nor superposition: the compound eyes of the chestnut leafminer *Cameraria ohridella*. 
2.3 Challenging limits: Ultrastructure and size-related functional constraints of the compound eye of *Stigmella microtheriella* (Lepidoptera: Nepticulidae).
Challenging limits: Ultrastructure and size-related functional constraints of the compound eye of *Stigmella microtheriella* (Lepidoptera: Nepticulidae).

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**Abstract**

With a body length of only 2 mm, the nepticulid *Stigmella microtheriella* (Stainton, 1854) is one of the smallest moths known to date. We investigated the optical design of its lemon-shaped compound eyes, which measure 83.60 µm in anterior-posterior and 119.77 µm in dorso-ventral direction. The eyes consist of about 123 facets, each of the latter just 9.9 µm in diameter. TEM investigations reveal an optical design with features intermediate between both apposition and superposition optics similar to that known from two other small species of moths (one Nepticulid and one Gracillarid). Size-related evolutionary adaptations of the ommatidial organization include 1) the involvement of only five rhabdomeres in the formation of the distal rhabdom 2) the complete absence of a rhabdomere of the eighth (= basal) retinula cell, 3) the “hourglass” shape of the rhabdom with a characteristic narrow waist separating distal from proximal portion and 4) the reduction to one single layer of tracheoles as an adaptation to the overall restricted space available in this minute eye.

**Key words:** miniaturization, Lepidoptera, ultrastructure, vision, insects, ommatidia
Introduction

For more than 100 years two different basic types of compound eye in Arthropoda, described as apposition and superposition eyes by Exner (1891), have been known. Since then, numerous investigations have dealt with the ultrastructural characteristics, functional mechanisms, biological roles, development and phylogenetic significance of these eye types (e.g. Nilsson et al. 1986; Nilsson 1989, 1990; Warrant & McIntyre 1993, Gaten 1998; Richter 2002). Moreover, several sub-types of arthropod eyes with apposition and superposition optics have been described (for review see e.g. Nilsson 1989, Land & Nilsson 2002).

Interestingly, irrespective of the fact that most insects are small, earlier eye investigations have focused primarily on insects with large eyes. In Lepidoptera, eyes of a wide range of taxa had been studied (Yagi & Koyama 1963, Tuurala 1954, Ehnbohm 1948), but small species like for instance tiny leafmining moths with wingspans below 10 mm (e.g. Nepticulidae: genus *Ectoedemia* and *Stigmella*; Gracillariidae: genus *Phyllonorycter*) were largely ignored. Perhaps because of their patchy abundance and practical problems with dissecting and fixing their tiny compound eyes, they still remain critically under-investigated. Yet, especially the smallest known specimens might be of special interest as miniaturization is usually accompanied by an increased potential for reductions, structural simplifications and morphological novelties (Hanken and Wake 1993). Moreover the extent of the neural system and numbers and sizes of neurons (Polilov and Beutel 2010) on the one hand and a general lower limit of cell body size of around 2 µm (Beutel and Pohl 2005) on the other are known to limit miniaturization. Thus, it is timely to examine how the evolution of small body size in insects could have affected the anatomy and function of the compound eye.

The aforementioned species of tiny moths were chosen for this investigation not only because of their small sizes, but also because Meyer-Rochow and Gál (2004) had calculated a minimal functional size for compound eyes of the superposition type, i.e., the typical eye design present in nocturnal beetles and moths (Warrant et al. 2003). Meyer-Rochow and Gál’s (2004) theoretical approach showed that an unlimited eye size reduction for an eye with
superposition optics is not possible and that an eye of this type needs to possess an eye radius > 250 µm to benefit from superposition optics.

For the nepticulid *Ectoedemia argyropeza* (Zeller, 1839) and the gracillarid *Cameraria ohridella* (Deschka and Dimic, 1986), two leafmining moth species with an eye-radius below this limit, Honkanen and Meyer-Rochow (2009) and Fischer et al. (2012) described an intermediate type of eye, which exhibited features of apposition (no extensive clear-zone; rhabdom in contact with the cone) and superposition eyes (radial gradient of refractive index in the cone and presence of a tracheal tapetum). For this study, we chose *Stigmella microtheriella* (Stainton, 1854), a parthenogenetic species (van Nieukerken 2006) of a total body length of no more than 2 mm and thus 2-3 times smaller even than *E. argyropeza*.

Being one of the smallest moths known to date, an ultrastructural examination of the compound eyes of *S. microtheriella* should provide us with useful data to test Meyer-Rochow and Gál's (2004) hypothesis. The study is also expected to shed light on general trends that accompany any size-related adaptations of the structural components of the eye and the possible functional implications that compound eye miniaturizations might have.

**Material und Methods**

Electron and light microscopic analysis

Hazelnut leaves (*Corylus avallana* L.) with mines of the parthenogenetic moth *Stigmella microtheriella* were sampled in autumn 2010 in the vicinity of Jacobs University Bremen (Germany) and stored in translucent plastic boxes. After pupation, the cocoons were removed from the leaves and stored in glass vials, kept outside the lab in a shaded place under natural light and temperature conditions throughout the winter months. In spring, the pupae were exposed to room temperature and a natural day/night rhythm (shielded from direct sunlight) until eclosion. The heads of the specimens to be examined, either in the light (3) or dark adapted state (3), were decapitated and split in half under room light during daytime hours and under red light at night (filter blocking light of wavelengths below 600 nm), respectively, before being fixed for 2-12 h in a
modified Karnovsky’s (1965) fixative solution, consisting of 2% paraformaldehyde and 2.5% glutaraldehyde solution, buffered with 0.1 M cacodylate (pH 7.4). After a 2h period of postfixation in 2% OsO4 solution, also buffered with 0.1 M cacodylate (pH 7.4), the samples were rinsed three times in the same buffer, dehydrated in a graded series of ethanol before being passed through three acetone/Epon mixtures (3:1, 1:1, 1:3, pure Epon) and embedded in pure Epon-812. The samples were then polymerized for three days at a temperature of 60°C.

For light microscopy (LM), semithin sections were cut on an ultramicrotome (RMC, Boeckeler Instruments Inc., Tucson, AZ) with a glass knife and stained with 0.5% aqueous solution of toluidine blue for 30 s on a hot plate. Ultrathin sections were cut with a diamond knife, picked up on formvar-coated copper slot grids and stained with lead citrate and 2% aqueous uranyl acetate for 20 min each (modified after Reynolds 1963). The sections were examined either under a Zeiss EM 900 or a Jeol JEM-1011 transmission electron microscope (TEM), operated at 80 KV. Before any measurements commenced, the TEM was calibrated with a calibration grid (Plano S104). For scanning electron microscopy (SEM), osmium-fixed heads were dehydrated in a graded series of ethanol and then critical-point-dried (EMITECH 850, Emitech Ltd., Ashford, UK). Dried samples were coated with an approximately 15 nm thick gold layer in a sputter coater (Quorum Q150T S, Quorum Technologies Ltd., East Grinstead, UK) and observed under a JEOL JSM-5900 SEM, operated at 20 kV.

Morphometric analyses

Scanning electron microscopy was used to determine dorso-ventral and anterior-posterior lengths of the eye (i.e., height and width, respectively), total number of ommatidia per eye as well as facet diameters. Three compound eyes of three different individuals were used for these measurements.

Longitudinal light microscopic (LM) sections were used to determine ommatidial lengths, radii of curvature of the eye and its facets as well as interommatidial angles. The radius of curvature of the eye was measured on the broadest anterior-posterior extension (eye width) of the eye, in sections cut in dorso-ventral direction, by placing a circular measurement tool over the eye surface in the image analyzing software. The interommatidial angles were measured at
the point of intersection of the normals to the eye surface through the ommatidial axes of adjacent central ommatidia. TEM micrographs of cross-sections were used to measure rhabdom diameters, shapes, and diameters of pigment granules of primary pigment (PPC) and secondary pigment cells (SPC) as well as retinula cells. Measurements of rhabdom diameters were taken at the distal tip of the rhabdom close to the transition between rhabdom and cone (distal rhabdom diameter = DRH)) and the plane of the distal tip of the tracheoles (proximal rhabdom diameter = PRH). Longitudinal sections were used to determine cone length, corneal thickness, and diameter and position of pigment granules within pigment and retinula cells. For examinations involving LM and TEM, six compound eyes of either light- (3) or dark-adapted states (3), were used. Breeding success in nepticulids is known to be highly variable (Johansson et al. 1989) and this limited us to the use of no more than three individuals for each of our experimental approaches. Our study involved only female individuals, male individuals have not been described in S. microtheriella and the species is considered parthenogenetic (Johansson et al. 1989, van Nieukerken 2006). Nevertheless, the sex of all our specimens was routinely tested by genital analysis and we confirmed that all our specimens were indeed females. Measurements were gathered by image analysis software (Image J, Rasband, W.S, U.S. National Institutes of Health, Berthesda, MD) and based on an identical number of data per specimen to avoid a weighting in the statistics later.

Statistical analyses

In each data set, normality of distribution was tested with the Shapiro-Wilk normality test. As normal distribution was found in all data sets, comparisons between light- and dark-adapted states were performed by independent t-test. The analytic software SPSS was used for all of the statistical tests.

Results

General organization of the eye

*Stigmella microtheriella* possesses „lemon-shaped“ eyes that measure 119.8 ± 3.7 µm in dorso-ventral direction (eye-height) and 83.6 µm in anterior-posterior direction (eye width) (Fig.1A-B; Tab. 1). Each eye consists of an average
of 123 ± 1 facets of hexagonal shape with a facet diameter of 9.9 ± 0.5 µm; regional differences in facet diameters and the presence of interfacetal hairs were not observed (Fig. 1B-C). High power magnified scanning electron micrographs of the cornea revealed the presence of regularly arranged corneal nipples (Fig. 1D). Based on semithin horizontal sections through the widest region of the eye, the eye’s radius was determined as 63.7 ± 2.4 µm.

**Fig. 1A-D:** Scanning electron micrographs of the outer appearance of the head and compound eyes of *Stigmella microtheriella*. **A.** Ventral view of the head and the two compound eyes somewhat concealed by cephalic scales and hairs. Note that some collapse of the facets has occurred during drying in this specimen. **B.** Lateral view of the left compound eye of a head split in half after the removal of surrounding scales. The eye is lemon-shaped and exhibits its longest extension in the dorso-ventral direction. **C.** Close-up of the same eye as shown in b. The facets, measuring on average 9.9 µm in diameter, are strongly bulging and possess a more circular rather than hexagonal shape. **D.** Surface detail of one facet, densely covered by corneal nipples. A: anterior; D: dorsal.

On average, an ommatidium of *S. microtheriella* measures 47.1 ± 1.4 µm in total length; the interommatidial angle is 11.3° ± 1.9°. To approach a theoretical exit angle of light, needed to cover the neighboring proximal rhabdoms for superposition optics (= in total 7 ommatidia), measurements were performed on longitudinal sections of the eye. Measurements taken from the axis at the tip
of the cone to the distal tip of the peripheral tracheoles of the neighboring ommatidia revealed an angle of 36.8° ± 2.5°.

**Tab. 1** Measured parameters of the eye of *Stigmella microtheriella*. *P* values, calculated by independent T-Test, are given in decimal numbers (significant differences in case of *p*<0.05) for differences between the light/dark-adapted states (*P*). For each parameter the parallels (Par) of measurements per specimen (n) are stated.

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<td>Length basal cell</td>
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Diameter pigment granules of the:
- **primary pigment cells**  µm  0.8 ± 0.1  25/3
- **secondary pigment cells** µm  0.6 ± 0.1  25/3
- **retinula cells**        µm  0.4 ± 0.1  25/3

| Parameter                                      | Unit    | **Light**       | Par/n | **Dark**       | Par/n | *P*
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Fine structural organization

Dioptric apparatus and primary pigment cells

The dioptric apparatus consists of a strongly bulging, biconvex corneal lens (= cornea), made up of 15-25 layers of equal thickness and stainability, and an eucone crystalline cone (Figs. 2A, 3A). The cornea reaches a thickness of 3.6 ± 0.4 µm in its center (Fig. 3B) and its outer radius of curvature is 5.5 ± 0.4 µm.

Below the innermost corneal lamella and above the crystalline cone, one often observes a subcorneal layer approximately 1 µm in thickness (Fig. 3B). Under the TEM, the cytoplasmic material of this layer appears condensed and more electron-dense than that of the cone sheath and primary as well as secondary pigment cells (Fig. 3A-B).

The eucone crystalline cone possesses an average length of 18.2 ± 0.9 µm. In the light-adapted state, its proximal end is elongated and tapered (Fig. 3A). The crystalline cone is built by four cone cells, each containing a prominent nucleus in its distal region (Fig. 3A). Cross-sections of the crystalline cone show a radial gradient in staining intensity (Fig. 3C), ranging from a brighter (= less dense) periphery to a highly osmiophilic center in the more voluminous distal part. A gradient such as this is less obvious towards the proximal end of the crystalline cone (Fig. 3D). Four short extensions, the so-called cone projections, emerge from the proximal tip of the cone and surround the most distal end of the rhabdom (Fig. 3E), where they terminate in blunt ends without accompanying the retinula cells down to the basal matrix (Fig. 4A). Two primary pigment cells (PPC), containing pigment cell granules of a diameter of 0.8 ± 0.1 µm, surround the cone from the cornea down to the proximal tip of the crystalline cone (Figs. 2A; 3A,C). The nuclei of the PPC show an elongated shape and are situated below the maximal diameter of the cone (Fig. 2A-B).

Secondary pigment cells

Six secondary pigment cells, with nuclei situated directly below the cornea (Fig. 3A), envelope the primary pigment cells and surround the retinula cells down to the basal matrix. A short distal process emanates from the soma of each secondary pigment cell, attaching it to the innermost lamella of the interommatidial cuticle. In the proximal region of the ommatidium, the
secondary pigment cells continue to occupy the interommatidial spaces, i.e., the spaces between the retinula cells of three neighboring ommatidia (Fig. 2E). The diameters of the secondary pigment cells decrease proximally to thin strands crammed in between tracheoles (Fig. 5D). The pigment granules of the secondary pigment cells measure about 0.6 ± 0.1 µm in diameter, are electron-dense and most prominent in the distal region of the cell (soma + distal process), where they are in a position to shield the dioptric apparatus. Below the soma, i.e. in the proximal extensions, shielding pigment granules are scarce (Fig. 2C-D), but closer to the basal matrix each extension enlarges again in size and forms a terminal swelling that may contain a few additional pigment granules (Fig. 5E).

Retinula cells and rhabdom

An ommatidial retinula in *S. microtheriella* consists of seven rhabdomere-bearing retinula cells and reaches from the proximal tip of the cone close to the basal matrix. Below the rhabdom an additional, basal cell is seen and termed “8th retinula cell”, according to the R8 typology recently described by Friedrich et al. (2011). This cell does not participate in the rhabdom formation and lacks a rhabdomere (Figs. 2A,F; 4E; 5C). Besides being richly equipped with highly electron-dense shielding pigment granules, measuring 0.4 ± 0.1 µm in average diameter, the cytoplasm of all eight retinula cells contains cisternae of both smooth and rough endoplasmic reticulum, various lysosomal bodies and mitochondria of the cristae type.

In the centrally fused, “hourglass-shaped” rhabdom one can distinguish a distal rhabdom of an average length of 8.0 ± 0.7 µm and a diameter of approximately 2.9 ± 0.3 µm from a broader proximal rhabdom of maximum diameter of 4.8 ± 0.3 µm and a length of 9.3 ± 0.9 µm (all measurements referring to the light-adapted state). A tracheal tapetum lucidum surrounds the ommatidial group of retinula cells from the proximal rhabdom downward towards the basal matrix (Figs. 2A; 4A,D; 5A-C). A thin “waist” region that bears no microvilli separates the distal from the proximal rhabdom over a distance of approximately 2.5 µm (Figs. 2A, 3A, 4B, 5A-B).
Fig. 2A-F: Semischematic reconstruction of an ommatidium of *Stigmella microtheriella*. 

**A.** Longitudinal view. **B-f.** Transverse views at different section planes as indicated in **A:**

- **B.** Cross-section through median region of the crystalline cone area crossing the nuclear level of the two primary pigment cells. Six secondary pigment cells envelope the primary pigment cells. **C.** The distal rhabdom is formed by rhabdomeres of five retinula cells; two irregular, weakly pigmented retinula cells, do not participate in the formation of the rhabdom. The slender secondary pigment cells are crammed in between neighboring ommatidia. **D.** Cross-section through the waist region coinciding with the nuclear region of the retinula cells. Note that the retinula cells do not form microvilli at this level. Because of the absence of the rhabdomeres in this region, numbering of retinula cells is not reliably possible here. **E.** Cross-section through the proximal rhabdom, surrounded by tracheal tapetum that consists of a single layer. The proximal rhabdom consists of rhabdomeres of 7 retinula cells with microvilli oriented in three mayor directions. Opposing cells have rhabdometric microvilli oriented parallel to each other; partner rhabdomeres (RC 1,2+5, RC 3+6, RC 4+7) are however not necessarily adjoined. The two irregular retinula cells R2 and R5 show the same
microvillus alignment, i.e., parallel to R1. F. Close to the basal matrix the pigmented eighth retinula cell is situated. With the exception of two retinula cells, all others turn into axons, before the eighth cell reaches its maximal diameter. C: cornea; CC: crystalline cone; CCN: nucleus of a crystalline cone cell; D: desmosome; IRC: irregular retinula cell; M: mitochondrion; N: nucleus; RC: retinula cell; RC8: eighth retinula cell; RCN: nucleus of a retinula cell; RCP: pigment granule of a retinula cell; SPC: secondary pigment cell; TR: tracheole.

Fig. 3A-E: Transmission electron micrographs of the dioptric apparatus and the cone-rhabdom transition zone in an ommatidium of *Stigmella microtheriella* in the light-adapted state. A. Longitudinal view of the distal half of an ommatidium, showing the dioptric apparatus and the “hourglass”-shaped rhabdom. The elongated cone is enveloped by primary and secondary pigment cells. Pigment granules of the retinula cells line the distal rhabdom, whose diameter is similar to that of the proximal tip of the crystalline cone. B. Longitudinal section of the dioptric apparatus featuring the multilayered cornea covered by corneal nipples. A wide amorphous layer is noticeable subjacent to the multilayered corneal lens. C. Cross-section of the crystalline cone on the nuclear level of the primary pigment cells. The crystalline cone, surrounded by the pigment granules of the primary pigment cells, exhibits a distinctive internal matrix gradient (arrow). D. Cross-section of the slender proximal part of the crystalline cone. Note the mitochondrion in the peripheral cytoplasmic sheath that envelopes the central matrix of the crystalline cone. E. Cross-section through the cone-rhabdom transition zone. The four cone cells’ projections separate, pass alongside the distal rhabdom for a short distance and then end bluntly. C: cornea; CC: crystalline cone; CCN: nucleus of a cone cell; CCP: crystalline cone projection; DRH: distal rhabdom; M: mitochondrion; N: nucleus; PPC: primary pigment cell; PRH: proximal rhabdom; RCN: nucleus of a retinula cell; SCL: subcorneal layer; SPC: secondary pigment cell.
In the region, where the proximal tip of the cone and the distal rhabdom contact each other, the distal rhabdom’s diameter is largest and matches that of the cone tip (Fig. 3A). At this level, the rhabdom is formed by the rhabdomeres of five retinula cells, termed regular retinula cells in the following (Figs. 2B, 4A). The number of seven desmosomes indicates the presence of the thin extensions of two additional (the irregular and much less pigmented) retinula cells. The five regular rhabdomeres are arranged in a repetitive fashion according to which each two opposing rhabdomeres, with microvilli measuring about 58.9 ± 4.4 nm in diameter, are oriented in the same direction. The two pairs in turn are positioned nearly perpendicular to each other (Fig. 2B). The fifth rhabdomere shows a microvillar orientation intermediate to the two pairs. In essence, three different planes of microvillar orientations, offset to one another by an angle of approximately 120°, are present.

The asymmetric arrangement of the regular retinula cells and their associated rhabdomeres is caused by the unpaired (“single”) rhabdomere located next to one of the irregular retinula cells. However, this asymmetry allows us to identify and number individual retinula cells, starting with the single regular retinula cell R1. The remaining retinula cells are counted in ascending order in the direction towards the irregular cell next to R1. Hence, the two irregular cells are named R2 and R5 and the pairs of retinula cells with parallel microvillar orientations are R3 + R6 and R4 + R7 (Figs. 2B, 4A).

The nuclei of both regular and irregular retinula cells (R1-7) are present together between the tips of the crystalline cones and the distal ends of the tracheoles (Figs. 2A,C-D; 3A; 4A-B; 5A). The region, where the nuclei of the retinula cells reach their maximal diameter, coincides with the “waist” region of the rhabdom (Figs. 4A, 5A).

Below the “waist” region, the proximal rhabdom expands in diameter, but is still only built up by the rhabdomeres of the five earlier mentioned regular retinula cells. At the distal tip of the tracheoles, the proximal rhabdom reaches its maximum diameter and occupies almost the entire space enclosed by the tracheoles. The rhabdoms of two neighboring ommatidia are separated by a single layer of tracheoles (Fig. 4D).
At the level, where the diameter of the proximal rhabdom is maximal, the two irregular retinula cells also start to contribute to the formation of the rhabdom (Figs. 2E; 4C-D). The irregular retinula cell R5 couples with the formerly unpaired R1 and, subsequently, becomes a partner in a pair of rhabdomeres with parallel aligned microvilli (Fig. 4C). The R2 exhibits microvilli oriented parallel to those of R1 and R5.

Further proximally, the retinula cells diminish in size and, except for two or three retinula cells, turn into axons before the 8th (basal) retinula cell reaches its maximum diameter (Figs. 2F, 4E, 5C). Not only are the rhabdomeres of distal and proximal rhabdom portions not entirely congruent, there is also a shift in orientation caused by the spatial dominance of the subjacent 8th retinula cell. Since R1 and/or R2 of all the retinula cells tend to turn into axons first, the earlier asymmetry then changes into a symmetric arrangement. That makes it almost impossible to individually identify the retinula cells along the lower part of the proximal rhabdom and, even more importantly, to reliably attribute an axonal process to one of the R1-7. Close to the proximal tip of the ommatidium, the retinular axons, including that of the 8th retinula cell, come together and form a distinct bundle that passes through the basal matrix (Fig. 4F).

The basal matrix is an extracellular secretion of various epithelial cells shaping the compound eye and delimiting the union of ommatidia against the optic neuropils (Odselius and Elofsson 1981, Fischer et al. 2012). In the compound eye of S. microtheriella, the basal matrix appears thin and rather inconspicuous (Fig. 5D). Two major portions in the basal matrix can be distinguished: 1. the cellular portion, which comprises all sorts of terminal cellular swellings (on the ommatidial side: secondary, i.e. interommatidial, pigment cells; on the subommatidial side: glial cells and basal pigment cells) and 2. the extracellular portion, the actual extracellular matrix (ecm), that contains the continuous basal lamina of all contributing cells and some fibrous interlayers (Figs. 4F, 5E). In the centre of each ommatidium, the basal matrix leaves a perforation for the corresponding retinular axon bundle.
Fig. 4A-F: Transmission electron micrographs of retinula cells in a light-adapted ommatidium (dark-adapted state shown in 4b) of *Stigmella microtheriella* at various section levels (from distal to proximal). A. Cross-section through the distal rhabdom, in which five regular and two irregular retinula cells are visible. Only the rhabdomeres of the five regular retinula cells form the rhabdom. Note the pigment-free cytoplasm of the two irregular retinula cells. B. Cross-section of the “waist” region of the retinula. Apical cell borders can be distinguished by the presence of desmosomes (*arrow*). In the thinnest part of the “waist” region, no microvilli are noticeable. Other than an identification of the irregular retinula cells, labeling of the retinula cells is not possible. C. Oblique section through the distal tip of the proximal rhabdom. In this plane, the irregular retinula cells RC2 and RC5 start to contribute to the rhabdom. D. Cross-section through the mid-area of the proximal rhabdom, where all seven retinula cells
clearly participate in forming the rhabdom. E. Portrait of an 8th retinula cell which almost completely occupies the intraommatidial space toward the basal end of the rhabdom. Its cytoplasm is rich in electron-dense shielding pigment granules and exhibits a large nucleus. F. Subommatidial cross-section directly below the basal matrix. The bundle of eight retinula cell axons is surrounded by processes of some basal pigment cells. AX: axon; DRH: distal rhabdom; IRC: irregular retinula cell; M: mitochondrion; PPC: primary pigment cell; PRH: proximal rhabdom; RC: retinula cell; RCN: nucleus of a retinula cell.

Dark / light adaptations

The eyes of *S. microtheriella* display very considerable differences between dark and light-adapted states. The differences involve the position of the screening pigment shield as well as a change in the shape and dimensions of the crystalline cone and the diameter of the proximal rhabdom (Tab. 1). In the light-adapted state (Fig. 6A), the highly osmiophilic shielding pigment granules of the primary and secondary pigment cells surround the tip of the crystalline cone that in this adaptational state has developed a thin and elongated proximal projection of the same diameter as the distal rhabdom it contacts. The pigment granules of the retinula cells envelope the entire distal rhabdom, populate the “waist” region and form a cap-like pigment layer above the top of the proximal rhabdom (Fig 5B).

In the dark-adapted state, the shielding pigment granules of the primary and secondary pigment cells migrate towards and aggregate between the crystalline cones (Fig. 6B). In this adaptational state the crystalline cones appear characteristically “bullet-shaped”, lacking the tapered proximal elongation of the light-adapted state. The crystalline cones, moreover, exhibit significantly smaller maximal diameters (7.3 µm, p<0.01) as well as significantly greater lengths (19.8 µm, p<0.01) than those of the light-adapted state. Under dark adaptation pigment granules of the retinula cells do not surround the distal rhabdom. Due to the longitudinal displacements of the shielding pigment granules in the retinula and accessory pigment cells, a small pigment-free zone is formed that extends from the proximal cone region to the distal tip of the proximal rhabdom. Finally, a statistically significantly smaller maximum diameter is a feature of the proximal rhabdom in the dark-adapted state (4.5 µm versus 4.8 µm in the light-adapted state, p<0.01).
Fig. 5A-E: Transmission electron micrographs of several longitudinal sections through the retinula of the ommatidia of *Stigmella microtheriella* in the light-adapted state. **A.** The distal rhabdom is in direct contact with the crystalline cone and tightly surrounded by the shielding pigment granules of the retinula cells. The nuclei are closest to the rhabdom in the “waist” region (white arrow). **B.** A dense layer of shielding pigment granule aggregations is present near the distal tip of the proximal rhabdom, at a level where one encounters the first tracheoles. **C.** Longitudinal view of the 8th retinula cell close to the basal matrix with no obvious contribution to the proximal rhabdom. Note the large nucleus dominating the cytoplasm of the 8th retinula cell. **D.** Cross-section of the proximal extensions of two secondary pigment cells (arrow) restricted to the contact zone of three neighboring ommatidia. **E.** Oblique section through the proximal region of the retinula close to the basal matrix. The proximal extensions of the secondary pigment cells terminate in terminal swellings. AX: axon; BM: basal matrix; CC: crystalline cone; DRH: distal rhabdom; PRH: proximal rhabdom; RCN: nucleus of a retinula cell; RCP: shielding pigment granule of a retinula cell; SPC: secondary pigment cell; TR: tracheole.
Fig. 6A-B: Structural differences between dark- and light-adapted compound eyes of *Stigmella microtheriella*. A. In light-adapted ommatidia the crystalline cones exhibit distinctively tapered tips. Each tip is surrounded by the shielding pigment granules of the primary and secondary pigment cells. The distal rhabdom adjoining the crystalline cone is enveloped by the retinula cell pigment granules, whose dense aggregations lead to a thick layer just above the proximal rhabdom. B. In dark-adapted ommatidia the crystalline cone is more “bullet-shaped” and lacks the slender proximal tip, found in the light-adapted state. Furthermore, the granular equipment of the primary and secondary pigment cells and the pigment granules of the retinula cells have now taken up positions in the distal spaces between neighboring crystalline cones. BM: basal matrix; C: cornea; CC: crystalline cone; DRH: distal rhabdom; PRH: proximal rhabdom; RC8: eighth retinula cell

**Discussion**

We examined the ultrastructure of the compound eye of one of the smallest moths known to date. Besides describing this eye’s anatomy and ultrastructure, another goal has been to test whether the design of superposition optics, known from large moths, is or is not of advantage for the function of the eyes in the smallest moths. Since our results have shown that in the eye of *S. microtheriella* the structural prerequisites to permit superposition are not fully met, the discussion will focus on the comparison of eye adaptations in other small moth species and specifically address the question of size-related adaptations and minimum limits of the structural entities of the eye.

General organization of the eye – external features

With an average facet number of 123 and given its morphometric dimensions (eye height: 119.8 versus eye width: 83.6 µm), the compound eye of *S. microtheriella* has to be considered the smallest eye of all Lepidoptera recorded
to date. It has considerably fewer than the 224 facets of the compound eye of *Ectoedemia argyropeza* (Zeller, 1839), for which Honkanen and Meyer-Rochow (2009) gave values of 206.4 µm and 152.3 µm for dorso-ventral and anterior-posterior lengths, respectively. It also has far fewer facets than the 200 mentioned by Yagi and Koyama (1963) for the eye of a male of an undetermined species of *Stigmella*. Although the facet diameter is also the smallest described so far for any Lepidoptera, it is worth mentioning that regardless of the much smaller body size of *S. microtheriella*, the facet diameter is not considerably smaller than that of other small moths like *Stigmella* sp. (12.5 µm, Yagi and Koyama 1963), *E. argyropeza* (13.8 µm, Honkanen and Meyer-Rochow 2009), *Leucoptera coffeella* (Guérin, 1842) (10 µm, Meyer-Rochow and Stringer 1993) and *Cameraria ohridella* (Deschka and Dimic, 1986) (11.5 µm, Fischer et al. 2012). It seems noteworthy to point out that the facet diameter of *S. microtheriella* fits the range of 9-10 µm that has been suggested as the minimal effective facet diameter on the one hand for apposition eyes in insects generally (Barlow 1952) and on the other hand for Lepidoptera in particular (Yagi and Koyama 1963).

Anatomical details

Regardless of the optical type established in a given compound eye, be it superposition or apposition, the ommatidia of both crustacean and hexapod eyes primarily consist of the same typical set up of cellular components (e.g. Paulus 1979, Melzer et al. 1997, Dohle 2001, Richter 2002). Thus, not surprisingly, the complete setup of two primary pigment cells, four cone cells, eight retinula cells, and six secondary (interommatidial) pigment cells is also present in the compound eyes of the smallest Lepidoptera (e.g., *E. argyropeza*: Honkanen and Meyer-Rochow 2009; *C. ohridella*: Fischer et al. 2012; *S. microtheriella*: this paper). Consequently, no size-related reduction in total cell types and numbers occurs. The diameters of the rhabdomeric microvilli (58.93 nm) and shielding pigment granules (primary pigment cells: 0.8 µm, secondary pigment cells: 0.6 µm, retinula cells: 0.4 µm) also show no obvious size differences from those in larger lepidopterans (Meinecke 1981; Kolb 1985; Shimohogashi and Tominaga 1986; Lau and Meyer-Rochow 2007, 2008; Lau et al. 2007) and similar results have recently been obtained from the minute parasitic wasp *Trichogramma evanescens* whose eyes possess a radius < 35
µm (Fischer et al. 2011). As suggested in an earlier paper (Fischer et al. 2011), it seems as if certain largely unknown functional and/or structural limitations prevent cytoplasmic organelles of retinula and pigment cells to become reduced in size beyond a critical minimum. Since ommatidial cell numbers in smaller lepidopteran compound eyes are no different from those encountered in larger species we propose that, based on our present results, a size reduction in ommatidia can only be achieved by reducing the rhabdom volume (number and length of rhabdomeric microvilli), sizes and placements of the contributing cells as well as the total number of shielding pigment granules. Ultimately, perhaps even some nuclei might be sacrificed (cf., Polilov 2011 on anuclear neurons in the neuronal make-up of the minute parasitoid wasp *Megaphragma mymaripenne*), but this has not been reported for retinula cells up to date.

The intermediate type of eye

The compound eye of *S. microtheriella* represents an intermediate type of eye, combining features of both apposition and superposition eyes, similar to what has recently been described also for the small compound eyes of the nepticulid *E. argyropeza* (Honkanen and Meyer-Rochow 2009) and the gracillarid *C. ohridella* (Fischer et al. 2012). To distinguish superposition from apposition optics in the lepidopteran compound eye, information on the nature, location and abundance of (sub-)cellular characters can be helpful (Fischer et al. 2012). Table 2 summarizes the most useful clues.

Although the lack of a wide gap between proximal cone tip and distal end of the rhabdom (known as the clear-zone and seen as an essential characteristic of eyes employing superposition optics) in the eye of *S. microtheriella* suggests apposition optics, features intermediate in nature between the two eye types are recognizable. For instance, a gradient in contrasts, visible on transmission electron micrographs of sections through the cone, indicates a gradient of refractive indices in the central matrix of the eucone crystalline cone (cf., Nilsson 1990). Other characters improving superposition are the existence of a tracheal tapetum and the nucleus of the 8th retinula cell in a position below the rhabdom. These characters are also seen in the unmodified superposition eyes of larger lepidopteran species (Tuurala 1954).
Tab. 2 Compilation of histological and ultrastructural characters typical for apposition, superposition and combined apposition/superposition i.e., intermediate eye type, modified after Fischer et al. (2012). Own results are marked (*), all other characters according to the cited authors.

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<td>tracheal tapetum (Exner 1891)</td>
<td>present*</td>
<td>present</td>
<td>not present</td>
</tr>
<tr>
<td>position of the nucleus of the 8th retinula cell (Tuurala 1954)</td>
<td>below the rhabdom*</td>
<td>below the rhabdom</td>
<td>Beside the rhabdom</td>
</tr>
<tr>
<td>rhabdom shape in cross sections (Yack et al. 2007)</td>
<td>roughly circular*</td>
<td>flower-shaped</td>
<td>not flower-shaped</td>
</tr>
<tr>
<td>rhabdom in contact with the cone (Exner 1891)</td>
<td>yes*</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>rhabdom shape in longitudinal sections*</td>
<td>bottle-shaped*</td>
<td>diameter decreases from tip to base</td>
<td>diameter decreases from tip to base</td>
</tr>
<tr>
<td>screening pigment migration (Autrum 1981)</td>
<td>longitudinal migration of retinula cell pigments*</td>
<td>longitudinal migration of secondary pigment cell pigments</td>
<td>radial migration of retinula cell pigments</td>
</tr>
</tbody>
</table>

Somewhat ambiguous characters, considered intermediate, are cone and rhabdom shapes and the migration of the pigment granules in the secondary pigment cells during dark-light adaptation. Dark/light-adaptational changes involving the shapes of the crystalline cones and especially their proximal tips appear to be even more pronounced in *S. microtheriella* than in *C. ohridella* and *E. argyropeza* (cf., Honkanen and Meyer-Rochow 2009; Fischer et al. 2012). To discuss possible functional consequences of these changes, measurements of the refractive index distribution within the cones would be required, but as yet no such measurements are available for any tiny moth eye.

As in *E. argyropeza* and *C. ohridella* (compare Honkanen and Meyer-Rochow 2009, Fischer et al. 2012), the almost circular shape of the rhabdom in transverse section, compared with the flower-shaped rhabdoms of larger moths with superposition eyes, can be interpreted as an adaptation to effectively utilize...
the available space in order to maximize the rhabdom's volume as far as possible under the given spatial restrictions of a miniaturized ommatidium.

Some processes occurring during dark/light-adaptation, such as the longitudinal pigment migration (exclusively taking place in the retinula cells in contrast to that of larger moths, in which the process is taking place in the secondary pigment cells), are deemed to be the result of the limited space available in the eye. Furthermore, a longitudinal pigment migration in the secondary pigment cells is not likely to be very effective, because the small diameter of the proximal extensions of the secondary pigment cells enveloping the retinula cells would not provide sufficient shielding (Fig. 2C-D).

Size-related adaptations and their functional consequences

Adaptations seemingly related to the small size of the eye and ommatidia of *S. microtheriella* include: 1) hourglass-shape of the rhabdom, 2) reduction of the rhabdomeres in the distal rhabdom to five, 3) absence of the rhabdomere of the 8th retinula cell and 4) reduction of the tracheolar envelope to one layer.

1. Hourglass-shape of the rhabdom

Rather prominent size-related adaptations are seen in *S. microtheriella* (this study) and in *E. argyropeza* as well as (Honkanen and Meyer-Rochow 2009). In these two nepticulids a “waist” region with no rhabdomeric microvilli separates the distal from the proximal rhabdom, whereas in the larger gracillarid *C. ohridella* (Fischer et al. 2012) the distal rhabdom merges into the basal rhabdom.

Relatively little space is available for the large nuclei of the seven retinula cells (five regular retinula cells and two irregular retinula cells in case of *S. microtheriella*), which are restricted to the zone around the distal rhabdom ranging from the proximal tip of the crystalline cone to where the tracheolar layer ends (= distal tip). Positioned in the plane of the “waist” region between the two rhabdom regions, the nuclei take away space of the rhabdom in *S. microtheriella* and *E. argyropeza* (Honkanen and Meyer-Rochow 2009). Therefore, one consequence of miniaturization in the eyes or, more precisely, the rhabdoms of *S. microtheriella* and *E. argyropeza* has to be the reduction of visual membrane material, i.e. rhabdomeric microvilli. In *C. ohridella* (Fischer et
al. 2012), however, a “waist” region does not occur, possibly because the distance between crystalline cone and circumretinular tracheoles is much greater and therefore is likely to provide sufficient space to accommodate the nuclei of the seven regular retinula cells in one single plane without the necessity to constrict the diameter of the distal rhabdom. In addition to the nuclei, space must also be allocated to the retinula cell pigment granules needed in the process of light/dark-adaptation. As gradual size reductions do not affect the diameters of the pigment granules, adaptations to save space are likely to involve reductions in the total number of pigment granules or the size of the rhabdom.

2. Reduction of the distal rhabdom to 5 rhabdomeres

A reduction of the primarily seven rhabdomeres down to five in the distal part of the rhabdom of *S. microtheriella* is unique among hitherto examined lepidopteran compound eyes of small size. Although the two irregular retinula cells do not participate in the formation of the distal rhabdom, the remaining rhabdomeres of *S. microtheriella* continue to exhibit a pattern of rhabdomeric microvilli (constant throughout the ommatidium), comprising three main directions, offset to one another by 120°. This arrangement fulfils the basic requirement for detecting linearly polarized light (Kirschfeld 1972, Horváth and Varju 2004). Generally, leafmining moths are restricted to single or few food plants (Johansson et al. 1989) and *S. microtheriella* is known to lay eggs only on *Corylus* and *Carpinus* (Johansson et al. 1989). As discussed in Honkanen and Meyer-Rochow (2009), the question arises how these moths are capable of choosing preferred plants from others. It was demonstrated by imaging polarimetry that leaves possess different reflection-polarization characteristics (Horváth et al. 2002) and that matte leaves can be visually distinguished from shiny, smooth leaves (Hegedüs and Horváth 2004). Polarized light might therefore play an important role in host plant recognition of *S. microtheriella*, at least at close range, just as it might do in orientation tasks.

3. Reduction of the rhabdomere of the 8th retinula cell

Owing to its lack of a rhabdomere, the non-participation of the 8th (basal) retinula cell in the formation of the rhabdom is another unique feature of the eyes of *S. microtheriella and E. argyropeza* (Honkanen and Meyer-Rochow
Since the rhabdomere of the 8th retinula cell is usually smaller than those of the regular retinula cells, even in the eyes of larger Lepidoptera (Lau et al. 2007, Lau and Meyer-Rochow 2008, Fischer et al. 2012), it seems that in the eyes of S. microtheriella and E. argyropeza a minimal limit is undercut at which a rhabdomere becomes too small to achieve an acceptable signal-to-noise ratio. It remains unclear if this situation in the eye of S. microtheriella is accompanied by a selective curtail of wavelength discrimination, reducing the range of the perceived wavelength spectrum in these eyes. Although possible, we hesitate to speculate, because spectral sensitivity measurements in Nepticulidae generally and information on the spectral properties of photopigments in the retinula cells and the 8th retinula cell in particular, are still lacking.

4. Reduction of one layer of the tracheal tapetum

A further modification in the ommatidia of S. microtheriella concerns the tracheal tapetum. In S. microtheriella, it consists of just one singular row of circumretinular tracheoles. In contrast, a double layer of tracheoles envelopes each ommatidium in other moth species examined earlier, including some with small eyes (e.g. Fischer et al. 2012). The reduction to one layer is likely to represent a compromise between either losing the effective reflective sheath completely or losing space that can be used to expand the retinula cells and the rhabdom. Since the rhabdom is short in S. microtheriella, an opportunity to have space for a slightly enlarged, i.e. wider rhabdom to increase the total volume of membrane material capable of housing photopigments, could have overridden the advantages of a thicker tapetum.

Minimal limits - functional consequences

In addition to limits of some structural entities, there exist optical limits that are in conflict with a theoretically unlimited size reduction (Barlow 1952, Warrant and McIntyre 1993), like for instance the minimal diameter of the corneal facet lens. With a diameter of 9-10 µm, the facet diameter in S. microtheriella seems to have reached the limit in miniaturization that cannot be undercut (Yagi and Koyama 1963, Barlow 1952). Besides some negative effects by diffraction patterns (known as the airy disc), which are increasing with decreasing facet diameter (see e.g. Warrant and McIntyre 1993 for review of optical limiting factors), the second main draw-back consequence of facet diameters this small,
is that less light can enter the ommatidium. Thus, the sensitivity of the eye, already limited by the diminished rhabdom size (= effective volume to absorb light), must further decrease. The total rhabdom length (distal + proximal part) of S. microtheriella is of a value that Meyer-Rochow and Gál (2004) had calculated as the minimally possible rhabdom length (i.e., 16 µm). This limit was based on the assumption that then still 10% of the light could be absorbed, not taking into account the effect of a reflective tapetum.

In connection with an eye’s sensitivity, it is of interest that the smallest moth species of the genus Stigmella have been mentioned to be mainly active during the day (Johansson et al. 1989), exhibiting a different time preference from the more typical moths of larger size, which use superposition optics and exhibit nocturnal lifestyles. Shifting to a diurnal lifestyle can effectively compensate for a small rhabdom size, because the difference between night and day light intensities amounts to several log units (Warrant and McIntyre 1993).

We believe that turning to a diurnal lifestyle paralleled the change from the phylogenetically inherited lepidopteran superposition eye to a functional apposition eye. At least in S. microtheriella the modification of the visual mechanisms must have taken place, but it may also apply to other and perhaps all Nepticulidae and/or other small taxa. Functional apposition optics in S. microtheriella are mainly established by the dense cover of retinula cell shielding pigments granules tightly surrounding the distal rhabdom, inhibiting cross-talk between adjacent light-adapted ommatidia. Since rhabdoms with larger diameters (rhabdom diameter > 2 µm) can function more efficiently, in respect to light absorption, as light guides than narrower rhabdoms (< 2 µm), which function as wave-guides (Snyder 1979; van Hateren 1989; Warrant and McIntyre 1993), the distal rhabdom of S. microtheriella ought to be able to absorb relatively more light than the distal rhabdom of the slightly larger moth C. ohridella (Fischer et al. 2012).

Pigment migration during dark-adaptation results in a pigment-free area extending from the crystalline cone to the proximal rhabdom, but to what extent this rather narrow pigment free clear-zone could assist superposition in S. microtheriella under low-light conditions is difficult to evaluate on the basis of structural data alone. Functional superposition involving at least a cluster of seven neighboring facets is not likely to be efficiently possible even in the dark-
adapted compound eye of *S. microtheriella*. Meyer-Rochow and Gál (2004) calculated as the maximally possible exit angle of light 28°, based on refractive index values for the crystalline cones, measured for several insect species (see Meyer-Rochow and Gál (2004)). Irrespective of whether this angle is achievable with the small dioptric apparatus of the *S. microtheriella* eye, measurements taken of it reveal that an exit angle of 36° would be needed for the incoming light rays to be fully distributed amongst neighboring ommatidia (= in total seven facets). If the minimal limit calculated by Meyer-Rochow and Gál (2004) would hold true also for *S. microtheriella*, then neighboring proximal rhabdoms can only be partly hit by the incoming light. An exit angle > 36° seems quite unlikely due to the curvature of the eye. The curvature sets the limit, because at a certain exit angle light will not be reflected back by the tapetum to the proximal rhabdoms of neighboring ommatidia. In addition, the large value of 36° is also unrealistic with regard to the small size of the dioptric apparatus, which is below the minimal theoretical limit of 32 µm, calculated by Meyer-Rochow & Gál (2004). Yet, in theory, we cannot entirely exclude superposition, as special adaptations of the refractive index distribution within the crystalline cone may considerably influence the exit angle of light leaving the proximal cone tip. However, the refractive index distribution within the crystalline cone was not measured to date for this or any other small lepidopteran species except *Ephestia kuehniella* (Hausen 1973).

**Conclusion**

Our study of the eye-design of the minute moth *S. microtheriella* gives an insight into one of the smallest eyes of recent Lepidoptera. It confirms the hypothesis of Meyer-Rochow and Gál (2004), according to which in compound eyes with a radius below 250 µm special adaptations are found to compensate for the absence of a wide clear-zone seen as a necessary pre-requisite for superposition vision. The outcome is a type of eye, intermediate between superposition and apposition that is characterized by a broad distal rhabdom of a diameter exceeding 2 µm, placed between the crystalline cone and the proximal rhabdom. Although the typical cellular components of the insect ommatidium (i.e., primary and secondary pigment cells, four cone cells, retinula cells) are still present in this minute moth eye, some modifications as a consequence of the reduced space in the ommatidium are noticeable: the
tracheolar sheath consists of a single layer, the distal rhabdom is made up of just 5 rhabdomeres, a constriction known as the waist separates the distal from the proximal rhabdom and offers space for the retinula cell nuclei, and the 8th retinula cell lacks a rhabdomere. As a consequence of the small size of the rhabdom and the inevitable reduction in sensitivity, *S. microtheriella* seems to have changed its lifestyle and unlike larger moths has adjusted its activity maximum to times of higher illumination levels, in which the possession of a functional apposition eye with a distal rhabdom of a diameter > 2 µm appears to be most appropriate.

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2.4 Miniaturization of the lepidopteran compound eye: a comparative morphological analysis.
Miniaturization of the lepidopteran compound eye: a comparative morphological analysis

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Abstract

As a rule moths were thought to possess compound eyes of the superposition type. However, since ultrastructural investigations on small compound eyes have recently shown the existence of an intermediate eye type combining features of apposition and superposition eyes, it seemed of interest to investigate additional species for a more general comparative analysis. In this study a total of 12 moth species with body sizes ranging from 1.88 to 6.03 mm and eye radii inbetween 63.67 and 184.94 µm were investigated by scanning and transmission electron microscopy. Measurements showed a positive correlation between body size and eye size and revealed size-dependent anatomical adaptations, which are discussed in the context of limiting factors and functional aspects. Although all of the investigated species exhibited the typical organization of compound eyes generally, three different eye designs were found: a) an apposition eye, b) an eye resembling apposition eyes, but featuring a unique cone structure, and c) eyes intermediate in structure between apposition and superposition eyes. The results reveal that in this cases, except the apposition eye of Micropterix aruncella, a clear division into apposition and superposition eyes is not feasible and that functionally a transition between superposition optics and apposition optics appears likely in the smallest lepidopteran eyes, probably found in the eye of Phylloņoryctet medicaginella.

Interspecies comparisons revealed a new type of basal matrix for the Lepidoptera and allowed us to speculate on the most likely number of retinula cells originally present in species of this order of insects.
Introduction

In 1891 Exner recognized that insect compound eyes can be seen to represent two basic types: apposition and superposition eyes. Insects considered crepuscular or nocturnal (like most moths) were thought to possess eyes of the superposition type. This indeed holds true for many species, especially those of medium to large size (Yagi and Koyama 1963), but exceptions were already noticed near to 60 years ago by Tuurala (1954), who termed the untypical eyes “atypical superposition eyes”. These eyes were described on the basis of light micrographs and found only in small-sized species of moths. Tuurala found that in these eyes the crystalline cones were in direct contact with the rhabdoms and that a clear-zone, essential for superposition vision, was therefore missing.

Tuurala’s findings did not find a great deal of acceptance and only recently interest in ultrastructural investigations of small lepidopteran eyes returned after Meyer-Rochow and Gál (2004) had published a theoretical paper, in which the two authors calculated lower size limits for eyes of the superposition type. Based on their results, possessing a compound eye of the superposition type cannot bestow any benefit to eyes with a radius < 250 µm: a limitless reduction in size of the optical structures is not possible (Meyer-Rochow & Gál 2004).

This raised the question about the functional design in species with eyes that have radii below this limit. In three ultrastructural studies of the eyes of two nepticulid (Honkanen & Meyer-Rochow 2009, Fischer et al. 2012b) and one gracillarid species (Fischer et al. 2012a) special adaptations combining features of apposition and superposition eyes (for discussion see Fischer et al. 2012a) were found.

Besides whether these adaptations can be generalised for all moths with an eye-radius smaller than 250 µm, two further question arose: a) are there any size-related adaptations, possibly conditioned by functional reasons and lack of space in the eye and b) is it possible to describe the functional impacts that compound eye miniaturizations might have? In order to answer these questions we carried out a comparative morphological (histological and ultrastructural) investigation of the compound eyes of several moth species of different sizes.
The study was also expected to generate data for future optical investigations into the question of the minimal limit of superposition in these types of eye.

We chose to investigate 12 different species of moths representing the taxa Nepticulidae, Tischeriidae, Gracillariidae and Yponomeutidae. Body sizes in the species investigated, ranged from 1.88 mm to 6.03 mm. Information on small moth eyes already available from Honkanen & Meyer Rochow (2009), Fischer et al. (2012a), and Fischer et al. (2012b), was incorporated into our investigation. For comparison one species of a phylogenetically basal moth, i.e. *Micropterix aruncella* (Scopoli, 1763) of the family Micropterigidae, was investigated. This species is thought to possess apposition optics (see personal comment by Kristensen in Warrant et al. 2003; Yack et al. 2007). Astonishingly, the eyes of *Micropterix* have not earlier been scrutinized by ultrastructural methods.

**Methods**

All species came from the vicinity of Jacobs University Bremen (Germany) and were collected as adults during spring and summertime (Micropterigidae: *Micropterix aruncella* (Scopoli, 1763); Tischeriidae: *Tischeria ekebladella* (Bjerkander, 1795); Gracillariidae: *Cameraria ohridella* (Deschka and Dimic, 1986), Yponomeutidae: *Argyresthia goedartella* (Linnaeus, 1758), *Argyresthia pruniella* (Clerck, 1759)) or were raised from mined leaves collected in late summer and autumn (Nepticulidae: *Stigmella microtheriella* (Stainton, 1854), *Ectoedemia hannoverella* (Glitz, 1872), *E. septembrella* (Stainton, 1849), *E. argyropeza* (Zeller, 1831); Gracillariidae: *Phyllonorycter coryli* (Nicelli, 1851), *P. maestingella* (Müller, 1764), *P. medicaginella* (Gerasimov, 1930), *P. nicellii* (Stainton, 1851), *P. robiniella* (Clemens, 1860), *P. esperella* (Goeze, 1783)). The mined leaves were stored in translucent plastic bags under natural temperature and light regimes over winter at a shaded place outside the lab. The bags were taken indoors to room temperature at the end of January and hatching started within the following weeks.

Transmission and scanning electron microscopy

Following maintenance of the insects under a natural day/night rhythm (shielded from direct sunlight), the heads of specimens to be examined were
decapitated, split in half either during daytime hours under room light conditions or at night in darkness under dim red light, fixed for 12-24 hrs in modified Karnovsky's (1965) fixative solution and postfixed for 2 hrs in 2% OsO4 solution, both solutions being buffered with 0.1 M cacodylate buffer (pH 7.4). For detailed description of the sample preparation see Fischer et al. (2012a). For light microscopy (LM), semithin sections were cut on an ultramicrotome (RMC, Boeckeler Instruments Inc., Tucson, AZ) with a glass knife and stained with 0.5% aqueous solution of toluidine blue on a hot plate for 30 s. Ultrathin sections were cut with a diamond knife and picked up on formvar-coated copper slot grids. The ultrathin sections were stained with lead citrate and 2% aqueous uranyl acetate for 20 min each (modified after Reynolds 1963). The sections were mainly examined under Zeiss EM 900 and Jeol JEM-1011 transmission electron microscopes (TEM), operated at 80 KV. The TEM was calibrated with a calibration grid (Plano S104) before any measurements commenced. For scanning electron microscopy (SEM), dried heads were split into half and glued on sample holders. The dried samples were coated with a layer of approximately 15-nm gold in a sputter coater (Quorum Q150T S, Quorum Technologies Ltd., East Grinstead, UK) and observed under a J EOL J SM-5900 SEM, operated at 20 KV.

Morphometric analyses

Total body sizes as well as lengths of the front wings were measured with a digital calliper. Scanning electron microscopy was used to determine dorso-ventral and anterior-posterior lengths of the eye (i.e., height and width, respectively), total number of ommatidia per eye and facet diameters.

Longitudinal sections for LM were used for measurements of ommatidial lengths, corneal thickness, crystalline cone lengths, extent of distal and proximal rhabdom moieties as well as length of the basal cell (measured as the distance from basal matrix to the distal tip of basal cell's nucleus). Radii of curvature of the eye and corneal facets and interommatidial angles were also measured from longitudinal sections. The broadest anterior-posterior extension (eye width) of the eye was used to measure the radius of curvature of the eye in sections cut in a dorso-ventral direction, by laying a circular measurement tool over the eye surface in connection with image analysing software. The
interommatidial angles were determined at the point of intersection of the normals to the eye surface through the ommatidial axes of central ommatidia.

TEM micrographs of cross-sections were used to measure rhabdom diameters, shapes, and diameters of pigment granules of primary pigment cells (PPC) and secondary pigment cells (SPC) as well as retinula cells. Measurements of rhabdom diameters were taken at the distal tip of the rhabdom and at the maximal diameter of the proximal rhabdom (in case of apposition eyes at the level of the occurrence of the 8th cell.). Longitudinal sections were used to determine cone length, corneal thickness, and shapes and positions of the pigment granules within the retinula cells. The eyes of three individuals (=6 eyes per species) were used for measurements under the SEM, but in the case for *Phyllonorycter maestingella* and *Tischeria ekebladella* the measurements were based on 2 individuals (= 4 eyes each). For examinations involving LM and TEM, three eyes of one sex (same sex as in SEM-investigation) in the light-adapted state were used and one or two eyes of dark-adapted states. To facilitate inter-species comparisons all measurements were gathered on light-adapted eyes using image analysis software (Image J, Rasband, W.S, U.S. National Institutes of Health, Berthesda, MD).

**Results**

*General organization of the eye – external features*

In total the external eye structures of 12 species with body size ranges of 1.88 mm to 6.03 mm (and front wing lengths of 1.72 to 5.7 mm, respectively) were investigated by scanning electron microscopy (Fig.1). The measurements are compiled in Tab. 1, supplemented by measurements from three additional small lepidopteran species, investigated earlier by Honkanen & Meyer-Rochow (2009, *Ectoedemia argyropeza* (Zeller, 1839)), Fischer et al. 2012a (*Cameraria ohridella* (Deschka and Dimic, 1986)) and Fischer et al. 2012b (*Stigmella microtheriella* (Stainton, 1854)). For the latter three species body sizes and lengths of the front wings were also measured.

All of the investigated species reveal a more or less oval to lemon-like eye shape with the longest extension in the dorso-ventral direction (Fig.1). In the case of *Phyllonorycter medicaginella* (Gerasimov, 1930) and *Tischeria ekebladella* (Bjerkander, 1795) a notch is found close to the base of the
antenna (Fig. 1c,d). The surface of the facets is covered by regularly arranged corneal protuberances, known as corneal nipples (Fig. 2). Interfacetal hairs (Fig. 2a,e), positioned in interfacetal spaces of the compound eyes, were only found in *Micropterix aruncella* (Scopoli, 1763) and *Argyresthia goedartella* (Linnaeus, 1758).

Eye sizes in the investigated species ranged from a mean 119.8 µm versus 83.6 µm (dorso-ventral versus anterior-posterior) in the nepticulid *Stigmella microtheriella* to 365.0 versus 314.6 µm in *Argyresthia goedartella*. These two species possess also the smallest and largest mean numbers of facets detected in the investigated species, namely 123 facets and 678, respectively.

In relation to body size (total body length, length of the front wing) a positive correlation between the parameters eye size and number of facets was apparent for the investigated species, both increasing with body size (Fig. 3). Also the facet diameter, ranging from 9.9 µm in *Stigmella microtheriella* to 14.0 µm in *Argyresthia goedartella* follows the general trend of an increase in eye size, facet numbers and body size. Contrary to the parameters eye length (dorso-ventral) and eye width (anterior-posterior), the correlation with the facet numbers proves to be less linear and more exponential with some variable values in the body size range of 4-4.5mm (Fig.3).

**Histology: eye-design**

All investigated eyes share the general composition of the ommatidia, which consist of a dioptric apparatus, comprising a multi-layered cornea and a crystalline cone, and the proximally situated retinula (Fig.4; Fig.5). The cornea is either bi-convex or plano-convex. The subjacent crystalline cone is usually bullet-shaped (Fig.4) and its proximal tip is especially tapered in the in light-adapted eyes of the nepticulid species *Ectoedemia hannoverella*, *Ectoedemia argyropeza*, *Stigmella microtheriella* and the micropterigid *Micropterix aruncella* (Fig.4a,e,f). In all cases the rhabdom is in direct contact with the crystalline cone, but the shape of the rhabdom differs: In *P. medicaginella* and *M. aruncella*, the rhabdom is of a columnar, rod-like shape that has its largest diameter distally and tapers proximally (Fig.4a,b; Fig.5c,d). Contrary to that, in all other species investigated (including *E. argyropeza*, *C. ohridella* and *S. microtheriella*) one can distinguish a distal rhabdom of smaller diameter from a
broader proximal rhabdom (Fig.4a-d; Fig.5a,b), the latter being surrounded by tracheoles that form a tracheal tapetum (see also chapter fine structural organization).

Fig.1 Scanning electron micrographs showing the different outer appearances of the compound eyes of selected investigated species. All pictures are oriented so that the dorsal part is facing up, the anterior side is on the right. a. *Micropterix aruncella*, b. *Argyrethia goedartella*, c. *Phyllonorycter medicaginella*, d. *Tischeria ekebladella*, e. *Phyllonorycter nicellii*, f. *Ectoedemia septembrella*. 
Fig. 2 Scanning electron micrographs showing the outer appearance of the facets of a representative number of investigated species, sorted by facet diameter (from large to small). In all eyes the facets are covered by corneal nipples but only two eyes possess interfacetal hairs (a,e). Note the strongly bulged facets in d and f, the irregularities on the surface are drying artefacts. a. Argyresthia goedartella, b. Tischeria ekebladella, c. Phyllonorycter maestingella, d. Phyllonorycter medicaginella, e. Micropterix aruncella, f. Stigmella microtheriella.
A tracheal tapetum is missing in Ph. medicaginella and M. aruncella. Whereas a continuous transition between distal and proximal rhabdom is present in most of the investigated species, distal and proximal rhabdom regions are separated by a narrow “waist” in the nepticulids Stigmella microtheriella, Ectoedemia argyropeza and E. hannoverella (Fig.4e,f; Fig.5b), leading to an “hourglass”-shaped rhabdom.

### Comparative measurements

Out of the 12 species investigated by SEM, internal eye designs were examined by light microscopy and transmission electron microscopy on a selection of species of different body sizes. Comparative measurements on the ommatidia (Tab. 2) were complemented by additional data available for E. argyropeza from Honkanen & Meyer-Rochow (2009), C. ohridella from Fischer et al. (2012a) and S. microtheriella from Fischer et al. (2012b).
Fig. 3 Correlation between body size and the measured eye-features eye height (dorsal-ventral), eye-width (anterior-posterior) and number of facets. Eye size parameters increase near to linearity, but the number of facets shows a trend to increase exponentially with body size.

As far as was deemed necessary and possible, additional measurements were taken for these species (marked in Table 2). The compound eyes of the investigated species possess eye radii ranging from a mean of 63.67 µm to 184.94 µm and are composed of ommatidia of mean total lengths of 46.42 µm to 107.79 µm (Fig. 6a). For radii as well as ommatidial lengths, the largest values are found in A. goedartella, but the smallest eye radius occurs in S. microtheriella and the shortest ommatidial length in P. medicaginella. Figure 6 reveals a positive correlation between total body size and eye radius and ommatidium length. The interommatidial angle exhibits a trend to decrease with body size and angles between 5.32° in P. esperella and 11.25° in S. microtheriella have been measured (Tab. 2).
Fig. 4 Light micrographs of longitudinal sections through light-adapted eyes of a selection of the investigated species. a. Micropterix aruncella, b. Phyllonorycter medicaginella, c. Phyllonorycter maestingella, d. Argyresthia goedartella, e. Ectoedemia hannoverella, f. Stigmella microtheriella.
Fig. 5 Longitudinal semi schematic reconstruction of different types of ommatidia investigated in this study. a. *Cameraria ohridella* as an example of most of the studied small moths, b. ommatidium typical for the nepticulids *S. microtheriella*, *E. argyropeza* and *E. hannoverella*, c. the ommatidium of *Phyllonorycter medicaginella*, d. *Micropterix aruncella*. C: cornea, CC: crystalline cone, CCN: cone cell nucleus, DRH: dorsal rhabdom, PPC: primary pigment cell; PRH: proximal rhabdom, RC: retinula cell, RC8: eighth retinula cell (basal cell), RCN: retinula cell nucleus, RH: rhabdom, SPC: secondary pigment cell, TR: tracheoles, W: waist.

Additionally to the interommatidial angle, also the angles inbetween the proximal tip of the crystalline cone and A) the tip of the tracheal tapetum of the same ommatidium and B) the neighbouring ommatidium were measured in order to estimate which angles have to be achieved by the dioptric apparatus for possible superposition involving the 6 closest, i.e., neighbouring ommatidia (A, first limit) or even more (B, second limit). Both measurements show an increase, parallel to the increase of the interommatidial angle and a decrease of the eye radius with decreasing body size. The smallest values of angles A and B were found for the largest investigated species *A. goedartella* with respective
angles of 5.30° and 16.27°; the largest values were measured for S. microtheriella with 16.33° and 36.75°. No measurements were taken on P. medicaginella and M. aruncella, i.e., species with columnar, rod-like rhabdoms, for they are in a variety of ways (see below) exceptional.

The two species P. medicaginella and M. aruncella do not fit into the general correlation, because they exhibit significantly smaller ommatidial lengths in relation to body size than any of the other investigated species. Due to the different anatomical make-up of the two species in question, also the ratios between the components of their ommatidia differ from those of the other investigated species (Fig. 6b,c).

The proportion between the dioptric apparatus (cornea and crystalline cone) and the retinula (including the basal cell, proximal rhabdom waist (if present) and distal rhabdom) ranged from 32/68 % (P. medicaginella) to 38/62 % (M. aruncella) (Fig. 6b, Tab.3). P. medicaginella shows the shortest dioptric apparatus of all the investigated species with a mean size of just 14.81 µm, of which the cone is responsible for the proximal 9.98 µm. The remainder is the cornea.

Table 2. (following page) Measured parameters of the eyes of the investigated species, sorted by increasing body size.

* not included in (Fischer et al. 2012a)
** in Honkanen and Meyer-Rochow (2009) the basal cell is included in the measurement of the proximal rhabdom. For the ratios the estimation of 5.45 µm for the basal cell was subtracted from the length of the proximal rhabdom.
*** The cornea is separated into two distinct layers, measured individually by Honkanen and Meyer-Rochow (2009), the sum of both mean values was used for the table.
<table>
<thead>
<tr>
<th>Species</th>
<th>Stigmella microcrotherella (Stainton, 1854)</th>
<th>Ectoedemia argyrolea (Zeller, 1839)</th>
<th>Ectoedemia hannoverella (Gilit, 1872)</th>
<th>Phyllonycteryta medicaginella (Gerisimov, 1930)</th>
<th>Phyllonycteryta maestingella (Müller, 1764)</th>
<th>Cameraria ohridella (Deschka and Dimic, 1986)</th>
<th>Micropterix aeruncella (Scopoli, 1763)</th>
<th>Phyllonycteryta esperella (Goeze, 1783)</th>
<th>Argyresthia goedartella (Linnaeus, 1758)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eye radius</td>
<td>μm  63.67 ± 2.44</td>
<td>79.37 ± 7.31</td>
<td>90.02 ± 0.80</td>
<td>89.67 ± 1.46</td>
<td>106.6 ± 6.6</td>
<td>116.8 ± 4.8</td>
<td>113.29 ± 1.85</td>
<td>133.93 ± 1.16</td>
<td>184.94 ± 6.01</td>
</tr>
<tr>
<td>interommatidial angle</td>
<td>deg. 11.25 ± 1.85</td>
<td>8.51 ± 1.17</td>
<td>7.86 ± 1.70</td>
<td>11.12 ± 2.13</td>
<td>6.08 ± 0.73</td>
<td>7.1 ± 1.0</td>
<td>8.41 ± 1.27</td>
<td>5.32 ± 0.70</td>
<td>6.10 ± 0.75</td>
</tr>
<tr>
<td>first limit exit angle of light</td>
<td>deg. 16.33 ± 2.70</td>
<td>-</td>
<td>9.48 ± 0.89</td>
<td>-</td>
<td>10.65 ± 1.31</td>
<td>9.21 ± 1.51</td>
<td>-</td>
<td>8.78 ± 1.58</td>
<td>5.30 ± 1.02</td>
</tr>
<tr>
<td>second limit exit angle of light</td>
<td>deg. 36.75 ± 2.46</td>
<td>-</td>
<td>28.25 ± 2.39</td>
<td>-</td>
<td>23.60 ± 1.77</td>
<td>22.51 ± 2.36**</td>
<td>-</td>
<td>20.41 ± 1.64</td>
<td>16.27 ± 1.06</td>
</tr>
<tr>
<td>ommatidium length</td>
<td>μm  47.13 ± 1.42</td>
<td>61.27 ± 3.80</td>
<td>67.67 ± 2.39</td>
<td>46.42 ± 1.94</td>
<td>76.40 ± 3.91</td>
<td>80.1 ± 3.4</td>
<td>67.45 ± 4.23</td>
<td>85.52 ± 5.52</td>
<td>107.79 ± 4.31</td>
</tr>
<tr>
<td>radius curvature cornea</td>
<td>μm  5.50 ± 0.40</td>
<td>5.40 ± 0.68</td>
<td>6.74 ± 0.34</td>
<td>7.24 ± 0.23</td>
<td>8.54 ± 0.30</td>
<td>7.8 ± 0.4</td>
<td>8.61 ± 0.52</td>
<td>7.8 ± 0.4</td>
<td>6.10 ± 0.75</td>
</tr>
<tr>
<td>thickness cornea</td>
<td>μm  3.56 ± 0.37</td>
<td>7.29 ***</td>
<td>4.72 ± 0.73</td>
<td>4.84 ± 0.54</td>
<td>5.14 ± 0.60</td>
<td>5.0 ± 0.4</td>
<td>5.26 ± 0.49</td>
<td>5.18 ± 0.47</td>
<td>7.10 ± 0.50</td>
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<tr>
<td>length cone</td>
<td>μm  18.19 ± 0.87</td>
<td>20.13 ± 1.46</td>
<td>26.87 ± 1.08</td>
<td>9.98 ± 0.82</td>
<td>29.96 ± 0.73</td>
<td>27.0 ± 1.4</td>
<td>18.46 ± 1.14</td>
<td>28.96 ± 0.92</td>
<td>36.05 ± 1.66</td>
</tr>
<tr>
<td>length distal rhadom</td>
<td>μm  8.03 ± 0.71</td>
<td>13.52 ± 1.38</td>
<td>12.94 ± 1.74</td>
<td>31.43 ± 2.33</td>
<td>15.01 ± 0.83</td>
<td>15.3 ± 1.7</td>
<td>38.07 ± 3.24</td>
<td>16.78 ± 1.19</td>
<td>23.10 ± 1.25</td>
</tr>
<tr>
<td>waist</td>
<td>μm  2.5</td>
<td>4.61 ± 1.23</td>
<td>4.5 ± 0.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>length proximal rhadom</td>
<td>μm  9.32 ± 0.89</td>
<td>15.45 ± 1.62</td>
<td>17.90 ± 2.95</td>
<td>-</td>
<td>17.43 ± 0.99</td>
<td>15.6 ± 1.8</td>
<td>-</td>
<td>26.04 ± 1.26</td>
<td>25.33 ± 2.48</td>
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<tr>
<td>length basal cell</td>
<td>μm  6.27 ± 0.53</td>
<td>not measured**</td>
<td>5.8 ± 0.95</td>
<td>-</td>
<td>6.15 ± 0.70</td>
<td>6.39 ± 0.70*</td>
<td>-</td>
<td>7.36 ± 1.26</td>
<td>10.46 ± 1.21</td>
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<tr>
<td>diameter distal rhadom</td>
<td>μm  2.86 ± 0.27</td>
<td>3.15 ± 0.47</td>
<td>2.45 ± 0.22</td>
<td>2.98 ± 0.50</td>
<td>1.95 ± 0.15</td>
<td>1.7 ± 0.1</td>
<td>1.67 ± 0.10</td>
<td>1.80 ± 0.22</td>
<td>1.78 ± 0.20</td>
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<tr>
<td>diameter proximal rhadom</td>
<td>μm  4.81 ± 0.33</td>
<td>3.82 ± 0.62</td>
<td>5.86 ± 0.56</td>
<td>2.26 ± 0.20</td>
<td>7.22 ± 0.44</td>
<td>5.7 ± 0.4</td>
<td>1.53 ± 0.08</td>
<td>7.33 ± 0.91</td>
<td>7.83 ± 0.69</td>
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<tr>
<td>ROR distal</td>
<td>%  29.82 ± 4.44</td>
<td>13.91 ± 3.91</td>
<td>29.04 ± 4.23</td>
<td>32.07 ± 4.27</td>
<td>31.09 ± 5.66</td>
<td>23.3 ± 2.3</td>
<td>26.48 ± 5.21</td>
<td>27.28 ± 3.34</td>
<td>20.48 ± 2.85</td>
</tr>
<tr>
<td>ROR proximal</td>
<td>%  89.02 ± 3.34</td>
<td>30.36 ± 8.41</td>
<td>92.40 ± 2.9</td>
<td>39.24 ± 7.67</td>
<td>86.94 ± 2.83</td>
<td>83.1 ± 5.7</td>
<td>21.7 ± 4.08</td>
<td>84.54 ± 5.06</td>
<td>90.89 ± 3.76</td>
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<td>number retinula cells</td>
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</tr>
<tr>
<td>diameter pigment granules prim</td>
<td>μm  0.79 ± 0.07</td>
<td>0.76 ± 0.12</td>
<td>0.67 ± 0.07</td>
<td>0.74 ± 0.09</td>
<td>0.89 ± 0.07</td>
<td>0.77 ± 0.09</td>
<td>0.76 ± 0.07</td>
<td>0.84 ± 0.05</td>
<td>0.69 ± 0.04</td>
</tr>
<tr>
<td>diameter pigment granules sec</td>
<td>μm  0.55 ± 0.05</td>
<td>0.65 ± 0.15</td>
<td>0.50 ± 0.05</td>
<td>0.53 ± 0.07</td>
<td>0.63 ± 0.05</td>
<td>0.48 ± 0.04</td>
<td>0.50 ± 0.05</td>
<td>0.56 ± 0.05</td>
<td>0.59 ± 0.05</td>
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<tr>
<td>diameter pigment granules ret</td>
<td>μm  0.43 ± 0.05</td>
<td>0.66 ± 0.10</td>
<td>0.42 ± 0.06</td>
<td>0.60 ± 0.07</td>
<td>0.56 ± 0.05</td>
<td>0.58 ± 0.05</td>
<td>0.56 ± 0.08</td>
<td>0.63 ± 0.07</td>
<td>0.4 ± 0.07</td>
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<tr>
<td>diameter microvilli</td>
<td>nm  59 ± 4</td>
<td>58 ± 7</td>
<td>60 ± 7</td>
<td>58 ± 6</td>
<td>53 ± 8</td>
<td>64 ± 6</td>
<td>63 ± 8</td>
<td>52 ± 7</td>
<td>68 ± 3</td>
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</tbody>
</table>
Fig. 6 Correlation and ratios of parameters measured for the investigated species. a. shows the positive correlation between body size and eye radius and ommatidium length of the investigated species. b. Comparison of the size of the components of the ommatidia of the investigated species, sorted by ommatidium length (smallest on the left). As the mean values of the components were summed in this figure, deviations in total length can occur in comparison to the measured total ommatidium length in Tab. 2. c. Comparison of the percentages of the different components within the ommatidium of the investigated species.
In species with distinct different distal and proximal parts of the rhabdom, the ratio between dioptric apparatus and retinula is close to 40/60 % in the largest species A. goedartella (42.0/58.0) and P. esperella (40.5/59.5) and in the range of 45/55-48/52 percent for the other species (Fig. 6c, Tab.3). Thus the portion of the retinula increases slightly with ommatidial length, while that of the dioptric apparatus decreases.

The cornea shows an increase in thickness with ommatidial length (with the exception of E. argyropeza) ranging from a mean of 3.56 µm in S. microtheriella to 7.1 µm in A. goedartella and representing 6.1-7.4% of the total ommatidium length in all samples.

Also the crystalline cone increases in length with total ommatidium length, but the percentage of the total ommatidium reveals a decrease in proportion. Disregarding E. argyropeza, the smallest values of 34.3 % (28.96 µm) and 35.0 % (36.05 µm) were found in P. esperella and A. goedartella, respectively. The crystalline cone of S. microtheriella with its mean length of 18.19 µm occupies 38 %.

The ratios between distal and proximal moieties in relation to the total rhabdom (except for Micropterix and P. medicaginella) are very similar, with slightly larger proximal than distal rhabdom regions (Tab.3). Exceptions are found in P. esperella and in the nepticulids, in the latter especially due to the waist separating distal from proximal rhabdom regions. The lowest ratios for the proximal rhabdom, namely 47.0 and 35.5%, occur in S. microtheriella and E. argyropeza, respectively.

The pigmented basal cell body, containing the nucleus of the eighth cell, is situated below the rhabdom, except for M. aruncella where it is found at the side of the rhabdom. In P. medicaginella only a part of the eighth cell is situated below the rhabdom.

The space not covered by the proximal rhabdom was taken as a measure for the extent of the eighth cell. These measurements reveal a ratio of 8.0-11% of the total ommatidium length, except for S. microtheriella, in which the basal cell occupies a slightly higher percentage (13.1%). In addition to the longitudinal extensions of the rhabdoms also their diameters were measured at the distal tips of both distal and proximal rhabdom.
Table. 3 ratios of the components of the ommatidia of the investigated species, sorted by increasing ommatidium length

<table>
<thead>
<tr>
<th>Component</th>
<th>P. medicaginella</th>
<th>S. microtheriella</th>
<th>E. argyropeza</th>
<th>M. aruncella</th>
<th>E. hannoverella</th>
<th>P. maestingella</th>
<th>C. ohridella</th>
<th>P. esperella</th>
<th>A. goedartella</th>
</tr>
</thead>
<tbody>
<tr>
<td>cornea</td>
<td>10.5</td>
<td>7.4</td>
<td>12.0</td>
<td>8.5</td>
<td>6.5</td>
<td>7.0</td>
<td>7.2</td>
<td>6.1</td>
<td>6.9</td>
</tr>
<tr>
<td>crystalline cone</td>
<td>21.6</td>
<td>38.0</td>
<td>33.0</td>
<td>29.9</td>
<td>36.9</td>
<td>40.7</td>
<td>38.9</td>
<td>34.3</td>
<td>35.0</td>
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<td>distal rhabdom</td>
<td>68.0</td>
<td>16.8</td>
<td>22.2</td>
<td>61.6</td>
<td>17.8</td>
<td>20.4</td>
<td>22.0</td>
<td>19.9</td>
<td>22.5</td>
</tr>
<tr>
<td>waist</td>
<td>-</td>
<td>5.2</td>
<td>7.6</td>
<td>-</td>
<td>6.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>proximal rhabdom</td>
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<td>19.5</td>
<td>16.4</td>
<td>-</td>
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<td>23.7</td>
<td>22.4</td>
<td>30.9</td>
<td>24.6</td>
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<tr>
<td>basal cell</td>
<td>-</td>
<td>13.1</td>
<td>8.9</td>
<td>-</td>
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<td>8.3</td>
<td>9.5</td>
<td>8.7</td>
<td>11.0</td>
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<td>dioptic apparatus</td>
<td>32.0</td>
<td>45.4</td>
<td>45.0</td>
<td>38.4</td>
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<td>47.6</td>
<td>46.0</td>
<td>40.5</td>
<td>42.0</td>
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<td>retinula</td>
<td>68.0</td>
<td>54.6</td>
<td>55.0</td>
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<td>proportion rhabdom</td>
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<tr>
<td>distal rhabdom</td>
<td>100.0</td>
<td>40.5</td>
<td>48.1</td>
<td>100.0</td>
<td>36.6</td>
<td>46.3</td>
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<tr>
<td>waist</td>
<td>-</td>
<td>12.6</td>
<td>16.4</td>
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<td>12.7</td>
<td>-</td>
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<td>proximal rhabdom</td>
<td>-</td>
<td>47.0</td>
<td>35.5</td>
<td>-</td>
<td>50.6</td>
<td>53.7</td>
<td>50.5</td>
<td>60.8</td>
<td>52.3</td>
</tr>
</tbody>
</table>

With 3.82 µm *E. argyropeza* had the largest rhabdom diameter in the distal rhabdom, followed by *P. medicaginella* with 2.98 µm. In general the smallest investigated species of the Nepticulidae possessed rhabdom diameters above 2 µm, whereas the other species had diameters below 2 µm in diameter. A linear decrease in rhabdom diameter with increasing ommatidium size is not obvious in the investigated species. However, measurements of the Rhabdom-Occupation Ratio (= percentage of the rhabdom of the total diameter of the retinula cells of one ommatidium measured in cross sections) showed the largest ratios to be present in *P. medicaginella* (32.7 %), *P. maestingella* (31.09 %), *S. microtheriella* (29.82 %) and *E. hannoverella* (29.04%). *E. argyropeza* differs from all other species with a value of nearly 14%.

In contrast to the distal rhabdom diameters, the largest value of 7.83 µm for the proximal rhabdom diameter is present in *A. goedartella*, the largest of the investigated species. The smallest values (2.26 µm and 1.53 µm) were measured in the smallest respective species like *P. medicaginella* and *M. aruncella* with columnar, rod-like rhabdoms. Rhabdom-occupation-ratios ranged from 83.1 to 92.40 % in most
species, but were only 30.36% in *E. argyroeza*, 21.7% in *M. aruncella*, and 39.4% in *P. medicaginella*, the species with columnar, rod-like rhabdoms.

**Fine structural organization**

Independently of the different eye designs described in the histological section, a homogenous ultrastructural set-up of the ommatidia can be described: four cone cells, two primary pigment cells, six secondary pigment cells and eight retinula cells.

**Dioptric apparatus and primary pigment cells (PPC)**

The transparent cornea of all investigated species is multi-layered and of plano-convex or bi-convex shape. The bulging outer surface is covered by corneal nipples, i.e., protuberances of the epicuticle (Fig. 7a). In some samples also a thin subcorneal layer was found (Fig. 8a,b). The adjacent cone sheath is attached to the cornea by microvillar plaques (Fig. 8b). The eucone crystalline cone, built up by 4 crystalline cone cells, is enveloped by 2 primary and 6 secondary pigment cells, the latter adjoining the retinula cells down to the basal matrix. Cross sections of the cones of all investigated species (except *M. aruncella* and *P. medicaginella*) reveal a radial gradient in colour/contrast in toluidine-stained semi-thin sections as well as in osmium-stained ultra-thin sections. This gradient is also present in the proximal strongly tapered tip of the crystalline cone of the nepticulid species (Fig. 7f). The cone of *M. aruncella*, however, appears more homogenous than that of the other species (Fig. 7e), and a so far unique structure was found in *P. medicaginella* (Fig. 8a,c,d). The short crystalline cone of the latter species, possessing a total length of 9.98 µm, contains a spherical structure of different staining intensity in the proximal tip of the cone (with osmium and toluidine). Ventrally, longitudinally-oriented endoplasmatic reticula (affiliated with the spherical structure), reach down as far as to the proximal tip of the crystalline cone (Fig. 8d,e,f). In all species investigated, the proximal tip of the cone is in the contact with the rhabdom (Fig. 7d) and reveals four short cone cell projections, which terminate in blunt ends a small distance below the cone (Fig. 8f). We never observed any cone projections in deeper layers, or found the swollen end of a cone cell close to the basal matrix.
Fig. 7 Transmission electron micrographs of the crystalline cone and the cone-rhabdom transition zone. General setup shown exemplarily on *Phyllonorycter maestingella* (a,b,c,d) a. The dioptric apparatus consists of a multi-layered cornea, covered by corneal nipples and the crystalline cone. The nuclei of the cone cells are situated close below the cornea. b. oblique section through the crystalline cone, exhibiting the nuclear planes of the primary and secondary pigment cells, surrounding the crystalline cone. Note the distinct horizontal gradient within the cone. c. oblique longitudinal sections of the cone reveal the more proximal position of the nuclei of the primary pigment cells and show parallel-oriented microtubule bundles in the cone sheath (arrow)d the crystalline cone is in direct contact with the distal rhabdom. e. cross section through the crystalline cone of *Micropterix aruncella* showing no distinct gradient. F. cross section of the tapered tip of the crystalline cone of *Ectoedemia*

Fig. 8 Transmission electron micrographs of the dioptric apparatus and the cone-rhabdom transition zone in an ommatidium of *Phyllonorycter medicaginella.* a. Longitudinal overview showing dioptric apparatus consisting of cornea and cone and the surrounding pigment cells. A round, more darkly stained structure is visible within the cone. The rhabdom is in direct contact with the crystalline cone. b. Contact zone cornea-cone, the cone sheath is connected to a sub-corneal layer, situated below the cornea, by microvilli (arrows). c. The nuclei of the cone cells, situated close below the cornea, are situated closely to the spherical shaped central region of the cone. d. Longitudinal-oriented endoplasmatic reticula are situated in between the osmiophilic and denser spherically-shaped central region of the cone and the proximal tip of the cone. e. Close up of the proximal tip of the crystalline cone f. Close up of the proximal tip of the cone showing two short blunt cone projections. C: cornea, CC: crystalline cone, CCN: cone cell nucleus, ER: endoplasmatic reticulum, N: nucleus, PPC: primary pigment cell, RC: retinula cell, RH: rhabdom, SPC: secondary pigment cell.
Two primary pigment cells envelope the crystalline cone down to the proximal cone tip (Fig. 7b,c). Their nuclei are positioned in the plane somewhat in the middle of the crystalline cone and their pigment granules exhibit in all investigated species the largest diameters of all pigment granule-bearing cells (Fig. 8a), measuring 0.66-0.89 µm.

**Secondary (interommatidial) pigment cells (SPC)**

The secondary pigment cells, numbering six in all of the investigated species, surround the primary pigment cells and the retinula cells further proximal down to the basal matrix. These cells harbour pigment granules of 0.40 – 0.66 µm in diameter, which are restricted to the distalmost region of the cell, where also the nucleus is located. The very thin proximal elongations (Fig. 9d,f) do not usually contain pigment granules until close to the basal terminal swellings (Fig. 12).

**Retinula cells and rhabdom**

In all species investigated, eight retinula cells are present. Seven of these are associated with the distal rhabdom and their nuclei are positioned in the distal region of the retina. The eighth cell together with its nucleus is situated at the proximal end of the ommatidium. The retinula cells contain pigment granules with diameters of between 0.41-0.65 µm, which are surrounding the distal end of the rhabdom in the light-adapted state (Fig. 8a; Fig. 9c,e; Fig. 10).

With the exception of *P. medicaginella* (Fig. 10) and *M. aruncella* (Fig. 11e), two of the seven retinula cells of the distal rhabdom do not bear pigment granules, or at least are less pigmented (Fig. 9c,d; Fig. 11a-d,f). The position of these two cells, opposite to each other and separated by two retinula cells on one side and three on the other, facilitated the identification of the orientation of neighbouring ommatidia and revealed the presence of an equator in the eyes of *C. ohridella* and *S. microtheriella* (Fig. 9c,d; Fig. 11a,b,c). At the equator the orientation of the rhabdomeres shifts by approximately 90°(Fig. 11a,b,c).

Not always 7 retinula cells contribute their rhabdomeres to the formation of the distal rhabdom: In *S. microtheriella* only 5 retinula cells participate with their rhabdomeres in the distal rhabdom (Fig. 9e) and in *P. medicaginella* the 7th cell starts to participate.
Fig. 9 Transmission electron micrographs of cross sections of the distal tip of the distal (left) and proximal (right) rhabdom. a-b. *Micropterix aruncella* (dark-adapted) c-d. *Phyllonorycter maestingella* e-f. *Stigmella microtheriella*. M: mitochondrion, RC: retinula cell, RC8: eighth retinula cell, RCN: retinula cell nucleus, RCP: retinula cell pigment, SPC: secondary pigment cell, TR: tracheole, black arrows: unpigmented retinula cells, white arrow: secondary pigment cell.
Fig. 10 Transmission electron micrographs of cross-sections of the rhabdom in regular ommatidia and ommatidia from the dorsal rim area of *P. medicaginella* (a-e) and *P. maestingella* (f). a. At the distal tip six retinula cells contribute to the rhabdom in *P. medicaginella*, with microvilli oriented perpendicular to each other. b. The orientation of the microvilli changes to three different planes once the seventh retinula cell joins in the participation with a rhabdomere shortly below the plane shown in a. c-d. Contrary to the situation found in the regular ommatidia, the perpendicular orientation is not disturbed in the dorsal rim area, exhibiting more rectangularly-shaped rhabdoms, after the 7th rhabdomere.
joins. e. At the proximal end of the ommatidium of *P. medicaginella* the 8th retinula cell is positioned, with its nucleus at least partly positioned beside the rhabdom. At this point only few of the other cells still bear small rhabdomeres. f. Cross section through the proximal rhabdoms of *P. maestingella*, slightly above the distal tip of the tracheoles; note the different shapes and microvillus orientations in the regular ommatidia (lower-left corner) and ommatidia of the dorsal rim area (upper-right corner). AX: axon, M: mitochondrion, RC: retinula cell, RC8: eighth retinula cell, RCN: retinula cell nucleus, RH: rhabdom.

a short distance below the cone (Fig. 10a-e). Whereas cross sections of the distal rhabdoms show them to be of circular outline in all of the investigated species throughout the entire eye, rhabdoms somewhat rectangular in cross sections are noticeable in *P. medicaginella* close to the dorso-posterior edge of the eye, which is indicative of a dorsal rim area (Fig.10c,d). As in the other investigated *Phyllonorycter* species, which also exhibits a dorsal rim area (DRA), the orientation of the microvilli is different in the regular ommatidia from those of the ommatidia of the DRA.

Whereas in the regular ommatidia 3 different planes, offset to one another by approximately 120°, are present throughout the distal and proximal rhabdoms (as is also the case in all other investigated species (Fig. 9), in *C. ohridella* 4 planes are present (Fig.11b)), the microvilli of the DRA showing a strict perpendicular orientation to each other (Fig.9b; Fig.10f). One of the planes is always oriented parallel to the border of the eye. The DRA is limited to 1-2 rows of ommatidia.

While a continuous transition between distal and proximal rhabdom is found in nearly all of the investigated species, the waist region of the nepticulids ranging from approximately 2.5 µm in *S. microtheriella* to 4.5-4.61 µm in *E. hannoverella* and *E. argyropeza* is at least partly free of any microvilli (Fig.11c,f). Whereas the proximal rhabdoms of the investigated species are generally of circular outlines in cross section, those of *A. goeadartella* and *P. medicaginella* exhibit more or less flower-shaped rhabdoms in cross sections (Fig.9; Fig.10f). Except for the species like *P. medicaginella* and *M. aruncella* with columnar, rod-like rhabdoms (Fig.9b), the proximal part of the rhabdom is generally surrounded by a tracheal tapetum. Usually neighbouring ommatidia are separated by two rows of tracheoles (Fig.9d), but in the case of *S. microtheriella* it is only one row (Fig.9f). Isolated tracheoles occur sporadically in the eyes of *P. medicaginella* and *M. aruncella*. 
Fig. 11 Transmission electron micrographs of cross sections of the distal rhabdom under light-adaptation. a. Equator in *Cameraria ohridella*. The orientation of the ommatidia is indicated by white arrows pointing at the non-pigmented retinula cells. b. Detail of one rhabdom of *C. ohridella* showing the two pigment free retinula cells. c. *Stigmella microtheriella*: evidence of the equator based on views from the distal rhabdom and the waist region. The orientation of the ommatidia is indicated by white arrows, the black arrow marks a secondary pigment cell. d. Two pigment free retinula cells (marked by white arrows) are also present in the eye of *Argyresthia goedartella*. e. In *Micropterix aruncella* all retinula
cells contain pigment granules. In the waist region of *Ectoedemia hannoverella* no rhabdomeres are present. RC: retinula cell, RCN: retinula cell nucleus, RCP: retinula cell pigment, RH: rhabdom, SPC: secondary pigment cell.

The eighth retinula cell is positioned close to the basal matrix (Fig.12a,b) and is participating only with a small rhabdomere (Fig.12a) to the proximal rhabdom (except for *S. microtheriella* and *E. argyropeza*, where no participation takes place). The nucleus of the eighth cell, positioned below the rhabdom, fills nearly the entire space of the most proximal tip of the ommatidium (Fig.12b). In contrast to all other investigated species the nucleus of the eighth cell is not situated below the rhabdom in *M. aruncella*, but joins the rhabdom laterally along its most proximal part (Fig. 9b). In *P. medicaginella* at least some part of the nucleus is located beside the rhabdom (Fig.10e). Towards its proximal end, the ommatidium is divided from the optical neuropile by the basal matrix (Fig.12b,c,d,f). On the retinal side the terminal swollen endings of the secondary pigment cells, containing several pigment granules (Fig.12b,c,d,f), and the 8th basal retinula cell are attached to the basal matrix (Fig.12b,c). Openings within the basal matrix, through which the axons of the retinula cells can pass, are found centrally in each ommatidium and in interommatidial spaces for tracheoles arising from tracheal cells below the basal matrix (Fig.12). In addition to tracheal cells there are also some subommatidial pigment cells that contribute to the basal matrix from the other side and contain large nuclei and electron-opaque pigment granules (Fig. 12e, f).

**Dark/light-adaptations**

During dark-adaptation the eyes of all investigated species show a distalward displacement of pigment grains within the retinula cells, leading to a nearly completely screening pigment free space (except for the basal 8th retinula cell) between the proximal tip of the cone and the basal matrix (Fig 13; Fig.9a-b). The pigment granules of the primary and secondary pigment cells are restricted to the level between the cornea and the proximal tip of the crystalline cone, but they too shift distalward. In addition to the displacements of the screening pigment granules in connection with the light- and dark-adapted states, distinct changes are also affecting the shapes of the crystalline cones. Whereas in the light-adapted state the crystalline cones are generally proximally tapered (this is a particularly pronounced feature in the nepticulids *S. microtheriella*, *E. argyropeza* and *E. hannoverella*), the proximal
tips of the cones change into blunter and rounder shapes under dark adaptation (Fig. 13).

Fig. 12 Transmission electron micrographs of longitudinal sections of the proximal rhabdom and basal matrix of *Phyllonorycter maestingella* in the light-adapted state. a. The eighth retinula cell, containing a few pigment granules, is situated close to the basal matrix, contributing to the rhabdom a small rhabdomere. b. Near to all the space inbetween the
basal matrix and the rhabdom is filled by the nucleus of the eighth cell. c. Detail of c. showing the terminal swellings of the secondary pigment cells and retinula cell axons passing through the basal matrix. c. Tracheal cells, situated below the basal matrix show large nuclei sizes. e. Detail of d. showing the nucleus of a subommatidial pigment cell and the pigment granules within this cell type. f. The subommatidial pigment cells contributes to the basal matrix (black arrow in f. and d.). AX: axon, BM: basal matrix, M: mitochondrion, N: nucleus, RC: retinula cell, RC8: eighth retinula cell, RH: rhabdom, SOPC: sub-ommatidial pigment cell, SPC: secondary pigment cell, TR: tracheole, TRC: tracheal cell.

Fig. 13 Light micrographs of longitudinal sections through dark-adapted eyes. a. Micropterix aruncella, b. Phyllonorycter medicaginella, c. Argyresthia goedartella, d. Stigmella microtheriella.

**Discussion**

The aim of this study has been to provide a comprehensive comparative description of the external and internal features of the compound eyes of 12 (external features, internal features: 6) different tiny species of moths. Some of the features were shared by all of the species examined and allowed us to predict certain general trends involved in eye miniaturization. However, with regard to the cellular organisation and
ultrastructure of the retinal cells considerable inter-species differences were apparent. These differences will be discussed in the light of adaptations assumed to have accompanied body and head size reductions in the smallest species.

**External features and eye size correlations**

In their comprehensive investigation, involving more than 400 species of medium to large-sized Lepidoptera, Yagi and Koyama (1963) correlated eye size positively with wing length. Our results corroborated the assumption of a positive, near to linear, correlation between eye and body size or front wing length also for small Lepidoptera. In general the eyes of the investigated species take up a substantial part of the head, indicative of the value that optical information must have for the species. In order to make the best use of the available space, in some cases the eyes are reaching close to and even partially around the antennae, leading to notches within the eyes (Fig. 1c,d).

As the dimensions of the head limit the available space for the eyes, the number of facets, but also their diameters decrease with smaller body size. The decrease in facet diameter is of particular interest, as it is accompanied by an increasing amount of diffraction (Warrant and McIntyre 1993). Therefore it can be assumed that the maintenance of a certain number of facets is needed to fulfil the individual optical needs of the species and that this option is favoured over a smaller number of larger, but less diffraction-impacted facets.

In contrast, measurements of facet numbers lead one to suspect that a more exponential rather than linear increase is taking place in connection with larger body sizes. In any case, while facet diameters are increasing, the consequences of diffraction are lessened. Concurrently especially the mid-sized species of our investigation show a diversity of different combinations of eye size, facet numbers and diameters, revealing a variety of options to optimise these eyes to the optical and visual needs of a species. Compound eyes are energetically expensive structures (Niven and Laughlin 2008) und thus have to be focal subjects of evolutionary forces. Different diameters of facets (determining the total number of facets of an eye) affect sensitivity and spatial resolution and since sensitivity and resolution cannot both be increased simultaneously in an eye of a given size (reviewed in Warrant and McIntyre 1993), a compromise has to be reached. Sensitivity and spatial resolution
can only be increased simultaneously through an enlargement of the eye as a whole (allowing for smaller interommatidial angles) and larger eyes, generally, possess more options in fine-tuning the structural correlates towards a species-specific optimal balance of spatial resolution and sensitivity.

Smaller eyes, because of the size limitations imposed on the structural elements and their arrangement in the eye, have fewer options and losses in spatial resolution to improve sensitivity are often the only choice. Further functional interpretations, involving also a discussion of internal eye features, will follow in the paragraphs below.

Eye types

In total three different eye types (Fig.5) were identified in the investigated species: A) apposition optics in *Micropterix aruncella*, B) a so far unique apposition eye design with a specialized cone in *Phyllonorycter medicaginella* and C) an intermediate eye type showing a combination of features of apposition and superposition optics.

**A) Apposition optics**

Noticed already by Kristensen in Warrant et al. (2003) *Micropterix aruncella* possesses apposition optics, which are mainly defined by the characteristics of a homogenous cone and a rod-like rhabdom in direct contact with the cone.

As is typical for apposition optics in Lepidoptera, the dioptric apparatus occupies only a rather short proportion of the total ommatidium (Yagi & Koyama 1963) and the nucleus of the eighth cell is positioned next to the rhabdom (Tuurala 1954). However, contrary to apposition optics described in other insects, the eyes of *M. aruncella* do show longitudinal pigment migrations within the retinula cells during dark-light adaptations. Usually radial pigment migrations occur in the retinula cells of apposition optics (Autrum 1981), but in the eye of the Cabbage Butterfly *Pieris brassicae* (Linnaeus, 1758) radial pigment migrations are confined to the distal retinula cells (Kolb and Schwarz 1980); pigments in the proximal retinula cells migrate longitudinally just like those in the secondary pigment cells. In the nymphalid *Aglais urticae* (Linnaeus 1758) the radial migration of the retinula pigment is combined with a slight vertical migration (Kolb 1985). It seems that also in other species of Lepidoptera with apposition optics, some longitudinal pigment migrations occur in the
retinula cells, although the longitudinal pigment displacements never reach the degree found in the smallest investigated species seen in this study.

**B) The optics of *Phyllonorycter medicaginella***

The eyes of *P. medicaginella* combine features of apposition optics (like small ratio of dioptric apparatus to retinula and rod-like rhabdom in contact with the cone) with a cone that is highly untypical for apposition as well as superposition optics. As this is the species with the shortest ommatidium and dioptric apparatus, one may suggest the cone to be an adaptation for the extremely small size and the need for a short focal length. As cornea and cone both have light-refracting functions, it could be useful to possess structures in the cone to help focus the impinging light, when the required short focal distance cannot be reached by the conventional design. For the time being, the unusual cone structure is therefore considered to represent a size-related adaptational feature of this extremely small eye.

It is, however, quite astonishing that a dorsal rim area with rectangularly-shaped rhabdoms, seen for the first time in small lepidopteran eyes, is present in the small eye of *P. medicaginella*. As in other insects (Anton-Erxleben and Langer 1988), the rhabdomeres of the dorsal rim area in *P. medicaginella* show a different orientation (perpendicularly oriented microvilli) in comparison to the regular ommatidia. Since all other investigated *Phyllonorycter* species show similar perpendicularly arranged microvilli within the dorsal rim area, this suggests that these insects can make use of polarized light (most probably for navigational tasks) and that this feature is kept in *P. medicaginella* although it reduces the total number of regular ommatidia.

As in the apposition optics of *Micropterix*, dark-light adaptation involves mainly longitudinal pigment displacements within the retinula cells. The combined action with pigment migrations in the primary and secondary pigment cells creates a pupil mechanism within the eye.

**C) intermediate eye design***

The majority of the investigated species exhibit an intermediate eye-design, which has been discussed in detail by Fischer et al. (2012a). These species combine features of apposition optics (the dioptric apparatus is in contact with the distal part of the rhabdom) with features typical for superposition optics (gradient within the
crystalline cone (see Nilsson (1990) for correlation of TEM-sections and optical measurements), tracheal tapetum and position of the nucleus of the 8th cell below the rhabdom). Other features include the bottle-shaped rhabdoms and an obvious longitudinal pigment migration within the retinula cells. These eyes show a distinct change in cone shapes between light- and dark-adaptation, and in combination with the pigment migrations this suggests different optical functional needs under light- and dark-adapted states.

**Size-independent ultrastructural features**

Altogether three size-independent aspects occurred in connection with the investigated eyes A) a consistent ultrastructural set-up of the ommatidia B) no size differences of pigment granules and microvilli and C) no cone projections reaching and contributing to the basal matrix.

*A) consistent ultrastructural set-up*

Irrespective of the optical type of the investigated species and the phylogenetic position as well as body size of the investigated taxa, ommatidia of all investigated species possess 4 cone cells, 2 primary and 6 secondary pigment cells, and 8 retinula cells, conforming to the ground pattern of the Tetraconata (Melzer et al. 1997, Dohle 2001, Richter 2002, cf. Paulus 1979, 2000). No reduction in cell numbers can be reported even for the smallest investigated species.

As also the basal group of the Micropterigidae reveals this cell number, and as it is furthermore found in several other groups (Tineidae: Faucheux 1987; Plutellidae: Wang and Hsu 1982; Tortricidae: Hämmerle and Kolb 1987), we consider the possession of 8 retinula cells to represent the original state for all the Lepidoptera.

However, there are lepidopteran species with more than eight retinula cells, e.g., some Pyralidae (9-13 retinula cells: Fischer and Horstmann 1971, Stone and Koopowitz 1976), Geometridae (15 retinula cells: Meyer-Rochow and Lau 2008), Noctuidae (in part 14-16 retinula cells: Horridge et al. 1977) and some day-active butterflies (usually 9 retinula cells: e.g. Ribi 1978, Eguchi 1982). As shown for the butterflies, the additional retinula cell is an R7-alike and can be explained to have evolved from the 8 retinula cell pattern (see Friedrich et al. 2011). In case of the pyralids, retinula cell as well as cone cell numbers varied even in a single eye
Nevertheless, all species examined have in common only one single basal cell and the higher number of retinula cells might therefore be a doubling of the set of the remaining seven retinula cells or could be due to duplications of certain cell-duets in the ommatidium.

Interesting in connection with the number of retinula cells is also the fact that the 8th retinula cell is not reduced for space-saving purposes in *S. microtheriella* and *E. argyropeza*, although this cell is not bearing a rhabdomere. It does, however, contain some screening pigment. A possible reason why this cell is not reduced could be the role that this cell plays during the differentiation of the ommatidium as shown for *Drosophila*. The eighth cell is the first retinula cell to develop during the formation of the ommatidium, and for this reason has also been named “founder cell” (e.g., Tomlinson and Ready 1987). Furthermore, it has been shown that this cell affects the development of the other retinula cells (Karpilow et al. 1996). Although somewhat speculative for Lepidoptera as similar experiments have not been performed for any species in this taxon, it is at least known that the basal cell in *Ephestia kuehniella* is the first retinal cell to develop (Umbach 1934). Friedrich et al. (2011) also speculated for the nocturnal cockchafer *Anoplognathus* sp., in which the 8th cell’s rhabdomere is also missing (Meyer-Rochow and Horridge 1975), that this cell’s essential role as a founder cell precludes a reduction of it.

**B) No size differences within pigment granules and microvilli**

In all of the species investigated the diameters of the screening pigment granules belonging to pigment and retinula cells lie in the range of 0.4-0.8 µm. With diameters of 60 ± 10 nm rhabdom microvilli in all eyes investigated also exhibit no correlation with body or eye sizes. Both values also do not differ in larger insects generally and lepidopterans in particular (Kolb 1985, Hämerle and Kolb 1987, Lau and Meyer-Rochow 2007, Lau et al. 2007, Meyer-Rochow and Lau 2008). Therefore we have to assume that these sizes are functionally optimal and applicable to eyes of all sizes. What determines this limit and whether there are chemical, optical or other physical constraints, remains unknown. An adaptation to the lack of space in small eyes therefore has to involve a reduction in the amount of rhabdion material, leading to less sensitive eyes and poorer vision (fewer microvilli = less light-absorbent material and fewer screening pigment grains = reduced potential to adapt to different light-intensities and protect cells from harmful radiation).
C) Cone cells not contributing to the basal matrix

Another consistent feature of all the investigated species are the short, blunt ending projections of the crystalline cone cells, not reaching down to the basal matrix (Fig.8, Fig.14).

In Hexapoda generally, proximal cone cell processes seem an essential feature to anchor the retinula relative to the optic axis of the ommatidium and to the basal matrix (e.g. Odselius and Elofsson 1981, Meinertzhagen 1991, Melzer et al. 1997). Odselius and Elofsson (1981) in their classification of the arthropod basal matrix always refer to the basal swellings of the proximal cone cell processes as an essential component of the basal matrix.

It is therefore surprising that this apparatus of proximal cone cell roots has never been described from the ommatidia of any lepidopteran (including the species of this study), regardless of phylogenetic position, ommatidial dimensions, lifestyle and optic type of eye. In Lepidoptera the crystalline cones end in a blunt tip; only occasionally the proximal cone tips diversify and form short extensions, which, however, never reach further down into the retinula (e.g. Nepticulidae: *Ectoedemia argyropeza*: Honkanen and Meyer-Rochow 2009; *Stigmella microtheriella*: Fischer et al. 2012b; Gracillariidae: *Cameraria ohridella*: Fischer et al. 2012a; Pyralidae: *Ephestia kuehniella*: Fischer and Horstmann 1971; Crambidae: *Acentria ephemerella*: Lau et al. 2007; Sphingidae: *Deilephila elpenor*: Welsch 1977; Hesperiidae: *Trapezites symmomus*: Horridge et al. 1972; *Parnara guttata*: Shimohigashi and Tominaga 1986; Pieridae: *Pieris brassicae* (Kolb 1977); Nymphalidae: *Aglais urticae* (Kolb 1985)). Given that despite numerous ultrastructural studies of eyes of larger Lepidoptera, proximal cone cell processes have also never been reported from the long and spacious ommatidia of the larger species, it seems unlikely that the cone cell processes should simply have been overlooked or been reduced in concert with the smaller eye size. We therefore hypothesize their early loss in the last common ancestor of the Lepidoptera, making this an additional apomorphy for the entire Lepidoptera. Consequently, a new type of basal matrix can be added to the six ones described by Odselius and Elofsson (1981): the lepidopteran type. This type of basal matrix is only formed by the secondary pigment cells, the retinula cells, tracheal cells and some subommatidial pigment cells (Fig.14).
Fig. 14 Schematic illustration of the basal matrix found in the investigated lepidoptera. From the top side the retinula cells and the swollen endings of the secondary pigment cells are in contact with the basal matrix (BM), which is perforated by the axonal extensions of the retinula cells and the tracheoles of the tracheal cells positioned below the BM. In addition to the tracheal cells, subommatidial pigment cells adjoin the BM from below. AX: axons; BM: basal matrix; RC: retinula cells; SOPC subommatidial pigment cells; SPC: secondary pigment cells; TR: tracheoles; TRC: tracheal cells.

Size related changes

The decrease in eye-radius and ommatidial length and the fact that the facet diameter is not decreasing linearly with the two aforementioned parameters, does not only have optical consequences, but also impacts on the available space to host the various cell types and cell components within an ommatidium. The increase of the interommatidial angle (as a result of the smaller ommatidial length in combination with a slightly decreased facet diameter) results in a compression of the ommatidium (Fig.15) and thus a decrease in the size of the dioptric apparatus and the retinula. The measured ratios revealed in the first instance no minimal size of any component that remained constant and was not undercut in the smaller species. In fact the ratios reveal that of an ommatidia 6.1-7.4 % is given to the cornea (exception E. argyropeza with 12%), 33.0-40.7% to the crystalline cone, 16.8-22.5 percent to the distal rhabdom, 19.5-30.9 % to the proximal rhabdom and 8-13% to the basal cell.

Similar values have been reported from the eyes of larger Yponomeutidae (Yagi & Koyama 1963), e.g., Plutella maculipennis (Linnaeus, 1758) with an ommatidium
length of 122.9 µm, with ratios to the cornea of 5.2%, cone 29.5%, the constricted part of the retinula (corresponding to the distal rhabdom in the smaller species) 30.7%, the thick part of the retinula (= proximal rhabdom) 34.6% and *Yponomeuta polysticta* (Butler, 1879) with an ommatidial length of 184.3 µm with ratios to the cornea of 5.2%, cone 28.9%, the constricted part of the retinula 31.2% and the thick part of the retinula 34.7%.

With respect to space, the plane of the distal rhabdom is of special interest as this is the only area providing space for the nuclei of the retinula cells (except for that of the basal cell) and for the pigment granules needed in dark-light adaptation. It is thus interesting, that the proportion of the distal rhabdom (including here also the waist area as a space to host the nuclei) amounts to around 20-28% in all investigated species and that thus the available space to host the retinula cell nuclei decreases with decreasing ommatidial length. To date nothing is known about dimensional differences of retinula cell nuclei in insect eyes of different sizes generally and whether there is constant ratio between cell volume and nucleus size. However, for apposition optics in the tiny parasitic wasp *Trichogramma evanescens* (Westwood, 1833) with ommatidial lengths of only 34.97 µm in females and 24.29 µm in males (Fischer et al. 2011) it was possible to show how space is allocated to the nuclei of the retinula cells of neighbouring ommatidia in alternating (tandem) positions. This differs from the arrangement in larger hymenopterans (e.g., Perrelet 1970; Skrzipek and Skrzipek 1971, Menzel 1972) and allows for a denser, more compact packing of ommatidia with small facet diameters. This reveals that the size of the nuclei (at least that of the retinula cells) affects miniaturization in small compound eyes.

The following discussion will deal specifically with adaptations found in the eyes of the species investigated in this study, starting with adaptations found in the largest and all other species and ending with adaptations only found in the smallest of the investigated species.

*A) pigment migration within the retinula cells*

In moths of larger sizes shielding between ommatidia during the dark-light adaptation is mainly accomplished by longitudinal pigment displacements within the secondary pigment cells (Warrant and McIntyre 1996). In the species investigated in this study, however, pigment granules of the retinula cells shield the rhabdom, exhibiting a
distalward longitudinal migration during dark-adaptation. Tuurala (1954) described this dark/light adaptation within the retinula cells for other small lepidopteran eyes as well and it has also been reported in *Ephestia kuehniella* (Horridge and Giddings 1971).

Most likely in contrast to the larger moths, the secondary pigment cells in the eyes of the small species do not completely envelope the retinula cell cluster and shading is consequently incomplete. Moreover, turning the retinula cells into the main pigment shield allows for a reduction in the diameter of the secondary pigment cells and in this way leaves more space for the retinula cells and the rhabdom. Indeed a decrease in the diameters of the extensions of the secondary pigment cells is generally observed in our species. In the smallest of the investigated moths, i.e., *S. microtheriella*, only very slender cell-extensions, almost to narrow to accommodate pigment grains, are found (Fig.11; Fischer et al. 2012b). It’s worth noting that the closer the pigment granules are to the rhabdoms, the greater their optical impact on the rhabdoms, for part of the propagating light travels outside a rhabdom when the diameter of the latter is below 2 µm (Snyder 1979) and would then be absorbed by the pigment granules. Therefore an optical functional change accompanies the change from secondary to retinula cell pigment migrations.

It remains an open question whether or not the lack of pigmentation in the two non/less pigmented retinula cells (exept of *P.medicaginella* and *M. aruncella*) is related to a particular function of these cells, but some impact on absolute (or spectral) sensitivities is likely. On account of the presence of such less pigmented retinula cells, this would also apply to the eye of *Plutella xylostella* (Wang and Hsu 1982) and probably to that of *Ephestia kuehniella* as well (Fig.5, Fischer and Horstmann 1970).

**B) Decrease in radius of curvature of the cornea**

The reduction in size of the dioptric apparatus goes hand in hand with decreasing ommatidium length. As the ratios show, corneal thicknesses amounting to 6.1-7.4 % of ommatidial length, remain rather similar across all species with the intermediate eye-type. In combination with facet diameter this results in a decreased radius of curvature of the cornea and, consequently, strongly bulging facets as seen under the SEM (Fig.2). As also shown for small scarab beetles (Caveney and McIntyre 1981) a
decrease in radius of curvature results in a stronger lens and enables light to be focused within the central part of the short crystalline cone. It is thus an adaptation to maintain (or safeguard) the function in the presence of a shorter dioptric apparatus. Given that the outer radius of the cornea influences the way in which incident light is refracted to the cone, a more convexly-curved corneal lens ought to allow light at larger incident angles to be transmitted to the cone, light that in facets with flatter corneal surfaces would be absorbed by the primary pigment cells. The need for the shorter focal length of the corneal lens might therefore also result in an increase of photons transmitted into the ommatidium, albeit at the expense of spatial resolution.

**C) increase in distal rhabdom diameter**

All of the smallest investigated species (i.e., the nepticulids) show distinctly enlarged rhabdom diameters compared to larger species with the intermediate eye type. On the one hand a greater rhabdom diameter increases the total volume of the rhabdom as the small proximal rhabdom cannot be increased further (its ROR is already close to 90%). On the other hand the increase in diameter leads to a change of the optical function of the distal rhabdom (Snyder 1979, Warrant and McIntyre 1993), now functioning as a light-guide (rhabdom diameter > 2 µm) and not as a wave-guide (rhabdom diameters < 2 µm). Since part of the propagated light travels outside the wave-guide (see above), the increase in photon absorption in the smallest species occurs in the distal rhabdom. Extremely tapered cones with similar diameters to those of the distal rhabdoms only occur in the eyes of day-adapted nepticulids and might thus be an adaptation for improved light transmission to the distal rhabdom (compare the mode coupling function found in afocal apposition optics in butterflies (Nilsson et al. 1988)).

**D) Hour-glass shaped rhabdom**

The nepticulids, already mentioned in C) possess hour-glass shaped rhabdoms with no microvilli present in the waist region of the rhabdom. On the one hand the waist region provides some space for at least some of the nuclei of the retinula cells and on the other it allows the retinula cell pigment granules in the light-adapted state to form a "shield" above the proximal rhabdom (Fig.4e-f; Fig.11f). As space for anything else but the distal rhabdom is limited below the cone, the only space available to accommodate the nuclei of the retinula cells is the waist region of the rhabdom. The
increase in diameter of the distal rhabdom in these species clashes with the need to host the nuclei and the pigment granules needed for the light adaptation mechanism. Thus the need for an increase in rhabdom volume in the distal region of the ommatidium seems to be curtailed by the total volume of the cell organelles in the retinula cells, given they cannot be reduced in size.

The maximal rhabdom diameter is furthermore limited by the maximal acceptance angle (\(= \frac{d}{f}\), with \(d=\) rhabdom diameter, \(f=\) focal length) in relation to focal length and interommatidial angle. In order to avoid a negative impact on spatial resolution by overlapping acceptance angles (Snyder 1979) of neighbouring rhabdoms, the acceptance angle should not exceed the angle separating neighbouring ommatidia, i.e., the interommatidial angle, by a wide margin.

**E) Basal cell not bearing a rhabdomere**

Only in the cases of *E. argyropeza* and *S. microtheriella* the eighth retinula cell (i.e. basal cell) is not bearing a rhabdomere. As discussed in Fischer et al (2012b) a possible reason for this might be, that a functional limit at which useful information can still be gathered, is undercut. As no information is available on the spectral sensitivity in these tiny moths, its impossible to judge whether the reduction of the basal cell’s rhabdomere is accompanied by a decrease in the range of the perceived wavelength spectrum.

**F) Reduction of participating retinula cells within the distal rhabdom**

The reduction to only 5 retinula cells, participating in the distal rhabdom of *S. microtheriella*, can be discussed in connection with C) and D). The non-involvement of some rhabdomeres in the distal rhabdom increases the available space for the remaining rhabdomeres, especially if space is limited by the need to have retinula cell pigments and nuclei near the area of the distal rhabdom. Interestingly the two retinula cells, not participating in the distal rhabdom are the two less pigmented cells, described also from the eyes of larger species. As the two cells, nevertheless, contribute to the proximal rhabdom with their rhabdomeres, the latter are not completely lost in these retinula cells. An additional function could be that the two cells in question profit from the presence of the distal retinula cells, filtering the incident light and and thus still possessing a specialized function.
**G) Reduction of one layer of tracheoles**

In all species scrutinized a double-layer of tracheoles is usually present to optically isolate neighbouring ommatidia. Only in *S. microtheriella* the tracheal tapetum is reduced to one layer, increasing in this way the available space for the proximal rhabdom without losing the reflective function of the tapetum totally.

**Functional interpretation eye size**

Rutowski et al. (2009) showed that a positive correlation between eye surface area and body size existed for butterflies and that visual acuity and sensitivity also increased allometrically with body size. This conclusion was reached, because increased facet diameters in combination with increased eye sizes resulted in interommatidial angle decreases (Rutowski et al. 2009). Conversely one should expect the opposite effects with decreasing eye size (Warrant & McIntyre 1990). Indeed, our study shows that total number of facets and facet diameters decrease with smaller body sizes. Due to the fact that facet diameters are not decreasing linearly with a decrease in eye radius, interommatidial angles have to increase with smaller eye sizes (Fig.15). Facet diameters do not fall below 9.9 \( \mu \text{m} \), which is close to the minimal limit of 9-10 \( \mu \text{m} \) suggested by Yagi and Koyama (1963) for Lepidopterans. As diffraction prevents an unlimited size reduction of the facets without optical draw backs (Barlow 1952), less available space for compound eyes on the heads of these tiny species has to result in a decrease in facet numbers and thus also in spatial resolution.

Even more important than spatial resolution has to be sensitivity, for resolution alone is useless for an eye if it is not sensitive enough to absorb sufficient photons to gain meaningful information above a certain noise level (Warrant and McIntyre 1993). Sensitivity largely depends on the facet diameter (responsible for the amount of light entering the ommatidium), rhabdom length/volume as the light-absorbing structure, photopigment density in the visual membranes, and the interommatidial angle in case of superposition optics impacting on the number of facets that can participate in the superposition process (reviewed in Warrant and McIntyre 1993). Additionally, a tracheal tapetum can increase photon capture by reflecting photons that have not initially been captured by the photopigment molecules in the rhabdom.
As all of these parameters, with the exception of the interommatidial angle and photopigment density, decrease with decreasing body size, sensitivity has to decreases as well, but it increases with increasing body size (Fig.15) as shown for butterflies by Rutkowski (2009). The probability for effective superposition (number of participating facets) diminishes with increasing interommatidial angles and reduced distances between the tips of the crystalline cones and the tracheoles (= distal tip of the rhabdom).

However, the loss in sensitivity is best demonstrated by the drastic decrease in mean rhabdom lengths from 48.43 µm in the largest (A. goedartella) to 17.35 µm (excluding the microvillus-lacking waist region) in the smallest (S. microtheriella) of the investigated species. This begs the question how the smallest species can compensate for the loss in sensitivity. Behavioural observations in the field revealed a shift to a more diurnal activity within the nepticulids and especially for the genus Stigmella (Johansson et al. 1989) and for C. ohridella (Kalinova et al. 2003). The same holds true for many Phyllonorycter species, which exhibit calling behaviour during mating in the photophase (Mozuraitis & Buda 2006), and therefore behave quite differently from the crepuscular and nocturnal larger moth species like Ephestia kuehniella, to name but one example (Xu et al. 2008). Only the largest of the species investigated in this study, A. goedartella, is mentioned to be also crepuscular or night-active (Agassiz 1996).

The shift to a diurnal activity and thus to times of higher illumination levels can compensate for the reduced sensitivity in the smallest moths. Simultaneously, in contrast to lower light levels, identical sensitivities may be reached by smaller facet diameters and thus increasing spatial resolution in the diurnal lifestyle. This is accompanied by the question of the minimal number of facets needed to fulfil the visual needs of the animal and how critical differences in diffraction are with regard to resolution in facets of small diameter.

Since in the light-adapted eyes of the investigated species longitudinal pigment sheaths in the retinula cells prevent superposition, the diurnal activity operates in connection with functional apposition optics. With a larger eye size and thus larger rhabdoms, activity at lower light levels is possible, but the question remains if superposition is at all possible in the smallest investigated species, even when fully dark-adapted.
Small eye size vs. superposition process

This question can be split into two: A) Is superposition possible at all and B) if it is possible, what benefit does it bestow on the small insect given the small rhabdom volume in the small insects’ eyes.

Whether superposition is at all an option depends on the dioptric apparatus able to produce an exit angle for the light that makes it possible for the light to reach at least the closest neighbouring proximal rhabdoms. As shown in the measurements taken on the species of this study, besides the decreasing eye radius and increasing interommatidial angle, it is the smaller distance between proximal tip of the cone and distal start of the proximal rhabdom (= distal tip of the tracheoles) that increases the exit angle needed, so that the light from neighbouring ommatidia can reach the central rhabdom.

Resulting from this is the fact that fewer ommatidia will be able to take part in the superposition process with decreasing eye size if no specific adaptations are present in the cone. Meyer-Rochow and Gál (2004) calculated a maximal exit angle of light of 28° and the main question is whether small crystalline cones with lengths of below 20 µm are still able to achieve this angle. Meyer-Rochow and Gál (2004) furthermore calculated a minimal length of 32 µm for the dioptric apparatus on the basis of refractive indices typical for insect eyes. This calculated minimal length for the dioptric apparatus is undercut in the smallest of our investigated species.

Since no interpretation of the properties of the dioptric apparatus is possible on the basis of morphological investigations alone, optical measurements are needed for further discussions. Unfortunately no refractive index data are available for any small moth eye. Only for *Ephestia kuehniella* (Zeller, 1879), a moth that exceeds the investigated species of this study several times in size, refractive indices for the complete dioptric apparatus have been published (Hausen 1973, Vogt 1974).

Measurements on the minimum angle needed for light to be passed to a neighbouring proximal rhabdom and decreasing with the radius of the eye, gave an angle of 16° in the smallest of our investigated species (*S. microtheriella*) and approximately 10° in the medium sized species. It is not unlikely that such an angle can be achieved also by very small dioptric structures, but it is less probable that the second exit angle limit (needed for the superposition process with more than the 6
directly neighbouring facets) is reached, the smaller the eye becomes. This means, that it is likely that 6 surrounding ommatidia are able to channel light to the proximal rhabdom of the focal (central) ommatidium also in the *S. microtheriella*, but that the effect of superposition (= increasing sensitivity by factor 6) can not compensate for the small rhabdom volume and thus a nocturnal lifestyle is not feasible if vision is required for it.

Fig. 15 Comparison of sensitivity impacting parameters for compound eyes of different eye-radius. With the exception of the interommatidial angle all other parameters increase with increasing eye-size. The dotted lines within the schematic drawings indicate the minimal exit angle needed to be exceeded by the crystalline cone, in order to make superposition possible.

The conflict between the need of an increase of the distance between cone and proximal rhabdom to keep the exit angle small (= maintain superposition including at least the neighbouring least 6 ommatidia) and the simultaneous need to maintain an acceptable rhabdom volume can be demonstrated by the very similar ratios for the
distal rhabdom (= distance cone tip-proximal rhabdom, and including the waist region for the smallest investigated species) within a range of 20-28 %. Thus this value seems to be the best working compromise and reveals an attempt to maintain some aspect of the superposition process, just like the change of the cone shape between light- and dark-adaptation does.

The diurnal lifestyle permitted the species to increase the volume of the distal rhabdom, which has little impact on the superposition process under dark-adaptation as only its nearest, direct neighbours channel light to the focal proximal rhabdom in the smallest investigated species. Contrary to that, with increasing size (=larger eye-radius, smaller interommatidial angle, longer ommatidia), the need of a distal rhabdom decreases due to larger proximal rhabdoms and a larger number of facets that can take part in the superposition process. Indeed the presence of distal rhabdoms should become counterproductive as cross light will be absorbed by rhabdoms of ommatidia inbetween the ommatidium through which the light is entering the eye on axis and the proximal rhabdom of the central ommatidium. Furthermore a decrease in diameter of the distal rhabdom increases the resolution of the eye. The close position of the retinula cell pigments to the rhabdom limit the wave-guide modes that are able to propagate within the wave-guide. The less modes are able to propagate, the more the acceptance angle of the ommatidium is decreased (Land and Osario 1990). The pigment migration within the retinula cells, instead of the secondary pigment cells as in larger moths, can thus be interpreted as an adaptation to the diurnal lifestyle resulting in an increased resolution within species not limited in sensitivity by size-related constrains.

Interestingly, also in compound eyes with a radius > 250 µm distal rhabdoms are often present, but never reaching the dimensions found in the smallest moths. In case of Ephestia kuehniella, for which superposition was shown by Cleary (1977), the distal rhabdom measures a little less than 1 µm in diameter (Fischer and Horstmann, 1971) and is thought not to inhibit superposition (Nilsson 1989) and influence it in only a negligible way (Kunze 1979).

However, optical investigations on the crystalline cones of our investigated species are needed to validate our conclusions and to show to what extent a certain exit angle can be sustained in the small crystalline cone and whether special adaptations are present. It will also be interesting to test how the change in cone shape affects
the light-guiding function of the crystalline cone. Special adaptations are not out of the question, as no limits for cone length (=minimal size that is not undercut) were found in the eyes of the intermediate eye type. The example of *Phyllonorycter medicaginella*, in which special structures, deemed to represent optical aids, were present in the proximal region of the cones are case in point.

*Phyllonorycter medicaginella*, a miniaturized superposition eye?

Finally, the question is whether the eye of *Phyllonorycter medicaginella* is expressing one step further into the miniaturization process than what is seen in *S. microtheriella*. The design of the crystalline cone and the intermediate eye design of the other investigated species of the genus *Phyllonorycter* suggest this direction to be more probable than that *P. medicaginella* evolved the crystalline cone structure from apposition optics. In terms of miniaturization the shift to a rod-like rhabdom might cross the limits shown for *S. microtheriella*: the diameter of the rhabdom, limited by the size of the nuclei of the retinula cells in *S. microtheriella*, can be increased due to the fact that the nuclei can be accommodated over a longer distance within an ommatidium that possesses a rod-like rhabdom. The gain in rhabdom length through a reduction in crystalline cone length in this small ommatidium can lead to an increase in rhabdom volume or at least maintain a volume deemed necessary for a hypothetical eye of the same size but with an intermediate eye design. As the retinula cell pigments densely shield the distal part of the rhabdom in the eye of *P. medicaginella*, a proximal separation of neighbouring rhabdoms by a tracheal tapetum might not be critically needed anymore. That a reduction occurs in cases of spatial constraints, was shown for *S. microtheriella* and thus a reduction to single rows of trachea needed for the air supply of the ommatidia is not unlikely. At least one case of a reduced tracheal tapetum is known for larger lepidopterans (Lau and Meyer-Rochow 2007) within the females of *Orgyia antiqua* (winged males, wingless females) revealing this character (possessing a tracheal tapetum) not to be very stable and more functionally conditioned.

However, *P. medicaginella* expresses, even more than the intermediate type of eye, that a functional transition between apposition optics and superposition optics is possible in small lepidopteran eyes. It has become increasingly difficult to hold up the older eye-type categorizations and a functional interpretation on the basis of characters derived from light microscopical information of samples fixed in only one
adaptational state (dark or light) can be errorprone. The comparative investigation of this paper shows how important for a detailed morphological description it is to also consider the ultrastructure of light and dark-adapted eyes; otherwise a functional interpretation of the eyes of small Lepidoptera is not possible. To validate the distribution of eye-types within the Lepidoptera it would therefore clearly be of interest to reinvestigate more of the taxa that had earlier been studied by light microscopical techniques alone (Ehnbohm 1948, Tuurala 1954, Yagi and Koyama 1963). Information obtained from such studies might then also shed more light on the original eye-type of this group of insects.

It is expected that the data base presented in this paper should be of value in future investigations on optics and refractive indices of the crystalline cones and their roles in setting the lower limit for superposition. A full understanding of the functional consequences of miniaturization in compound eyes is impossible without a thorough investigation of the optical properties of the eyes’ components. That is what is still lacking and what is the challenge of the future.

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2.5 Studying nano-structured nipple arrays of moth eye facets helps to design better thin film solar cells.
Studying nanostructured nipple arrays of moth eye facets helps to design better thin film solar cells

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Abstract

Nipples on the surface of moth eye facets exhibit almost perfect broadband anti-reflection properties. We have studied the facet surface micro-protuberances, known as corneal nipples, of the chestnut leafminer moth Cameraria ohridella by atomic force microscopy, and simulated the optics of the nipple arrays by three-dimensional electromagnetic simulation. The influence of the dimensions and shapes of the nipples on the optics was studied. In particular, the shape of the nipples has a major influence on the anti-reflection properties. Furthermore, we transferred the structure of the almost perfect broadband anti-reflection coating to amorphous silicon thin film solar cells. The coating that imitates the moth-eye array allows for an increase of the short circuit current and conversion efficiency of more than 40%.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Long before humans began constructing artificial nanostructures to control the propagation of light, insects like moths were using such structures to improve their vision systems. By covering their eyes’ facets with nanostructured nipple arrays, moths create an artificial layer, which acts as an almost perfect broadband anti-reflection coating. The protuberances on the eye’s facets act as an optically matched layer between the facets and the surrounding air. This layer greatly reduces reflectivity and maximizes the incoupling of light in the eye. Photonic structures such as these can be used as an inspiration to improve the in- or outcoupling of light in optical sensors, solar cells and light emitting diodes (LEDs) [1–3].

Bernhard and Miller were the first to seriously occupy themselves with corneal nipple structures in insect compound eyes [4]. Arrays of corneal nipples, radial or parallel ridges and even corneal hairs have, since then, been observed in a variety of insects [5–7] and a few crustaceans [8, 9]. Corneal nipples are particularly common in nocturnal moths, where they were thought to have a variety of roles to play [5, 10, 11]. In order to utilize and mimic the nanostructured corneal nipple arrays for usage in technological applications, it is imperative to understand the propagation of light in such a system and then to further optimize it to achieve the best performance. The influence of the nipple array on the vision of moths has been investigated by several biologists (e.g. [12, 13]), and effects of nipple height, periodicity and spacing of the nipples on the wave propagation have been investigated by scientists and engineers trying to replicate the structures [14–16]. The influence of the shape of the corneal nipple arrays on the optics of insect eyes has so far been studied only by very few authors (e.g. [11]). Stavenga and coworkers analysed the dimensions and shapes of the nipple arrays by atomic force microscopy (AFM) and fitted the surface profile to determine the effective refractive index of the nipple array. The effective refractive index was used as input parameter to simulate the wave propagation by a
one-dimensional transfer matrix multiplication method [11]. However, such a model is only valid for wavelengths distinctly larger than the dimensions of the nipples. To the best of our knowledge no full electromagnetic study of nanotextured nipple arrays has been carried out to date.

In section 2, we introduce the insects investigated in this study. Moreover, a brief description on the optical model used to solve the Maxwell’s equations in three dimensions is also presented in this section. The results of the optical modelling are described in section 3. In the first part of the results section, we look into the optical properties of corneal nipple arrays for different geometries of the nipple arrays. In the second part, the nanotextured moth-eye nipple arrays were used as the template to construct the amorphous silicon solar cell in substrate configuration. The surface texture reduces the reflectivity of the solar cell and enhances the absorption in the silicon diode layer.

2. Samples and methods

2.1. Insect sample

Corneal nipple arrays of the chestnut leafminer *Cameraria ohridella* (Deschka and Dimic, 1986) were examined by scanning electron microscopy (SEM). The chestnut leafminer (shown in figure 1(a)), known as a widespread pest species in Europe (e.g., [17] and papers cited within), is a moth of small body size, i.e. 4–5 mm. For the SEM-investigation, dried samples of the chestnut leafminer were coated with a layer of approximately 15 nm gold in a sputter coater (Quorum Q150T S, Quorum Technologies Ltd, East Grinstead, UK) and observed under a JEOL JSM-5900 SEM, operated at 20 kV.

An overview of the head morphology is shown in figure 1(b). One compound eye, consisting of about 400 hexagonal facets, is located on either side of the scale-covered head. The facets are arranged regularly in lines (figure 1(c)). Higher magnifications of the corneal surface reveal the presence of highly organized corneal protuberances, the so-called corneal nipples (figure 1(d)). In order to describe the extent and shape of these corneal nipples in more detail, uncoated samples were investigated by AFM. The AFM (Nanosurf Mobile S) was operated in the tapping mode and cantilevers for dynamic scans were used (ACLA-type).

2.2. Optical modelling

An atomic force microscope image of the nipple arrays on the moth eye facet is shown in figure 2. The arrays are closely packed and arranged in a hexagonal grid. A line scan of surface profile from the nipple array in figure 2(a) is shown in figure 2(b). The one-dimensional profile exhibits an almost periodic arrangement of protuberances, with an average diameter of 200 nm. Each nipple has an almost parabolic outline with a height of 70–80 nm. The height profile, $h(x, y)$, of a nipple can be described by

$$h(x, y) = h_0 \left(1 - \frac{x^2}{r_n^2}\right) \left(1 - \frac{y^2}{r_n^2}\right),$$

(1)

where $h_0$ is the maximal height of a nipple and $r_n$ is the radius of a nipple. The equation is valid for $x^2 + y^2 \ll r_n^2$. In order to investigate the influence of the nipples on the optical
properties we numerically modelled the facets using different shapes. By simulating the optics of the different shapes, we investigated the reflectivity of parabolas, cones and pillars. Simulations were performed by solving Maxwell’s equations in three dimensions using a finite difference time domain (FDTD) algorithm. Perfectly matched layer (PML) boundary conditions were set in the direction of the propagation of light. Periodic boundary conditions were assumed in the other two boundary directions. In other words, we assumed a periodic nipple array in the x- and y-directions. A sketch of the hexagonal simulation grid and isometric projections of the different nipple array shapes are shown in figure 3.

The refractive index of the nipples was set to $n_n = 1.52$, which is similar to that of most insect corneae [18–20]. The diameter of the nipples was kept fixed at $d_n = 2r_n = 200$ nm, while the height of the nipples, $h_0$, was varying from 0 to 250 nm. The nipple arrays were positioned in a hexagonal grid where $r_i$ and $r_o$ represent the inner and outer radius of a hexagonal cell, respectively. In the case of maximal packing, the nipple radius $r_i$ is equal to the inner radius of the hexagonal grid. In this case 90% of surface is covered by nipples. For the pillar-shaped arrays, the radius of the pillar was taken such that the proportion of the pillars covers 50% of the area of the unit cell.

3. Results

3.1. Reflectivity of corneal nipple arrays

The anti-reflection properties of the nipple arrays were investigated by varying the shape of the protuberances on the moth eye facet. The reflectivity from such surfaces as a function of the incident wavelength is shown in figure 4. Reflectivity plots for the pillar, conical and paraboloids are shown in figures 4(a)–(c). The reflectivity plot for nipple arrays modelled as circular pillars is shown in figure 4(a). The dashed curve is shown as a reference for reflectivity of a flat substrate. The reflectivity was calculated assuming perpendicular incidence of light, $R = (n_n - 1)/(n_n + 1)^2$. The reflectivity from circular pillar arrays exhibits strong modulations with changing heights of the pillar. 50% of the hexagonal unit cell base area is not covered with the pillars; as a result the interference of reflections from top and base of the pillars causes the maxima and minima of the curves. For a specific height of the pillar, we observe that the reflectivity is reduced to almost zero, albeit only for a very small band of the incident wavelengths. Therefore, circular pillar arrays will not be ideal for applications where a low reflectivity for a broader band is needed (e.g. optical sensors, solar cells). The reflectivity from cone-shaped arrays is shown in figure 4(b). The modulation of reflectivity as seen for arrays with pillars is not seen for cone-shaped arrays. Moreover, the reflectivity steadily decreases as the height of the cone array is increased. For array heights higher than 200 nm, reflectivity is almost zero for wavelengths up to 500 nm. By changing the shape to parabolic arrays, the reflectivity can be minimized for a much broader spectral range. The reflectivity curves in figures 4(c) for parabolic shapes of arrays are almost zero for array heights larger than 200 nm. By adjusting the height of the parabola the reflectivity of the broadband anti-reflection layer can be tuned.

The height of the nipples on the surface of the moth eye was approximately 70 nm. In such a case the reflectivity is mainly reduced for short wavelength down to 300 nm. All investigated moths so far show a spectral sensitivity of around 300–600 nm with peaks in the UV (345–360 nm), in the blue (440–460 nm) and the green (515–530 nm) [21, 22]. A limited number of moths possess an additional fourth peak, positioned...
Figure 4. Reflection as a function of the incident wavelength from moth eye facets covered with corneal nipple arrays with three different shapes—(a) pillar, (b) cone and (c) parabola. The dashed line represents the reference for a moth eye facet without corneal nipples.

in the red at 560–600 nm [21]. Assuming *Cameraria ohridella* possesses the same spectral sensitivity as other investigated moths, the reduced reflectivity by the corneal nipple arrays would have its greatest effect on wavelengths of the UV-band, although reflectivity is also reduced in the blue and green and thus should increase the total amount of light that can be absorbed by the retinae of the moth’s eyes.

For nipples with periods smaller than $\lambda/(2n_{n})$ the propagation of light can be explained by using effective medium theory [23, 24]. The periodic array acts as a refractive index gradient and allows the incident light to couple into the propagating medium with reduced reflection losses. In figure 5, the effective refractive index of an air/nipple interface is shown as the incident light passes through the nipple array. The effective refractive index, $n_{\text{eff}}$, can be calculated based on

$$n_{\text{eff}}(h) = n_a \times \frac{A_n(h)}{A_{\text{base}}} + n_{\text{air}} \times \frac{A_{\text{base}} - A_n(h)}{A_{\text{base}}},$$

where $A_n(h)$ is the area of the nipple as a function of the height of the nipple. $A_{\text{base}}$ is the base area of a nipple. $n_a$ and $n_{\text{air}}$ are the refractive index of the nipple and the surrounding media, which is in this case air. In the case of a parabolic-shaped nipple the effective refractive index is given by

$$n_{\text{eff}}(h) = \frac{\pi}{2\sqrt{3}} \times \left(1 - \frac{h}{h_0}\right) \times \frac{r_n}{r_i} \times (n_a - 1) + 1,$$

where $h$ is the height of the nipple, $h_0$ is the maximal height of the nipple, $r_n$ is the radius of the nipple and $r_i$ is the inner radius of the hexagonal grid. In figure 5 it is assumed that the radius of the nipple is equal to the inner radius of the hexagonal grid. The straight line for the parabola-shaped nipples represents a PML resulting in the lowest possible reflection. Therefore, the reflection properties, as seen in figure 4, are consistent with the refractive index gradient arising from the shape of the nipple arrays. Due to the abrupt change of the refractive index observed for the circular pillar arrays, the reflectivity from such a surface is higher compared to a cone or parabola-shaped nipple array.

Reflection properties from moth eye facets covered with a nipple array were so far discussed for the normal incidence of light. The reflectivity for a nipple array as a function of the incident angle is shown in figure 6.

The heights of the paraboloid nipple arrays were 50, 100 and 200 nm with their period fixed at 200 nm. The reflectivity was calculated for a monochromatic wavelength of 400 nm. The polarization of the incident light was assumed to be circular. Similar to what is observed for normal incidence, the reflectivity is reduced as the height of the corneal nipples is increased. The reflectivity does not change much up to an angle of incidence of 25°.

Hence, nipple arrays on the facet of moth eyes act as an anti-reflection coating. The shape of the nipples allows for almost optimal anti-reflection properties over a broad spectral
range. The parabolic nipple arrays are superior to numerous technical solutions, where simply arrays of pillar-shaped (or tapered pillar) structures are used to minimize optical reflection [25]. Furthermore, the reflection is reduced for all angles of incidence.

3.2. Solar cells with nipple arrays

Reducing the cost and increasing the conversion efficiency is a major objective of research and development on solar cells. In this study we will focus on the improved incoupling of light in a silicon thin film solar cell by utilizing anti-reflection coatings. As a consequence the absorption of the solar cell is increased, which results in an increased short circuit current and conversion efficiency. Most anti-reflection coatings (ARC) are based on quarter wavelength thick layers. Ideally the refractive index of an optimal quarter wavelength anti-reflection coating is given by \( n_{\text{ARC}} = \sqrt{n_1n_2} \), where \( n_1 \) and \( n_2 \) are the refractive indices of the two materials involved. Furthermore, the extinction coefficient of the anti-reflection coating should be as low as possible, so that almost no light is absorbed in the anti-reflection coating. The operation principle of the quarter wavelength layer is based on the destructive interference of waves being reflected at the interface between medium 1/ARC and ARC/medium 2. However, the condition for destructive interference is only fulfilled for a narrow band of wavelengths. For other wavelengths the reflection distinctly increases. The relationship between the thickness of the anti-reflection layer and the optimum wavelength is given by \( 4d_{\text{ARC}}n_{\text{ARC}} = \lambda \), where \( d_{\text{ARC}} \) is the thickness of the anti-reflection coating and \( \lambda \) is the incident wavelength. A significant increase of the reflection is observed for wavelengths different from the optimal wavelength.

The investigation discussed in the previous subsection has shown that parabola-shaped nipple arrays exhibit excellent anti-reflection properties over a broad spectral range. In this subsection we discuss the influence of a moth-eye surface texture on the wave propagation and the absorption of light in an amorphous silicon thin film solar cell. The optical properties of hydrogenated amorphous silicon (a-Si:H) and aluminum-doped zinc oxide (ZnO:Al) layers, which form the diode and front electrode layers of thin film silicon solar cells, are shown in figure 7. In order to illustrate a comparison between amorphous silicon and classical crystalline silicon (c-Si), the optical properties of crystalline silicon are also highlighted in figure 7. The optical data for crystalline silicon have been reproduced from [26]. The refractive indices (or the real part of the complex refractive indices) of a-Si:H, c-Si and ZnO:Al as a function of the incident wavelength are shown in figure 7(a). For wavelengths larger than 400 nm, the refractive indices of a-Si:H and c-Si are comparable. The refractive index of ZnO:Al remains mostly unchanged for varying wavelengths of the incident optical spectrum. In figure 7(b) the absorption coefficients of a-Si:H, c-Si and ZnO:Al as a function of the incident wavelength are shown. The absorption coefficients of a-Si:H and c-Si exhibit large differences. For incident wavelengths of 400–700 nm a-Si:H has a significantly higher absorption coefficient than c-Si. Thus, compared to the thickness of classical wafer-based mono- or multi-crystalline silicon solar cells (around 200–300 \( \mu \)m), the thickness of amorphous silicon solar cells can be much thinner (around 300–400 nm only). The absorption coefficient of ZnO:Al is very low which allows for high transmission into the silicon cell.

The layer sequence of the solar cell used in this study is consistent with amorphous silicon solar cells prepared in industry and academia [27–29]. We modified the classical layer sequence of the solar cell by introducing a back contact.
with a nipple texture. The n-i-p amorphous silicon solar cell in substrate configuration (light enters the solar cell through the front transparent conductive oxide layer) is conformally prepared on top of the textured back contact. A schematic cross section of such a textured amorphous silicon solar cell is shown in figure 8(a). The surface profile of the individual nipples on the surface was assumed to be parabolic. The back contact of the solar cell consists of an 80 nm thick zinc-oxide layer along with a perfectly reflecting metal contact. Following the back contact, the solar cell consists of a 300 nm thick (n-i-p) hydrogenated amorphous silicon solar cell. The n-layer and p-layer both were assumed to be 10 nm thick. Finally, in our simulation study, the solar cell structure is completed with a 500 nm thick aluminium-doped zinc-oxide layer as the front contact electrode. Experimentally, the zinc oxide layer is prepared by sputtering and the silicon layers are prepared by plasma-enhanced chemical vapour deposition (PECVD) [27]. The quantum efficiencies of the solar cells with varying period and height of the parabolic nipple arrays are shown in figures 8(b).

The quantum efficiency is defined as the ratio of the power absorbed by the absorber (i-layer) of the solar cell to the total power incident on the unit cell. The quantum efficiency was calculated using the equation

\[ QE(\lambda) = \frac{1}{P_{eq}} \int \frac{1}{c} \varepsilon_0 \alpha |E(x, y, z)|^2 \, dx \, dy \, dz, \]

where \( c \) is the speed of light in free space; \( \varepsilon_0 \) is the permittivity of free space; \( \alpha \) is the energy absorption coefficient \( \alpha = 4 \pi k/\lambda \), with \( n \) and \( k \) being the real and imaginary parts of the complex refractive index; \( \lambda \) is the wavelength; \( E \) is the electric field and \( P_{eq} \) is the optical input power. Based on the quantum efficiency, the short circuit current can be calculated as

\[ I_{SC} = \frac{q}{hc} \int_{\lambda_{min}}^{\lambda_{max}} \lambda \cdot QE(\lambda) S(\lambda) \, d\lambda, \]

where \( q \) is the elementary charge, \( h \) is Planck’s constant and \( S(\lambda) \) is the weighted sun spectrum (AM 1.5 spectral irradiance). A more detailed description of these calculations for the quantum efficiency and short circuit current density is given in [30]. The quantum efficiency of a 300 nm thick amorphous silicon solar cell deposited on a smooth substrate is also shown in figure 8(b). The quantum efficiency on a smooth substrate is used as a reference. The modulation of the quantum efficiency of the solar cell on the smooth substrate is caused by the optical interference of light in the layers of the amorphous silicon solar cell. Along with it, the quantum efficiencies of solar cells with parabolic nipple arrays with period of 200 nm and heights of 70 and 200 nm are shown. These values for the heights were chosen since the moth eyes exhibit nipple heights in the range of 70 nm (which have good anti-reflection properties for shorter wavelengths < 400 nm), whereas our investigation from the previous section has shown that broadband anti-reflection property (up to 800 nm wavelength) was achieved for nipple heights higher than 200 nm. With the introduction of the texture, an increase in the quantum efficiency up to a wavelength of...
The calculated short circuit current values are given in Table 1. The corresponding quantum efficiencies of the solar cells are shown in Figure 8(b). Table 1. Calculated short circuit values of a-Si:H thin film solar cells deposited on a smooth substrate and with parabolic nipple array texture with heights of 70 and 200 nm. The corresponding quantum efficiencies of the solar cells are shown in Figure 8(b).

<table>
<thead>
<tr>
<th>Substrate Type</th>
<th>Short Circuit Current (mA cm⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smooth substrate</td>
<td>12.00</td>
</tr>
<tr>
<td>Parabola texture (P-200 nm, H-70 nm)</td>
<td>15.20</td>
</tr>
<tr>
<td>Parabola texture (P-200 nm, H-200 nm)</td>
<td>17.65</td>
</tr>
</tbody>
</table>

600 nm is observed. For shorter wavelengths (<600 nm), the enhancement is achieved due to better incoupling of the incident light into the solar cell. Similar to what was observed in Figure 4(c), the higher height of 200 nm of the nipple array resulted in better incoupling of the incident light compared with the nipple array of height of 70 nm. A slight increase of the quantum efficiency in the longer wavelength region is also observed. The reflections from the simulated solar cells on a smooth substrate and with parabolic nipple arrays of two different heights are also shown in Figure 8(c). Consistent with the quantum efficiency plots, the reflectivities from the solar cells exhibit opposite behaviour. The solar cell with parabolic nipple arrays of height of 200 nm shows the least reflectivity up to the wavelength of 630 nm. The solar cell on a smooth substrate, on the other hand, reflects the most since it does not have any optical design for better incoupling of the incident light. The reflectivity for all three solar cells increases significantly as the incident wavelength gets close to the optical bandgap of a-Si:H (around 720 nm). Based on the quantum efficiency, the achievable short circuit current density was calculated assuming a standard (AM1.5) sun spectrum. The calculated short circuit current values are given in Table 1.

Compared with the short circuit current density of 12 mA cm⁻² for a solar cell on a smooth substrate, a gain of 27% and 47% is achieved for solar cells with parabolic nipple arrays of 200 nm periodicity and heights of 70 and 200 nm, respectively. The gain in the short circuit current is caused by improved incoupling of the shorter wavelengths.

In terms of fabrication of such textured arrays, several research groups have already demonstrated that moth-eye anti-reflection coatings grown on silicon wafers or transparent conductive oxide can greatly reduce the reflection losses from the surface [31–34]. Such moth-eye anti-reflection coatings can be of major importance for solar cells where the overall optical wave propagation is dominated by incoupling of light and not by diffraction and scattering of the incident light. This is the case for amorphous silicon solar cells (as discussed herein) and organic solar cells. In both cases the thickness of the solar cell is small or very small in comparison to the wavelength of the incident light. Thus, the proposed concept of integrating the moth-eye structures into the solar cells provides a promising route to enhance the performance of thin and ultra-thin solar cells.

4. Summary

Moths can efficiently couple light into their eyes with the aid of the corneal nipple arrays on their eye facets. Corneal nipples with a parabolic shape create an artificial coating on the facet, which shows broadband anti-reflection properties. These are achieved due to the linear change of the refractive index arising from the geometry of the protuberances’ shapes. In the next step parabolic nipple arrays were applied for texturing amorphous silicon solar cells. The nipple arrays cause a reduction in the reflectivity and an increase of the quantum efficiency for the shorter wavelengths. Compared with the solar cell on a smooth substrate, quantum efficiencies and the short circuit current of the textured solar cells were significantly enhanced.

References

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Chapter 3: Discussion

In order to carve out factors that limit miniaturization in compound eyes and to determine specific functional adaptations in eyes of small insect species, a tiny hymenopteran wasp, *Trichogramma evanescens* (Westwood 1833) (chapter 2.1) and several species of very small Lepidoptera (chapter 2.2; 2.3; 2.4) were investigated in this work. In addition an interdisciplinary approach was utilized to test the optical function of corneal surface structures (so-called corneal-nipples) of *Cameraria ohridella* (Deschka and Dimic, 1986) as photon absorption enhancing structures for solar cells (chapter 2.5).

In the following, the results shall be discussed in an over-arching way, combining and generalising the optical, ultrastructural, functional and phylogenetic data gathered in this doctoral study. In order to address these different aspects, the discussion is split into the following four chapters: I) Correlation of eye-size and body size, the need and costs of visual information, II) Factors limiting eye miniaturization, III) gaining information with small eyes, functional constraints of miniaturization and IV) Ultrastructural set-up of lepidopteran compound eyes and phylogenetic considerations.

3.1 Correlation of eye-size and body size, the need and costs of visual information

Yagi and Koyama (1963) correlated eye size positively with wing length, which also holds true for those lepidopteran species investigated in this study (chapter 2.4). As presented by Yagi and Koyama (1963) and, later on, by Rutowski et al. (2009), it is not only the number of facets that increases with eye size, but also facet diameter. Resulting from this, sensitivity and spatial resolution have to increase with eye size (Rutowski et al. 2009). Contrariwise the facet diameter does not decrease unlimitedly with smaller eye size; the smallest facet diameter so far known from eyes of Lepidoptera was found in *Stigmella microtheriella*, measuring up only to 9.9 µm (chapter 2.3), and thus confirming the minimal limit of 9-10 µm for facets in lepidopterans earlier proposed by Yagi and Koyama (1963). However, as the investigated Hymenopteran *Trichogramma evanescens* (chapter 2.1) showed, even smaller diameters can be found in compound eyes of insects in the natural (versus genetic or laboratory) environment. Although many different species have small eyes
with facet diameters not decreasing below the mentioned size of approximately 10 \( \mu \text{m} \), there are at least some aphids with facet diameters down to 8.3 \( \mu \text{m} \) (\textit{Tinocallis takachihoensis} (Higuchi, 1792), thorax length 539.8 \( \mu \text{m} \)). Nevertheless, also in aphids it is obvious that facet diameters come close to 10 \( \mu \text{m} \) in species with body sizes of around 1 mm (Döring & Späthe 2009). The negative drawback of increased diffraction and less light entering the eye because of smaller facets seems, thus, only to be acceptable in cases in which a certain number of ommatidia can not be undercut.

Yagi and Koyama (1963) furthermore showed that the eyes of male lepidopterans are usually larger than those of females. As insects in which the males are attracted to females mainly by pheromones (Cardé and Minks 1997), larger eye size in males is believed to represent the need for better vision during the flight phase so that obstacles on the way can be detected and avoided (Meyer-Rochow and Lau 2008). For \textit{Manduca sexta} (Linnaeus, 1763) it was actually shown that this insect species needs some visual input to be able to follow odour-plumes (Willis et al. 2011), since specimens with painted compound eyes were not able to track pheromone plumes at all, neither walking nor flying.

Lau and colleagues (Lau and Meyer-Rochow 2007; Lau et al. 2007; Meyer-Rochow and Lau 2008) showed that besides external features (e.g., eye size and facet diameter) also internal parameters are of importance in species with pronounced sexual dimorphisms, which in moths are the special cases of brachypterous (wingless) females. Besides different dimensions of the clear-zone within the two sexes (females have smaller clear-zones in all investigated species) females of \textit{Orgyia antiqua} (Linnaeus, 1758) also lacked a distinct tracheal tapetum (Lau and Meyer-Rochow 2007).

The only small lepidopteran species in the present work for which both females and males were investigated, \textit{Cameraria ohridella} (chapter 2.2) also showed significantly larger eyes (eye height) and a larger number of facets in the males, but no differences in facet diameters. The ultrastructure of the eyes, as also the dimensions of the ommatidia, did not differ between the sexes. Both female and male eyes can therefore be considered to be quite similar with regard to their optical quality. Contrary to that in the hymenopteran \textit{Trichogramma evanescence} (chapter 2.1) differences did occur between males and females. The males, of smaller body size
than the females, possess smaller eyes. With 24.29 µm the total ommatidium length of the male is even smaller than the already exceptionally small ommatidium of the female, measuring 34.97 µm in length. Males of *Trichogramma evanescens* are known to have a smaller activity range than the females (Romeis et al. 1998), which probably also need a considerable input from the optical system for finding host species to lay their eggs on.

That a certain need for optical information is reflected in eye size can be noticed also for other hymenopteran taxa. Gronenberg (2008) showed for the harvester ant *Pogonomyrex rugosus* that workers, females and males had different eye sizes. The eye size differences in this ant were paralleled by a smaller volume of the optical lobes and the brain in general and correlated well with the different complex behaviour repertoires (Gronenberg 2008). Behaviour is certainly not only vision-controlled and other factors are involved, too. However, especially for ants, different adaptations to different activities and visual needs were reported in the past (see Gronenberg 2008) and even a reduction/shrinkage of the optical neuropil was shown for female ant queens after finishing their flight phase (Julian and Gronenberg 2002). Whereas visual information is needed during the flight, the young queens later exhibit negative phototaxis and dig into the soil. In addition to the evolutionary pressure on controlling the importance/need of visual information for a species (reviewed in Niven and Laughlin 2008), the latter example underlines the high cost of gathering visual information, so that even during one insect’s lifetime drastic changes occur when visual information becomes less useful or is not at all of use any longer. Neural tissue, including the afferents of various sense organs (including eyes), is metabolically expensive to develop and to keep in proper action as shown by Laughlin and colleagues (Laughlin et al. 1998, Laughlin 1998, Niven and Laughlin 2008). Furthermore Howard et al. (1987) showed that approximately 8% of the total energy budget of blowflies at rest is expended for the maintenance of the photoreceptors.

The ground principle of functional morphology implies the positive interdependence of developmental/metabolic investment, optimized function and evolutionary success of a given organ. In relation to vision in small insects this reveals that non-functional eyes should not occur in nature due to the decreased fitness through wasted energy irrespective of the size of the animal. It is difficult to define a minimal need/cost, as also theoretically a high cost of “simple” information might be indispensable.
information to successfully reproduce and assure the survival of the species. Thus one can generally assume some visual function to be present as long as eyes are present.

Nevertheless in connection with eye reductions it is certainly an interesting question to explore what happens when a certain threshold is undercut. What triggers the reduction and which structures are affected first on an ultrastructural level? Is it likely that also non-functional, plesiomorphic characters, retained from an ancestral eye design, are found in at least some species?

However, it is possible by conducting optical calculations, behavioural test and investigations of the optical neuropile to validate the general conclusions made on the basis of the morphological description of the eyes, but it would nevertheless be an interesting approach to test in a comparative investigation for similarities and differences in the optics of miniaturized species and species with reduced optics (like for instance cave species).

In case of *Trichogramma evanescens*, the smallest species investigated in this Ph.D study, the functionality of the optical system was proven by behavioural studies performed in the 1950s, which revealed positive phototaxis in this species (Quednau 1958).

### 3.2 Factors limiting eye miniaturization

As it was possible to show that eye size decreases with decreasing body size (chapter 2.1, 2.4) also in small insects, the questions arises how far theoretically an eye reduction is possible and which factors in the miniaturization process can be considered of major impact.

That even landmarks can be used for navigational tasks by insects having eyes of only 60 ommatidia (thus near to half the number of ommatidia found in *Trichogramma evanescens* (chapter 2.1), was demonstrated for the workers of the ant species *Leptothorax albipennis* (McLeman et al. 2002). Although the number of facets is a good parameter to estimate the possible resolution of eyes (Warrant 2001), a small number of ommatidia does not have to be necessarily be correlated directly with lesser function. Furthermore the question of the minimal possible number of ommatidia number needed to maintain a certain function generally depends on the
design and anatomy of the ommatidia as well as the value of the interommatidial angles. Regrettably, McLeman et al. (2002) did not mention the dimension of the facet diameters or other optical parameters for *Leptothorax* and therefore a detailed interpretation is not possible.

It seems reasonable to separate the question of limiting factors of eye miniaturization into a theoretical, optical functional part and an ultrastructural part that focuses on the cellular level and features impacting on the optimal functional use of the available space in the ommatidium.

**Optics and retinula**

Generally optical limitations and the wave-nature of light result in limits that cannot be undercut. The facet diameter limits the amount of light entering the eye, although slight adjustments are possible even at fixed facet diameters. Corneal nipples, found with few exceptions (Razowski and Wojtusiak 2006) in nearly all lepidopterans, are known to act as an antireflective layer (Stavenga et al. 2006), increasing the amount of light entering the dioptric apparatus by up to several percent. The efficiency is size dependent and as the present study shows (chapter 2.2, 2.5) the maximal efficiency is not gained in case of *Cameraria ohridella*. A possible reason for this might be the diurnal activity of this species. The small hymenopteran *Trichogramma evanescens* does not possess such corneal structures (chapter 2.1) and the facets reveal a smooth surface. Within all the investigated species there is no sign that the efficiency of corneal nipples is increased in species with small facets.

The facet diameter itself also affects resolution in two ways: A) by limiting the total number of facets in an eye of given size and B) by increasing the impact of diffraction with decreasing facet diameter (Warrant and McIntyre 1990). It is obvious that facets with a larger diameter will lead to an overall reduction of the total number of facets possible in an eye of a given size and thus affect resolution, although such eyes are less affected by diffraction than smaller facets. It is therefore an interesting question to ponder about the best possible compromise between losing resolution (in terms of fewer but larger facets = larger pixel size) and maintaining resolution provided by a certain number of smaller facets, which are, however, impacted more strongly by diffraction. Resulting from this is the question about the importance of diffraction in optics with poor resolution, as small structures cannot be resolved due to the large
pixel size. In an eye of a given size, in which the minimally needed number of facets is undercut (required to fulfil a certain resolution), it could be favourable to maintain the low number of facets, even if they are of smaller sizes and thereby suffer more from diffraction than larger facets. Supposedly the threshold has to be defined separately for each species in relation to the need of the visual information.

Irrespective of the dimension of the rhabdom diameter and the focal length, a larger facet diameter decreases the size of the airy disc and thus increases resolution. The dimension of the rhabdom diameter in relation to the distance to the aperture (facet) is another factor to be considered, if the airy disc is covering the receptor or if it exceeds its dimensions. In the best possible optics the airy disc should be as small as possible, lying within the dimensions of the receptor. As long as this criterion is fulfilled, two points seen through two facets, separated by one facet from each other, can be resolved (Barlow 1952) and this can be defined as the minimal limit. If there is an overlap within the airy discs on the receptors, the two points can not be resolved anymore, resulting in a drastic decrease in resolution (Barlow 1952), which should be critical in small eyes with a limited number of facets.

Both mentioned aspects can be described as “diffraction limit” but shall not be confused with each other. The latter diffraction limit can be calculated after Snyder (see chapter 2.1) and refers to the best possible optics limited only by diffraction (= better optics are only possible by larger facet diameters). As calculated, *Trichogramma evanescens* does not reach this point and thus the eyes are not diffraction limited in this relation. However, the conclusion taken in chapter 2.1 must be revised in that way that the eyes of *Trichogramma* still are nevertheless effected by the blurring effect of diffraction, increasing the smaller the facets get.

Besides the described features of the cornea and the facet diameter, also the optical properties of the dioptric apparatus have a limiting effect on eye miniaturization, as the dioptric structures need to fulfil the function of focusing the light on the rhabdom. The shorter the crystalline cone becomes, the higher the power of the focusing element must be in order to maintain the focal point on the apex of the rhabdom.

This interaction applies equally well to apposition eyes like that of *Trichogramma evanescens*, in which a focal misalignment will produce a blurred picture, but it is also of interest in superposition eyes in terms of the question if a certain exit angle
from the crystalline cone to the central rhabdom can be achieved to enable and maintain the superposition process. Both questions have in common that a qualified answer is not possible alone on the basis of morphological investigations, just as they were done in part in the present work. Optical investigations are needed, which in the case of *Trichogramma evanescens* are near to impossible to perform due to the small size of this insect’s dioptric apparatus. Irrespective of the practical problem to remove single cones from ommatidia in such a tiny eye, reliable measurements cannot be obtained by interference microscopy in such small objects for the complete cone (Hausen 1973).

In apposition eyes the minimal possible size of the crystalline cone, i.e. approximately the smallest possible focal length, will define the maximal length of the rhabdom in an ommatidium of a given size. As discussed in chapter 2.4, in superposition optics the optical function of the refractive crystalline cone in correlation with the interommatidial angle and the distance to the proximal rhabdom determines the minimal limit of superposition. It is therefore of high interest to examine if adaptations are found within the gradient of refractive indices within the crystalline cone, adapting the dioptric apparatus to the larger angles between neighbouring ommatidia and the shorter distances between the dioptric apparatus and the retina.

As the results of this work reveal (chapter 2.2, 2.3, 2.4), the conclusions reached by Meyer-Rochow and Gál (2004) on the basis of theoretical calculations are proved right, in that special adaptations occur in eyes of small size and that a superposition eye design is not sufficiently beneficial in combination with small eyes to allow species with such eyes to possess a nocturnal lifestyle. An as yet unanswered question is how the inability to perform efficient superposition in an eye with a radius below 250 µm is related to the properties of the crystalline cone. In their calculations Meyer-Rochow and Gál (2004) used fixed values to describe the properties of the cone and a minimal length of the dioptric apparatus; this is undercut in the smallest investigated animals in the present work (chapter 2.3, 2.4). Thus, two different scenarios are possible: A) similar refractive index gradients (as described for larger species) are found within the smaller cones or B) specific adaptations occur. However, even if the same refractive indices were present in the centre of the crystalline cone, already the smaller diameter of the latter should result in a steeper gradient and thus change the light guiding properties. Therefore a different optical
behaviour (in terms of the achievable exit angle) is likely, but it is not possible to say whether the predicted optical change can compensate for the small size of the dioptric apparatus and the short distance to the rhabdom. Without having optical data and further calculations (based on measurements of the refractive indices) at hand no definitive answer can be given.

With regard to sensitivity, minimal limits are set by the dimensions of the rhabdom as it was pointed out by Meyer-Rochow and Gál (2004). Yet, also with small rhabdoms an increase in sensitivity is possible within a certain range. As shown for the Lepidoptera generally (chapter 2.4), a shift of activity times to a diurnal behaviour can increase the light available to be perceived by the eyes by several log units (Land and Nilsson 2002) and a certain sensitivity should therefore also be maintainable in a small eye with short ommatidial lengths if the main activity is shifted to times of higher illumination, i.e. daytime hours. The limit is, of course, set by the maximal radiation available during the day. Further to a behavioural shift in order to maintain a certain sensitivity, the dimensions of the rhabdom could increase parallel to a reduction of the eye’s diameter. As the rhabdom length is limited by the total length of the retinula, it is the diameter of the rhabdom which has to increase. This is indeed seen in several night-active insect species with apposition optics (Greiner et al. 2004, 2007), adapting in this way to lower light levels. In case of very small eyes structurally impacting (see next chapter) and optical factors come into play. The rhabdom diameter affects the rhabdom’s acceptance angle, but an overlap of neighbouring facets needs to be avoided in order to ensure adequate resolution quality. This means that the maximal rhabdom diameter is dependent on the minimal possible focal length (acceptance angle = d/f) and the interommatidial angle. Additionally, space for the retinula cells is decreasing and the limit will be set by the minimal ratio of rhabdomere-to-retinula cell soma, enabling the eye to gather usable visual information under given illumination levels. Thus, the total number of facets in an eye of a given size has to decrease in order to allow larger facets, if minimally needed sensitivity cannot be gathered.

The upper conclusions are valid only for ommatidia functioning and without considering those mechanisms enhancing sensitivity such as optical superposition and spatial as well as temporal summation (Warrant 2004). As shown by Meyer-Rochow and Gál (2004) and also by the comparative investigation on small
lepidopteran eyes in this Ph.D study (chapter 2.4), optical superposition becomes more and more unlikely as an option, the smaller an eye gets and cannot be expected to play a role in combination with the smallest possible functional eye (and ommatidium, respectively). The same should hold true for spatial summation, as resolution will suffer critically as the pixel size already is quite large.

Contrary to the other two mechanisms enhancing sensitivity, temporal summation is possible and can help to increase sensitivity by increasing the integration time (e.g. Warrant 2004). However, even this mechanism has an upper limit. As a matter of fact, long integration times make it impossible to gain precise optical information during movements (flight control) or to detect fast moving objects (predator avoidance), because in both cases visual acuity will be affected by critical motion blur and objects may not be detected at all. Usage of temporal summation can be tested by electrophysiological methods, measuring the cell’s response to very short and dim flashes of light (Pinter 1972, Howard et al. 1984, Frederiksen and Warrant 2008). This method was used in a comparative approach dealing with differences between the dark-adapted and light-adapted eyes of the diurnal blue morpho Morpho peleides (Kollar, 1850) and the crepuscular owl butterfly Caligo memnon (C. & R. Felder, 1866) and made it possible to detect for both species a lower temporal resolution in dark-adapted eyes (Frederiksen and Warrant 2008). It is thus likely that temporal summation is present also in smaller species. Unfortunately, especially in the smallest investigated species studied in the present work, electrophysiological methods seem to be nearly impossible to perform.

**Structural impacting factors**

Size reductions of facet diameters and the eye radius result in a decrease in the total volume of an ommatidium and the available space for all participating components. This stands in contrast to the need of maximal use of space for the rhabdom to permit as much absorption of photons as possible especially when the interommatidial angle becomes too large for superposition. These space-related problems are further increased by the fact that some cellular components cannot be reduced in size (see chapter 2.3, 2.4). Therefore, how can additional space be gained in an insects compound eye without losing functionality?
Generally, it may be tough to clearly identify ommatidial characters that got modified from a reliably reconstructed ground pattern due to the process of miniaturization and did not evolve because of a different, so far unknown functional constraint. One example for the structural consequence miniaturization may cause, as found in *Stigmella microtheriella*, is the elimination of one layer of tracheoles (chapter 2.3). This increases the available space for the proximal rhabdom, without losing the reflective function of the tracheal tapetum (Warrant and McIntyre 1993) altogether and thus the separation of neighbouring ommatidia can be upheld.

Another eye character commonly encountered in small lepidopteran and hymenopteran eyes (chapter 2.1, 2.3, 2.4) and qualified to document deep structural impact of miniaturization concerns the secondary pigment cells. In small lepidopteran eyes, proximal processes of the secondary pigment cells do not bear pigment granules at the level of the rhabdom. In *Trichogramma evanescens* these proximal processes are even reduced completely below the cone level. In terms of functionality, migration of screening pigment granules within the retinula cells is sufficient in relation to dark/light adaptation in both cases and actually is needed in lepidopterans due to the possession of the distal rhabdom. As pigment migration is a feature also found in larger lepidopteran eyes (e.g., *Argyresthia goedartella* (chapter 2.4) and *Ephestia kuehniella* (Umbach 1934)) with more voluminous secondary pigment cells, the primary need for the shift of pigment migration from secondary pigment cells to the retinula cells seems an obvious consequence of the functional need to shade the distal rhabdom (chapter 2.4). Nonetheless, this shift results in a gain of available space for the retinula cells in the smaller species.

In terms of maximising the light absorbing material, i.e. microvilli, another cellular component becomes of interest with regard to the space available: the cell nuclei. Being an indispensable cell component of an eukaryotic cell, the nucleus must be present in each cell and sufficient supply of space is needed to host it. Of particular interest in this context are the retinula cells, bearing after all the visual pigment containing rhabdomeres.

The impact of the cell nuclei in relation to space can be shown by the example of the nepticulids *Ectoedemia argyropeza* (Honkanen and Meyer-Rochow 2009) and *Stigmella microtheriella* (chapter 2.3), both possessing hour-glass shaped rhabdoms in which the plane of the waist coincides with the level the retinula cell nuclei can be
found in. As the nuclei can only be stored within the region ranging from the proximal tip of the cone to the distal tip of the tracheoles, the hour-glass shape seems to be the only possible solution to retain a proximal rhabdom of large diameter and not to change it into a rod-like rhabdom with sufficient spaces for the nuclei as seen in apposition optics.

The possession of a rod-shaped rhabdom increases the distance over which nuclei can be stored within an ommatidium. Space within the retinula can thus be used in a more efficient way by distributing the nuclei to different planes along the rhabdom. In the eye of \textit{Trichogramma evanescens} space is used with even higher efficiency (see chapter 2.1) by alternating the nuclei position of neighbouring retinula cells tandem-wise and creating a line of staggered nuclei. Since somata of neighbouring ommatidia also intertwine, nuclei of retinula cells appear firmly aligned in longitudinal rows. Packaging nuclei as accurate and dense as this, allows the retention of the cone-shape of the ommatidia and their extreme closeness to each other. It also shows that this eye has apparently reached the limit of using the available space, because a further decrease in ommatidium size is incompatible with maintaining sensitivity, given that the diameter of the rhabdom should not become reduced.

Connected to this limit is the question about minimal cell sizes in general and minimal sizes of nuclei in particular. Beutel et al. (2005) estimated ca. 2 µm to represent the minimal limit for cell body size. Recently, Polilov (2012) even reported a case of anucleate neurons for a hymenopteran insect being even smaller than \textit{Trichogramma evanescens}. However, the minimal limit mentioned by Beutel et al. (2005) is not reached in any of those compound eyes investigated within this Ph.D thesis. It might be expected that the limit for functional retinula cells lies considerably above the postulated limit, as the retinula cell soma in addition to the various cytoplasmatic organelles must also bear the rhabdomere. The minimal functional size of the retinula cells should thus be determined by the minimal functional size of the rhabdomere to gain useful information and a minimal cell size to give additionally enough space to host the nucleus. It can be assumed that especially the basal 8\textsuperscript{th} retinula cell is particularly of interest to define the smallest possible size for a retinula cell. Main reason for focusing on the 8\textsuperscript{th} retinula cell as a study model ist that its dimensions are usually the smallest among all retinula cells in an ommatidium, irrespective of the total ommatidial length (Kelber 2006, Honkanen and Meyer-Rochow 2009,
chapter 2.1, 2.2, 2.3). As the comparative morphological investigation (chapter 2.4) shows, the length of the basal 8th retinula cell does not decrease proportionately with decreasing ommatidial length. In those two species, no longer containing rhabdomeres in this cell type, the length of the basal eighth retinula cells is around 5-6 µm, its diameter is approximately 1.0-1.5 µm and the cell space is nearly completely occupied by the nucleus.

As new technologies now allow high-resolution 3D-reconstructions on the basis of serial sections, a comparative investigation of ommatidia of different sizes seems promising to gain further information on minimal limits, as this technique also allows to calculate volumes of the different cells participating in an ommatidium (and their associated cell organelles) and furthermore permits to calculate ratios between cell sizes and the sizes of the corresponding nuclei.

3.3 Gaining information with small eyes, functional constraints of miniaturization

As presented in the previous chapters, sensitivity is restricting small eyes to higher light intensities and thus a diurnal lifestyle, and only a larger eye size allows its owner also activities at lower light intensities. But what do the eyes tell us about their performance and the visual information that can be gained by them?

First of all it is worth to note that the typical design of a compound eye is also present in the smallest of the investigated species, e.g. *Trichogramma evanescens*, and that the eyes do not consist of a smaller number of separated ommatidia as seen in some other small species like male scale insects (Buschbeck and Hauser 2009) or have evolved a small number of large eyelets as in Strepsiptera (Buschbeck et al. 1999). The latter taxon in particular reveals an extreme adaptation and shows a functional design strongly different from that normally associated with compound eyes. The fact that a certain number of closely packed ommatidia exist in all of the species investigated in this Ph.D study, suggests that a certain level of resolution is maintained.

The resolution will not reach the one of larger insects like the 1° in *Apis mellifera*, which corresponds roughly to a pixel size of a fingernail of the small finger held at arm length (Land and Nilsson 2002), but it will allow to discriminate larger objects/areas in order to control flight direction and to avoid obstacles and to gather
important visual information in the course of following odour-plumes in search of females or host plants. This, for sure, is only possible if temporal summation is not increased to a maximum. As a small body size should limit flight speed, slightly longer integration times ought to be tolerable.

In case of *Trichogramma evanescens* the small body size and the behaviour they display while on the wing let one assume that controlled flight is not possible. My own observations reveal that the “flight” of *Trichogramm evanescens* should be more considered an escape behaviour, seemingly initiated by a jump, rather than being a controlled, directed flight. Probably the small size of the wings also plays an important role in relation to the relatively short time the animals spend in the air. This is quite different in all of those lepidopteran species examined in this Ph.D study. These Lepidopera showed an ability to control their flights in terms of speed and direction.

Ocelli (present in almost all Hymenoptera, including *Trichogramma evanescens*, compare chapter 2.1) are believed to take part in controlling flight stability (Wilson 1978). With the exception of *Micropterix aruncella* (Scopoli, 1763), ocelli are not present in the investigated lepidopteran species (chapter 2.2, 2.3, 2.4) and control over space and for orientation must be gained in a different way.

Information provided by the E-vector (= degree of polarized light) is often used by insects for orientational and navigational tasks (reviewed by Labhart and Meyer 1999). In such cases it is usually a certain dorsalmost area in the compound eye, the so-called dorsal rim area (Labhart and Meyer 1999), which is involved in perceiving linearly polarized light. With regard to the investigated species all *Phyllonorycter* species possess such a dorsal rim area. This even applies to the smallest investigated *Phyllonorycter* species, *Phyllonorycter medicaginella* (chapter 2.4). In this case the dorsal rim area consists of only a small number of facets (20-25). This proves that even in the smallest compound eyes inherited, functionally highly specialized regions are conserved, although their presence decreases the total number of regular ommatidia, occupying the rest of the eye. The dorsal rim area can be expected to be still functional in this case, although confirmation by behavioural tests are yet lacking.
Besides chemical information, polarized light might also be involved in the task of finding the host plants – at least in case of leafmining lepidopterans. These are generally monophagous, thus feeding only on one or a restricted number of different plant species (e.g. Johansson et al. 1989), but to date it is unknown which senses are primarily used to identify the specific host plants. Horváth et al. (2002) demonstrated that leaves exhibit different reflection-polarization characteristics and that shiny, smooth leaves can visually be distinguished from matte leaves (Hegedűs and Horváth 2004). Thus, visual information gleaned from patterns generated by polarized light might be part of the signals available to the moths to detect their host plants, given that all of the investigated lepidopteran species posses the requirements to detect polarized light according to the criteria defined by Kirschfeld (1972).

Many visual tasks needed by insects can already be performed by achromatic vision. It is known that the detection of motion and polarization patterns as well as phototactic orientation do all occur purely on the basis of achromatic vision (reviewed in Kelber 2006). However, information on colour clearly increases information available about the environment. It is thus of interest that information on colour does not depend critically on high spatial resolution. Although high resolution increases the total amount of gained information, certain structures/areas might be recognizable on basis of their specific coloration also for eyes with low resolution. The detection of the direction and colour of the light alone can result in useful information. For example, the colour of terrestrial habitats (green-brown) differs considerably from that of the blue sky and also areas in the sky, because the solar half contains more long-wavelength light than the anti-solar half (Kelber 2006), might be distinguishable. In addition UV light is known to be used by insects to detect open areas, when entrapped by foliage (Mazokhin-Porshnyakov, 1969).

Unfortunately so far nothing is known about colour perception in those species that became part of this Ph.D study and if real colour vision (= detection of colours apart from the spectral peaks of the receptors) is possible. In order to separate information about light intensity from colour information, at least two different colour receptors are needed and both receptors need to scan the same visual field (Kelber 2006). The fused type of rhabdom, found in all investigated species, fulfils the latter need.
Larger Lepidopterans and Hymenopterans are known to be at least trichromatic, some species also possess an additional red receptor (Eguchi 1982, Briscoe and Chittka 2001) or may even be pentachromatic (Arikawa 1987). For *Ephestia kuehniella*, the smallest so far investigated moth species in relation to colour vision, only two peaks are recognizable in electrophysiological measurements (called electroretinogram recordings). One is found in the yellow-green around 546 nm and one lies in the UV around 350 nm (Gilburt and Anderson 1996). In addition this investigation also showed the presence of regional differences within the UV sensitivity. Ommatidia located in both dorsal and ventral regions of the eye were more sensitive to UV light than those placed in the equatorial belt.

We may assume that also the smallest moths possess some colour vision, at least on the basis of two different receptors. It is, however, difficult to evaluate the impact of the missing rhabdomere of the 8th basal retinula cell in relation to colour vision, as it is found in *Stigmella microtheriella* (chapter 2.3) and *Ectoedemia argyropeza* (Honkanen and Meyer-Rochow 2009). Generally, knowledge of the role that the rhabdomere of the 8th retinula cell plays in colour vision is relatively scarce due to problems concerning intracellular recordings from these rather small retinula cells (Kelber 2006).

Unfortunatly, small cells are generally problematic to handle in intracellular recording experiments. It is therefore especially difficult if not impossible to perform such recordings in such tiny species. Indeed, extracellular electroretinograms might possibly shed some light on spectral sensitivity peaks in medium sized species, but cannot resolve the question of colour vision and are therefore also not ideal. Besides behavioural tests, the determination of the presence of different opsins in the retinula cells could be one possibility to gain information about the receptors; microspectrophotometrically recorded absorption scans of teased out, individual receptor cells could be another.
3.4 Ultrastructural design features of the lepidopteran compound eye and phylogenetic considerations

Eye types in Lepidoptera – the intermediate eye type

Supplementing the findings of Tuurala (1954) and Yagi and Koyama (1963), the present work enlarges the knowledge about the eyes of Lepidoptera and underscores the importance of information on their ultrastructural organization. For a complete description of the various eye-types, but also for the description of the fine structural architecture of the compound eyes only a combination of light microscopical and transmission electron-microscopical investigations can meet the challenge. Details in relation to the presence, position, shape, and dimensions of the distal rhabdom are particularly essential to draw substantial hypotheses on functional adaptations of investigated ommatidia.

In agreement with earlier work on compound eyes the importance of including dark- and light-adapted eyes in the investigation has been confirmed. Without it the longitudinal pigment migration in the retinula cells, important information for the functional interpretation, as well as the changes in shape that the crystalline cones are capable of would not have been detected.

Understandably and resulting from the comments above, findings purely based on light microscopical studies have to be treated with caution and should be validated by TEM investigations. At least for two examples, Ectoedemia septembrella (Stainton, 1849) and Tischeria ekebladella (Bjerkander, 1795), which were mentioned by Tuurala (1954) to possess apposition optics and superposition optics, respectively, the intermediate eye type was found to be present when re-investigated by TEM (Fischer unpublished). Actually, Tuurala (1954) himself already noted that in the smallest eyes he investigated in the 1950s a detailed description was not possible on the pure basis of his light micrographs.

Nevertheless, as the present results show (chapter 2.2, 2.3, 2.4) Ehnbohm (1948) and Tuurala (1954) were certainly not mistaken with their description of an atypical superposition type of eye in small lepidopteran species, although both contain uncertainties and (as we now know) wrongly interpreted the eyes of some small small species as eyes with apposition optics. In Tuurala’s (1954) description the
presence of a tracheal tapetum was not reported in most of the eyes with the atypical eye type. An examination of the valid species names of species studied by Tuurala revealed, with one exception a small body size with wingspans of 13-26 mm in species with the atypical eye type, belonging to the *Glyphipterigidae*, *Tortricidae*, *Momphidae*, *Geometridae* and *Noctuidae*.

In addition to the results of this Ph.D study (chapter 2.4), the intermediate eye type is also found in the diamondback moth (*Plutellidae*, Wang and Hsu 1982) *Plutella xylostella* (Linnaeus, 1758) and most likely also at least in some of the species mentioned by Tuurala (1954). Based on present comparative morphological data, the presence of the intermediate eye type seems to be restricted to small ommatidia in principle. As miniaturization has taken place in several lepidopteran ingroups, intermediate optics co-evolved with small compound eyes many times independently, regardless of phylogenetic inheritage. Based on the eye size to body size correlations (described in chapter 2.4), it seems legitimate to conclude that the intermediate eye type should also be present in additional groups, provided they contain species with wingspans below 15-20 mm (compare Fig. 5).

In the following the idea shall be discussed, how functional needs may have led to the evolution of the intermediate eye type.

Accepting the fact that eye size is correlated to body size, small body size results in an eye not sensitive enough for a nocturnal life style, irrespective of whether apposition or superposition type being used. This is based on the assumption that photopigment density cannot be increased within the rhabdomeric microvilli, which is likely, as no changes in the microvillar diameters were discovered (chapter 2.1, 2.4). The shift to a diurnal lifestyle and thus access to more light would affect the options to adapt an eye for sensitivity and resolution optimally.

Assuming the best scenario in which the optics of the investigated moth eyes focus the incoming light to a point on the apex of the distal rhabdom (light-adapted state), the focal length will then be of a dimension close to the length of the crystalline cone (e.g. 36.05 µm for *Argyresthia goedartella*). Using the measured dimensions given in chapter 2.4 and combining the data with the sensitivity function (see introduction) reveals a sensitivity for the largest investigated moth *Argyresthia goedartella*, which is close to that of *Apis melifera* ($S_w = 0.22$, Warrant et al. 1996), namely $S_w = 0.28$. 
This value is also not critically changed, when the estimation of the focal length deviates of around $\pm$ 5 $\mu$m ($S_w = 0.22 - 0.38$). According to Yack et al. (2007) this sensitivity value (around 1/100 of that of nocturnal lepidopterans) fits the range in which diurnal butterflies operate. Having a similar sensitivity value as that shown for *Apis, A. goedartella* is no light-limited under day-light conditions. The small diameter of the distal rhabdom can be furthermore interpreted as a reduction of the acceptance angle of the rhabdom (small $d$ in relation to $f = \text{increasing spatial resolution}$) in comparison to a similar eye with a missing distal rhabdom (= longer focal length $f$ and broader rhabdom diameter $d$, both impacting on the acceptance angle $\Delta \rho$). This might thus explain, why distal rhabdoms in contact with the crystalline cone do also occur in larger eyes of diurnal species like *Phalaenoides* (Horridge et al. 1977).

In case of larger eyes, the diameter of the distal rhabdom is decreased down to a diameter of around 1 $\mu$m (Fischer and Horstmann 1971, Wang and Hsu, 1982, Bernard et al. 1984, Lau et al. 2007), probably a compromise between remaining minor optical information during the day and reduced impact during superposition at lower light levels. The longitudinal pigment migration inside the retinula cells leads to a sleeve screening and enveloping the rhabdom (the latter functioning as a waveguide), and guarantees optimal performance in that no stray light can cross between neighbouring ommatidia. With increasing eye size (not necessarily implying that larger eyes evolved from smaller eyes), the distal rhabdom becomes increasingly crucial and even counter-productive as the eyes are sufficiently sensitive for crepuscular/nocturnal activities due to smaller interommatidial angles and an increasing number of facets participating in the superposition process. The opposite situation is characteristic for ommatidia in miniaturized compound eyes. In order to maintain the minimally needed sensitivity in the light-adapted state, the distal rhabdom diameter increases as superposition is not possible due to the pigment sheath. As superposition, if possible at all, will only concern neighbouring ommatidia, larger diameters of the distal rhabdom are not as critical as in the larger eyes. Anyway, larger diameters will increase the acceptance angle and decrease spatial resolution. The smaller the eyes becomes, the more the distal rhabdom diameters approach values typical of the proximal rhabdoms. As the appearance of the hourglass shaped rhabdom seems to be linked directly to the space needed for the retinula cell nuclei, it would make sense to possess a rod like rhabdom in smaller
eyes. But in the studied nepticulid eyes the relation between sensitivity/resolution seems optimal with this hourglass-shaped rhabdom design.

Until now one aspect was not included in the deliberation: the shape of the crystalline cone. In the second smallest investigated eye, i.e. that of *S. microtheriella*, a tapered elongated cone shape is developed, but this is not the case in the smallest eye studied, i.e. that of *P. medicaginella*. The latter possesses a crystalline cone that features an electron-dense, spherical structure in its middle. With a tapered cone design, the light-guide starts in the cone, thus making the dioptric apparatus probably more efficient in terms of light loss and thus allows keeping the acceptance angle small. Maybe even similar mode-coupling effects as in butterfly eyes are taking place in these tapered cones (compare Nilsson et al. 1988). Contrary to that, the extremely short cone of *P. medicaginella* (measuring half of the size of that of *S. microtheriella*) is too short to maintain its proper function and simultaneously possess a tapered design. The distance between the spherical, dense structure within the cone and the distal tip of the rhabdom is so short that it seems difficult to catch sufficient light without an increase of the rhabdom diameter.

Based on the latter examples, it seems arguably more economic that the spherical cone structure has derived from a superposition cone, found in all other small investigated eyes of the taxon *Phyllonorycter*, and not a new “invention” from an apposition eye (not saying that superposition eyes are the original eye type of all lepidopterans). The rod-shaped rhabdom might therefore be the logic outcome of a need to broaden the distal diameter of the rhabdom for sensitivity/efficiency reasons and simultaneously to provide sufficient space to host the nuclei. As presented earlier the nuclei can be stored over a longer distance in case of a rod-shaped rhabdom in comparison to an ommatidium of similar size with an intermediate eye design, as on the later case near to all the space within the proximal part is blocked by the proximal rhabdom. As superposition is no longer an option in this kind of eye and, thus, no separation of the proximal parts of the rhabdom is needed anymore and the absence of a distinct tracheal tapetum in *P. medicaginella* is probably explained as well.

The presented results reveal that the established categories of apposition and superposition lose more and more of their meaning: first through the description of
afocal apposition optics (Nilsson 1984), then by the findings of intermediate eye types, and even more so by the description of the more or less non-categorizable compound eye of *P. medicaginella*. The investigation shows that in order to adapt to small size and a diurnal lifestyle, a change in the eye design from superposition to a functional apposition eye is possible. The boundary between pure superposition optics and the intermediate eye type depends on the properties of the cone, its distance to the proximal rhabdom and the diameter of the distal rhabdom. In order to describe the minimal limit for superposition optics, optical investigation and calculations on the basis of accurately measured refractive indices and quantitative morphological data (chapter 2.4) are needed in the future.

**Eye types in Lepidoptera – apposition versus superposition**

Already for decades the unsolved question which eye type is the ancestral one in Lepidoptera, apposition or superposition, has kept scientists pondering. A convincing interpretation in favour of one of these two principles of vision is hindered by the fact that evolution of different eye types is believed to have taken place several times independently (Yack et al. 2007). Yack et al. (2007) recently reviewed what is actually known about the distribution of apposition and superposition optics among the basal lepidopteran ingroups and listed their activity times based on the best available information (Fig. 6).

**Figure 6. Activity times and eye types found in the basal groups of Lepidoptera (dendrogram adapted from Yack et al. 2007).**


On basis of mainly diurnal activity times and apposition optics (except for the Agathiphagidae), Yack et al. (2007) concluded that the last common ancestor of the Lepidoptera to be a diurnal moth with apposition optics. The nocturnal kauri moths (Agathiphagidae) were suggested to have evolved superposition optics probably via afocal apposition optics through a gradual transition to adapt to an increasingly...
nocturnal lifestyle. The Lophocornoidae and Neopseustidae (almost certainly in possession of superposition optics: see personal comment of Kristensen in Yack et al. 2007) might, on the author’s (Yack et al. 2007) opinion, have evolved in the same way.

As shown in the present study (chapter 2.4), a diurnal lifestyle cannot be easily connected with the fact that these animals need to possess apposition optics. Furthermore, many other species with the intermediate eye type also show a diurnal lifestyle. It can also be expected that several more groups like for example Heliozelidae, Glyphipteriginae, Castniidae and Choreutidae mentioned to be diurnal (Kristensen 1998) do not necessarily have to possess apposition optics and should be therefore investigated by histological and ultrastructural methods. However, are perhaps also eyes of the intermediate type to be found within the basal groups? To answer this question it seems necessary to take a closer look at the data basis used for the conclusions mentioned above.

The description of apposition optics for the Micropterigidae was confirmed in this present study (chapter 2.4) by ultrastructural investigations. However, respective TEM data are so far lacking for all other groups. At least light microscopical data are available for the Eriocraniidae (Ehnbohm 1948; Kristensen 1968). A review of Fig. 52 presented by Kristensen (1968) reveals that apposition optics are present in the Eriocraniidae. Picture material for the Heterobathmiidae was not published so far, and the description of the eye type is based on a personal comment of Kristensen given in Warrant et al. (2003).

The analysis of the present morphological data basis discloses the urgent need for more detailed information and a comparative ultrastructural investigation of the compound eyes in the basal groups. Ultrastructural investigations, however, will be limited to single groups as access to living material is near to impossible for the Agathiphagidae, Acanthopteroctetidae and Lophocoronidae. In case of the Acanthopteroctetidae, for example, only few single specimens were collected, within the last 20 years (personal comment Niels Peder Kristensen).

Still, already high-resolution light micrographs of existing embedded museum material can provide further important information, especially for a detailed comparative histological study. Such a comparative investigation was recently
planned, because there are indications that the Acanthopteroctetidae exhibit the intermediate eye-type (Kristensen, Rota and Fischer, in preparation). Thus, there are signs of eyes with apposition and superposition characters, but also of the intermediate eye type to be found in the basal groups of Lepidoptera. The situation might not be that explicit and the question arises whether characters described for the intermediate eye type can help to distinguish the polarity in eye evolution. Alternatively, one may ask: has the intermediate eye type to be considered only a miniaturized type of superposition eye or does it represent a possible transitory stage in the evolutionary transformation from apposition to superposition optics?

All intermediate eyes described in the present study (chapter 2.4) are functional and thus both directions are possible in theory. However, there are more indications/characters supporting the view that miniaturization has been the driving force transforming a superposition eye into an apposition eye (see previous chapter and chapter 2.4). In addition, one also has to keep in mind that the nepticulids are the smallest known Lepidoptera so far, being smaller than all other members of the recent basal groups.

When breaking down to basic criteria of superposition, two characters turn out to be indispensable prerequisites: 1) a gradient of refractive indices is needed within the crystalline cone and 2) a distantly positioned proximal rhabdom. A distal rhabdom of small diameter may also be present (compare *Ephestia kuehniella*, for which superposition was proven by Cleary et al. 1977). The present investigations on lepidopteran compound eyes and also the available literature reveal the ability of retinula cells to produce rhabdomeres of different shapes and at different positions along the ommatidial longitudinal axis. Due to the connection of the retinula cells to the crystalline cone, be it via a cell strand or by cellular parts containing rhabdomeres, gradual changes of rhabdom shapes are possible in the course of adaptions with modified eye designs.

All other characters typical for superposition optics, like for instance the tracheal tapetum, enhance sensitivity and resolution, but are not an indispensable feature for superposition from scratch as long as pigment granules do not completely absorb the light inbetween neighbouring ommatidia. In addition, it is questionable how robust the character “tracheal tapetum” is in terms of evolution. How is it induced in the eye development and how easily can it be lost? Not only in cases of miniaturized eyes
one can find species devoid of a tracheal tapetum (chapter 2.4), but at least also within one larger lepidopteran species, i.e. *Orgyia antiqua* exhibiting a gender-related eye dimorphism, a distinct tracheal tapetum is absent in the female (Lau and Meyer-Rochow 2007).

Based on the before mentioned considerations, the most critical adjustments leading from apposition to superposition optics (and vice versa) have to take part within the crystalline cone. As afocal apposition optics are more efficient than focal apposition, it is indeed more reasonable to expect that focal apposition optics did not evolve from afocal ones (Nilsson 1989, Land and Nilsson 2002). However, did afocal apposition optics evolve from apposition optics by simply producing a gradient within the cone stalk, or did it evolve from refractive superposition optics? An interesting question deals with the development of the gradient of the refractive indices in the crystalline cone. Is the gradient produced directly from scratch or in a secondary tanning process (i.e., like the extraction of water resulting in a denser packing of proteins and an increase in refractive index) after a homogenous cone was produced also in refractive superposition eyes? If the latter is the case, how easy can such a process be gained or lost? The general development of the ommatidium in insects seems well conserved and evolutionary robust, independent from the optical type (apposition or superposition) (see Buschbeck and Friedrich 2008 and works cited within). It appears therefore questionable if it would not be more parsimonious to expect a secondary change within the cones to have happened, if afocal apposition and superposition eyes evolved from apposition optics. A secondary tanning process does not have to be performed in the same manner in all species. On the contrary, different mechanisms leading to the same result would even support the hypothesis that superposition eyes evolved several times independently from each other from apposition eyes (Land and Nilsson 2002). In dung beetles a change in the gradient of refractive indices of the crystalline cone is found during maturation even after the insect hatched (Warrant et al. 1990). Such a process was not noticed in a skipper butterfly with eyes of the superposition type (Schlamp 1989). Indeed the development of the cone deviates in between the investigated species (a dung beetle and a skipper butterfly) by Schlamp (1989), but so far it is not possible to discriminate on the available data basis, if the gradient is build directly during the formation of the cone or if secondary effects like tanning processes for example are also involved. Schlamp described a sponge-like structure during cone development in the skipper
butterfly eye. In the following new proteins bind to this structure ("fill the sponge") and the cone becomes a dense granular deposit (Schlamp 1989). It seems possible that a refractive index gradient can be generated by the use of different cone proteins, attached in a certain pattern to this scaffold. Nevertheless it is not proven so far and it does not exclude other factors to be included in the process of building up the refractive index gradient of the crystalline cone. An investigation of the development of apposition cones found within the Lepidoptera might be of interest in the future as a comparison in order to examine how crystalline cones without refractive gradients are build and if similar sponge-like structures as the ones described by Schlamp are involved.

Further investigations on the eyes of basal groups integrating histological, ultrastructural and functional (optical) examinations are clearly required to gain a larger data basis for a sound evolutionary discussion. Further investigations giving attention to the process (or processes), involved in producing the gradient of refractive indices within the cone of superposition optics and afocal apposition optics, might yield additional insights.

It might also be of interest to include the sister group of the Lepidoptera, the Trichoptera, which are known to also include some very small species with front wing length of about 3-4 mm (Malicky 2007). So far, compound eyes of Trichoptera have not been the target of comprehensive TEM studies. Therefore, as an outgroup comparison is not yet possible, there is no supportive information giving evidence to the plesiomorphic nature of apposition optics in the lepidopteran clade. Information on the architecture of trichopteran compound eyes is strictly grounded on histology and literally limited to three publications: Ast (1920), Umbach (1943) and Yagi and Koyama (1963). It is, however, known that most of the caddiesflies are crepuscular or nocturnal and according to Ast (1920) and Yagi and Koyama (1963) they do possess eyes of the superposition type. Umbach (1943) mentioned parallels to the atypical eye type found in some lepidopterans. Later on Yagi and Koyama (1963) presented one drawing (1963, Fig. 163D) illustrating a rhabdom attached to the dioptric apparatus. The drawing also reveals elongated, tapered cone shapes. Furthermore Ast (1920) noticed longitudinal pigment migrations within the retinula cells, as reported for the intermediate eye type (chapter 2.2, 2.3,2.4), which is also shown in one figure by Yagi and Koyama (1963, Fig. 162b).
Without doubt Lepidoptera did not evolve from a recent group of Trichoptera (Grimaldi and Engel 2005). Since nothing is known about the compound eyes of the basal groups within the Trichoptera the question remains so far unsolved, which eye type may have been present in the ground pattern of Lepidoptera and Trichoptera or, one level farther basal, in the ground pattern of monophyletic Amphiesmenoptera, a taxon comprising Trichoptera and Lepidoptera as sister groups.

**Number of retinula cells in the eyes of Lepidoptera**

The TEM observations made during this Ph.D project (chapter 2.2, 2.3, 2.4) reveal the presence of a homogenous ultrastructural organization across ommatidia, which represent the ground pattern of the Tetraconata proposed by various authors (Melzer et al. 1997, Dohle 2001, Richter 2002, cf. Paulus 1979, 2000). Besides 4 cone cells, 2 primary and 6 secondary pigment cells, 8 retinula cells are present in all of the investigated species, thus recalling the plesiomorphic state of Hexapoda and Tetraconata, respectively. This common ommatidial architecture is also valid for the most basal lepidopteran taxon Micropterigidae, the sister group of all other recent lepidopteran taxa (Kristensen & Skalski 1998, Wiegmann et al. 2002, Kristensen et al. 2007). Therefore, the ground pattern described by Paulus (1979) can be assumed to have been mainly retained in the ground pattern of the Lepidoptera, as well. As mentioned in an earlier chapter, little is known about the compound eyes of Trichoptera, the sister group of the Lepidoptera. However, Ast (1920) noted that the trichopteran species *Rhyacophila dorsalis* (Curtis, 1834) and *Halesus interpunctatus* (Zetterstedt, 1840) possess 8 retinula cells in their ommatidia. Seven of these retinula cells are distributed throughout the ommatidium, whereas one is restricted to the proximal part of the ommatidium. Thus, ommatidia with 8 retinula cells might also be present in the Amphiesmenoptera, as also the corneal nipples that are developed as well in both trichopteran and lepidopteran species (Bernhard et al. 1970).

Yet not all Lepidoptera bear ommatidia with only 8 retinula cells, even numbers of up to 15-16 have been encountered in certain species (Meyer-Rochow and Lau 2008, Horridge et al. 1977). In case of *Ephestia kuehniella* the number of retinula cells varies within one eye from 9 to 12 (Fischer and Horstmann 1971). Including all available information published so far, the number of retinula cells known for different families is given in Table. 2.
Table 2. Compilation of literature accounts dealing with the eye type and the number of retinula cells found in various lepidopteran families. The systematic classification is according to Kristensen et al. (2007). The numbers in brackets (if given) reflect the contribution of the retinula cells to form the rhabdom from the distal to proximal position.

<table>
<thead>
<tr>
<th>family</th>
<th>species</th>
<th>eye type</th>
<th>number of retinula cells</th>
<th>source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micropterigidae</td>
<td>Micropterix aruncella</td>
<td>APP</td>
<td>8 (7+1)</td>
<td>chapter 2.4</td>
</tr>
<tr>
<td>Nepticulidae</td>
<td>Ectoedemia argyropeza</td>
<td>INT</td>
<td>8 (7+1)</td>
<td>Honkanen &amp; Meyer-Rochow (2009), chapter 2.4</td>
</tr>
<tr>
<td></td>
<td>Stigmella microtheriella</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ectoedemia septembrella</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ectoedemia hannoverella</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tischeriidae</td>
<td>Tischeria ekebladellia</td>
<td>INT</td>
<td>8 (7+1)</td>
<td>Fischer unpublished</td>
</tr>
<tr>
<td>Tineidae</td>
<td>Tineola bisselliella</td>
<td>SUP</td>
<td>8</td>
<td>Faucheux 1987</td>
</tr>
<tr>
<td>Gracillariidae</td>
<td>Cameraria ohridella</td>
<td>INT</td>
<td>8 (7+1)</td>
<td>chapter 2.2, 2.4</td>
</tr>
<tr>
<td></td>
<td>Phyllonorycter esperella</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phyllonorycter maestingella</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phyllonorycter medicaginella</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yponomeutidae</td>
<td>Argyresthia goedartella</td>
<td>INT</td>
<td>8 (7+1)</td>
<td>chapter 2.4</td>
</tr>
<tr>
<td>Plutellidae</td>
<td>Plutella xylostella</td>
<td>INT</td>
<td>8 (7+1)</td>
<td>Wang &amp; Hsu 1982</td>
</tr>
<tr>
<td>Lyonetidae</td>
<td>Lyonetia clerkella</td>
<td>INT</td>
<td>8 (7+1)</td>
<td>Fischer unpublished</td>
</tr>
<tr>
<td>Tortricidae</td>
<td>Adoxophyes reticulana</td>
<td>INT/SUP</td>
<td>8 (7+1)</td>
<td>Hämmerle &amp; Kolb 1987 (dorsal eye region)</td>
</tr>
<tr>
<td>Pyralidae</td>
<td>Ephesia kuehniella</td>
<td>SUP</td>
<td>10 (9+1)</td>
<td>Horridge Giddings 1971</td>
</tr>
<tr>
<td></td>
<td>Amyelois transitella</td>
<td>INT/SUP</td>
<td>9-12</td>
<td>Fischer &amp; Horstmann 1971</td>
</tr>
<tr>
<td></td>
<td>Galleria mellonella</td>
<td>SUP</td>
<td>12-13</td>
<td>Bernard et al. 1984</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>13 (12+1)</td>
<td>Stone &amp; Koopowitz 1976</td>
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<tr>
<td>Crambidae</td>
<td>Acentria ephemerella</td>
<td>SUP</td>
<td>9 (7+2)</td>
<td>Lau et al. 2007</td>
</tr>
<tr>
<td>Bombycidae</td>
<td>Bombyx mori</td>
<td>SUP</td>
<td>11 (10+1)</td>
<td>Eguchi 1962</td>
</tr>
<tr>
<td>Saturniidae</td>
<td>Antherea polyphemus</td>
<td>SUP</td>
<td>9 (2+6+1)</td>
<td>Anton-Erxleben &amp; Langer 1988</td>
</tr>
<tr>
<td>Sphingidae</td>
<td>Cechenena lineosa lineosa</td>
<td>SUP</td>
<td>9 (2+6+1)</td>
<td>Eguchi 1982</td>
</tr>
<tr>
<td></td>
<td>Macroglossum pyrrhosticta</td>
<td>SUP</td>
<td>9 (8+1)</td>
<td>Eguchi 1982</td>
</tr>
<tr>
<td></td>
<td>Cephenodes hyalas</td>
<td>SUP</td>
<td>9 (2+6+1)</td>
<td>Eguchi 1982</td>
</tr>
<tr>
<td></td>
<td>Deilephila elpenor</td>
<td>SUP</td>
<td>9 (2+6+1)</td>
<td>Welsch 1977</td>
</tr>
<tr>
<td>Hedyliidae</td>
<td>Macrosoma heliconia</td>
<td>SUP</td>
<td>8 (4+3+1)</td>
<td>Yack et al. 2007</td>
</tr>
<tr>
<td>Hesperiidae</td>
<td>Pamara guttata</td>
<td>APP</td>
<td>9 (2+6+1)</td>
<td>Shimohigashi &amp; Tominaga 1986</td>
</tr>
<tr>
<td>Papilionidae</td>
<td>Papilio aegus</td>
<td>APP</td>
<td>9 (4+4+1)</td>
<td>Ribi 1987</td>
</tr>
<tr>
<td>Pieridae</td>
<td>Pieris rapae</td>
<td>APP</td>
<td>9 (4+4+1)</td>
<td>Ribi 1978</td>
</tr>
<tr>
<td></td>
<td>Pieris brassicae</td>
<td>APP</td>
<td>9 (4+4+1)</td>
<td>Kolb 1977</td>
</tr>
<tr>
<td>Lycaenidae</td>
<td>Polyommatus icarus</td>
<td>APP</td>
<td>9</td>
<td>Sison-Mangus et al. 2008</td>
</tr>
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<td></td>
<td>Lycaena rubidus</td>
<td>APP</td>
<td>9</td>
<td>Sison-Mangus et al. 2006</td>
</tr>
<tr>
<td>Nymphalidae</td>
<td>Aglais urticae</td>
<td>APP</td>
<td>9</td>
<td>Kolb 1985</td>
</tr>
<tr>
<td>Geometridae</td>
<td>Operophtera brumata</td>
<td>SUP</td>
<td>15 (14+1)</td>
<td>Meyer-Rochow and Lau 2008</td>
</tr>
<tr>
<td>Noctuidae</td>
<td>Orgyia antiqua</td>
<td>SUP</td>
<td>8 (7+1)</td>
<td>Lau et al. 2007</td>
</tr>
<tr>
<td></td>
<td>Phalaenoides tristifica</td>
<td>SUP</td>
<td>14-16</td>
<td>Horridge et al. 1977</td>
</tr>
</tbody>
</table>
On the one hand, the list reveals a lack of knowledge concerning several taxa, especially those taxa branching off the lepidopteran tree between rooting of the Tortricoidea and Pyraloidea (compare Fig. 5) and, on the other hand, that usually 9 retinula cells are present in the more highly evolved true butterflies (Fig. 5). Furthermore, the number of more than 8 retinula cells is not dependent on 1) apposition or superposition optics, 2) a diurnal or nocturnal lifestyle or 3) restricted to the true butterflies.

In which way a functional advantage is achieved because of the presence of more than 8 retinula cells and how the evolution of such an augmented apparatus of retinula cells might be explained, shall be discussed on the basis of the concept of retinula cell homologies, as most recently introduced by Friedrich et al. (2011).

**Retinula cell homologies and the application of the key provided by Friedrich et al. (2011)**

Based on data on eye development in Drosophila and the chronology of retinula cell development, Friedrich et al. (2011) recently proposed the homology of photoreceptors within insects and presented a key for the identification of retinula cell duets. The authors differentiate inner and outer photoreceptor cells based on the organization found in the open rhabdom type of Drosophila, but also identifiable by the axonal connections of the retinula cells to the optic neuropils. The inner cells (R7 and R8) project long fibres connecting to the second optical neuropile, the medulla. All outer receptor cells connect to the lamina, the first optical neuropile. This differentiation, however, is the first problem when one tries to apply this determination key, as the reconstruction and assignment of axonal connections to individual retinula cells by far exceed the standard methodological approach used for ultrastructural eye descriptions. Furthermore, the key only allows to identify cell duets, but not single cells within them. Especially, for a detailed morphological description an unambiguous assignment of individual retinula cells is important. So far, the key ignores those features, used as reference points in classic morphological descriptions of compound eyes in arthropods, as for instance the proximal projections of the crystalline cones (e.g. Meinertzhagen 1991). Connecting the proximal tip of the crystalline cone with the basal matrix (Odselius and Elofsson 1981), these proximal processes can be used in eyes with uneven numbers of retinula cells to identify single cells throughout the whole extension of the
ommatidium. As presented in this work (chapter 2.2, 2.3, 2.4), in lepidopteran ommatidia the proximal cone projections terminate in blunt ends shortly below the tip of the cone and cannot be consulted as a character of reference. The lack of extended proximal cone cell processes in Lepidoptera results in a basal matrix, which is unique for winged insects in relation to organization and formation. The lepidopteran type of an ommatidial basal matrix was not covered by the only existing, comprehensive reference work of Odselius and Elofsson (1981). Thus, in this doctoral thesis it was described in detail for the first time (chapter 2.4). Nevertheless, it remains to be investigated, if in eyes with cone cell projections, the position of the projections can be correlated to a certain position within the cell duet cluster and if, therefore, the key given by Friedrich et al. (2011) can be combined with the classic ultrastructural characters mentioned above.

According to Friedrich et al. (2011) the positions of the retinula cells to each other are in many cases giving sufficient information to identify the retinula cell duets also without information about their axonal connections. In all studies of eye development performed in insects so far, the 8th (basal) retinula cell is the first one to develop and it was shown for Drosophila that this is critical for inducing the development of the other retinula cells (Karpilow et al. 1996). In the following, three cell duets develop: first R2-R5, followed by the duets R3-R4 and R1-R6. The singular R7 is built last and its rhabdomere is taking a distal position within the rhabdom, whereas the R8 keeps its proximal position and participates only at the most proximal part of the rhabdom with a rhabdomere (e.g Tomlinson and Ready 1987). The R7 and R8 cell bodies are forming a median line, separating the members of each of the three duets (see Meinertzhagen 1991, Friedrich et al. 2011).

Although the key is based on a rhabdom of the open type, several examples presented in Friedrich et al. (2011) show that this system can also be applied to rhabdoms of the fused type. Nevertheless, to apply this key to the eyes examined in the present study is at least complicated and especially so when in the small eyes the rhabdomere of the 8th retinula cells is missing. It is therefore difficult to define the median axis between R7-R8 and it is questionable if the orientation of the rhabdomeric microvilli, which is usually different from all other cells in the R7 (often for this reason referred to as “singular cell”), is a sufficient character to determine the R7. In ommatidia of small-sized Lepidoptera the distal rhabdom is built by 7 cells and
therefore theoretically each cell can isolate three retinula cells on either side. Thus, at least in these cases axonal connections to the medulla need to be confirmed to reliably identify and distinguish inner and outer receptor cells (= R7/R8 vs. R1-6). Additional information is available from some larger moths species by looking at the distal tip of the rhabdom: one retinula cell joins the rhabdom with a rhabdomere in a deeper plane than the other six retinula cells, as for example in *P. medicaginella* (chapter 2.4). It might therefore be true that this cell conforms to the singular R7, according to the cell homologies advocated by Friedrich et al. (2011). However, in contrast to the larger ommatidia of bigger Lepidoptera, tracing cells in the small eyes of small species is hindered by the reduced space in the proximal part of the rhabdom: some cells have already turned into axons, while others may have slightly changed their microvillar orientations slightly due to cell displacements (chapter 2.1). Therefore, careful tracings of retinula cells are needed in a medium-sized lepidopteran ommatidium, to confirm the orientation of inner and outer retinula receptor cells as predicted by Friedrich et al. (2011).

**Functional use of more than eight retinula cells**

Based on current knowledge of different neuronal connections and different visual pigment opsins, it is possible to determine that the 9th retinula cells in butterflies are of an R7-like type (reviewed in Friedrich et al. 2011). In this case 3 long fibres project to the medulla (for review see Yack and Homberg 2003).

It is not a cell duplication in the actual sense, as the second R7 (the R7-alike) is built from a precursor cell already present in the pre-ommatidial cell cluster and not by a cell division of the R7 (see Friedrich et al. 2011). As mentioned before, the major part of these conclusions is based on studies on *Drosophila*, not at least due to a large number of mutants. However, the whole process of eye development with all so far known influencing factors is too complex and clearly beyond the scope of this summary. In this respect, I refer to Kumar (2011), who reviewed the development of the different cell types constituting the ommatidium of *Drosophila* and described various mechanisms and factors. In the following, only those aspects relevant for this discussion have been regarded.

The way how the different cell types develop from precursor cells may certainly help to shed light on the different numbers of retinula cells seen in the geometrid...
Operophtera brumata (14+1), the noctuid Phalaenoides tristifica (14-16) and the pyralids Ephestia kuehniella (9-12), Amyelois transitella (12-13) and Galleria mellonella (12+1).

Interestingly Operophtera brumata and Phalaenoides tristifica (probably also all other named examples) possess only a single basal retinula cell, which can be addressed quite certainly as R8 homolog according to Friedrich et al. (2011). It supports the generally accepted function of this cell to induce the fate of all other retinula cells in the pre-ommatidial cell cluster and it is therefore often referred to as the “founder cell” (e.g. Tomlinson and Ready 1987). This is also very likely the reason, why this cell is not reduced in the small eyes even if it lacks a rhabdomere (chapter 2.3, 2.4).

The remaining 14 retinula cells in the eye of Operophtera brumata might therefore be explainable as the result of a “duplication” of the R1-7 set or an added set of single duets. A detailed investigation of the axonal connections of the retinula cells in ommatidia comprising more than 9 retinula cells as well as the identification of the opsins could be of help in this regard in the future. At least, the number of cells making direct contact to interneurons in the medulla should allow us to determine the number of R7-alikes in this eye.

In the eye of Ephestia kuehniella it is of interest to note that Umbach (1934) described cell number irregularities not only for the distal retinula cells, but also within the crystalline cone cells (3-5). The question not answered so far is, if both are found within the same ommatidium. A comparison with Drosophila shows that more than 4 cone cells are found in mutants that possess additional retinula cells. However, there are also cases known, in which a lower number of retinula cells is accompanied by additional cone cells (Kumar 2011). This example illustrates the complexity of impacting mechanisms and factors driving eye development. Because of the complexity of impacting factors it is likely that additional retinula cells evolved several times independently.

It is nevertheless an interesting fact that more than 8 retinula cells are found mainly in higher evolved groups of lepidopterans. The non-monophyletic term “Macrolepidoptera” refers to the larger sized species of the Lepidoptera, but is it only the larger size of the eyes, or can a visual function/need be combined with the presence of additional retinula cells? The pyralid Ephestia (and thus not positioned
within the “Macrolepidoptera”) is relatively small in comparison to a typical macrolepidopteran species and yet it possesses more than 8 retinula cells. Size alone, therefore cannot be the only determinant limiting the presence of additional cells.

Just recently a new classification for the Lepidopterans was published by van Nieukerken et al. (2011), which largely follows that in the Handbook of Zoology (Kristensen 1998), and the later update (Kristensen et al. 2007). Main novelty is the position of the butterflies (Papilionoidea), now situated close to the Pyraloidea due to new molecular studies. Lepidopteran families possessing 9 retinula cells, so far situated mainly within the “Macrolepidoptera”, are therefore now splitted from each other. As the butterflies (9 retinula cells) are no longer part of the former “Macrolepidoptera” this clade is now termed “Macroheterocera”, the latter still containing the Bombycidae, Saturniidae and Sphingidae (all 9 retinula cells). Irrespective, if this new classification is approved and accepted by the scientific community in the future or not, this reveals the interest of investigations on compound eyes of lepidopteran groups situated phylogenetically inbetween the Tortricoidea and the Pyraloidea to gather more information about retinula cell numbers. A broader data basis might bring further information also for phylogenetical analyses.

But what is the evolutionary advantage of having additional retinula cells in an ommatidium? This question might be answerable by referring to the true butterflies. Optical information and colour vision have been proven to be important for flower detection and discrimination, mate recognition and the detection of oviposition substrates (Kelber 1999). Therefore optical tuning is not remarkable, and especially for mate choice a co-evolution of the spectral properties of the photoreceptors with the wing coloration has been assumed by different authors (Stavenga and Arikawa 2006, Sison-Mangus et al. 2006, Briscoe et al. 2010). Several different mechanisms have been described for butterflies helping to tune optical information, like multiple opsin strategies and different filter strategies (Stavenga and Arikawa 2006, Wakakuwa et al. 2007, Zaccardi et al. 2006). For both strategies additional retinula cells are of advantage. In a fused rhabdom, an additional, distally positioned retinula cell can filter light coming in from dioptic apparatus and reaching rhabdomeres of other, subjacent retinula cells (Snyder et al. 1973). Also in several sphingids, like
Cechenena lineosa (Walker, 1856), two distal retinula cells are positioned on top of all other cells (Eguchi 1982). Based on the position of the axons of these cells in relation to the basal cell, it appears obvious that these two distal retinula cells correspond to the R7 and R7-alike retinula cells classified by Friedrich et al. (2011). The subjacent photoreceptors might be even further tuned by the impact of specialized screening pigments, like red pigment granules functioning as red transmitting filters (= blue and green-absorbing pigment), as described in *Pieris rapae* (Ribi 1979). Tuning is also induced by additional or different opsins gathered within the photoreceptor membranes (Kitamoto et al. 1998). In this case, additional retinula cells are of advantage, as their presence increases the tuning possibilities. It was shown for the Small White butterfly *Pieris rapae crucivora* that several different variants of ommatidia are found in the same compound eye (Wakakuwa et al. 2007), named ommatidial type I-III.

The different ommatidial types are mainly characterized by different opsin combinations (Wakakuwa et al. 2007). Friedrich et al. (2011) listed the retinula cell numbers according to the proposed cell homologies and this reveals that the main differences between the three ommatidial types (I-III) are found within the R7 and R7-alike retinula cells. An additional retinula cell therefore permits a higher specialization and diversity of visual tasks in relation to information gained by only the regular 8 retinula cells. Furthermore it allows regional differentiation within the eye by accumulating different ommatidial types in different eye areas.

Interestingly different ommatidial types are also found in the eyes of Hymenopterans, which also posses 9 retinula cells and as in the Lepidoptera the 9th cell is a R7-alike (Friedrich et al. 2011). To the best of my knowledge no different types of ommatidia found in one eye have so far been reported for compound eyes with only eight retinula cells. This could simply be due to missing investigations, as so far nothing was known about the number of retinula cells of ommatidia found in the compound eyes of the more basal situated groups of Lepidoptera (chapter 2.2, 2.3, 2.4), but it would be of interest to test the hypothesis whether different ommatidial types are restricted to eyes with an additional R7-alike retinula cell. Furthermore, correlated with the different opsin distributions in the R7 and R7-alike retinula cells, different visual information may be projected to the medulla in the three ommatidial types. It remains to be investigated if this is also accompanied by changes within the circuitry.
of colour information. It seems likely that in order to process information of different ommatidial types, distributed throughout the compound eye, adaptations are needed to process the additional information. However, our understanding on the way (or ways) colour information is processed in insects in general and in Lepidopterans in particular is incomplete (see review by Kelber 2006).

**Conclusion**

Miniaturization is a fascinating topic as it highlights principles of functionality of systems found in nature, being under structural reconstruction of phylogenetically inherited sensory designs due to size limitations. With regard to photoreception it reveals mechanisms about adaptations that achieve the best possible functional use of small optics. The investigation of different small lepidopteran compound eyes did not only allowed to correlate eye size to body size, but also opened up the possibility to describe specific adaptational changes that occurred parallel to a decrease in size. It reveals that a functional change, perhaps even inter-change, between superposition and apposition optics is possible in connection with miniaturization and that the borders, which formerly delineated the categories of both functional eye types, are increasingly less useful.

The intermediate eye type, found in many of the small eyes investigated in this Ph.D study, displays a functional and anatomical mixture of characters found in both apposition and superposition eyes. Furthermore, the intermediate eye type confirms the conclusions reached by Meyer-Rochow and Gál (2004) on the basis of their theoretical investigation of superposition thresholds, namely that specific adaptations are to be expected in eyes with a radii below 250 µm. Smaller eyes simply cannot work properly as functional superposition eyes efficient enough for a nocturnal lifestyle.

The eyes of the intermediate type are adapted for higher resolution and serve their bearers to lead a predominantly diurnal lifestyle through the possession of a well developed distal rhabdom. Theoretically superposition remains possible under lower ambient light intensities, at least allowing some medium-sized species like *Argyresthia goedartella* some crepuscular activity. With decreasing body and eye size, overall some general features of the superposition eye are retained, so that increased sensitivity during low light conditions is an option. Even if the superposition
process includes no more than 6 neighbouring facets, this is still resulting in an increased sensitivity by factor 6. The minimal functional limit for superposition, involving the closest neighbouring ommatidia (6), cannot be determined by morphological investigations alone and requires a multi-methodological approach including optical investigations. As the smallest moths are mainly day-active, the increase of the distal rhabdom’s diameter seems to be negatively correlated with the insect’s size. This change in lifestyle is accompanied by an adaptation that affects the dark-light mechanism, i.e., the distribution of the screening pigments within the retinula cells, as the pigments granules found in the secondary pigment cells cannot sufficiently shade the rhabdom due to the small diameter of the extensions of these cells. The maximal rhabdom diameter is controlled by sensitivity/resolution constraints, i.e, the minimal possible size of the dioptric apparatus and limiting factors at the cellular level. Especially the size of the retinula cell nuclei make an impact on the minimal rhabdomere to retinula cell soma ratio, as also the special organization of the nuclei showed in *Trichogramma evanescens*. If minimal sensitivity cannot be reached under a given illumination level, the number of facets has to decrease in order to make space for a smaller number of facets with larger diameters. The absolute minimal threshold and tradeoff of sensitivity and resolution-needs will be set individually by each species.

Apart from miniaturization, the comparative investigation of the compound eyes of several lepidopteran species furthermore revealed similarities like the unique type of basal matrix and the homogenous number of 8 retinula cells in the ommatidia. The number of 8 retinula cells per ommatidium is constant across eyes of species representative of mainly the basal groups and represents a plesiomorphic feature with respect to the eye ground pattern of Hexapoda and Tetraconata, respectively. The higher number of retinula cells in some larger lepidopterans and the different types of ommatidia found in certain species may be interpreted as the result of an anti-miniaturization: i.e., maximizing and extending the available visual information that can be gained from the environment. Contrary to the eyes of small size, limited by sensitivity, resolution, and diffraction, the aforementioned limitations vanish the larger an eye becomes. The then-gained resolution by possessing an increasingly large number of facets that are less impacted by diffraction allows resolving increasingly fine details and smaller and smaller objects, so that the extended visual information increase will be of mounting interest. Not until this benefit is reached,
maximising visual information make any sense in terms of energy allocation. Thus indirectly also in this aspect size is an impacting factor, as high resolution cannot, as shown in this study, be reached in small eyes.

**Perspective**

The present Ph.D raises several questions, as in part already mentioned in several passages within the discussion. Until recently, biologists have just begun to study the phenomenon of miniaturization in connection with animal eyes in general and insect eyes, in particular. To date, our knowledge of structural implications, functional constraints and evolutionary pathways of miniaturization is still fragmentary and many questions remain unanswered. To strengthen, deepen and proceed with investigations into photoreceptors of small animals with (or without) compound eyes, it will be needed to focus on different ultrastructural, optical, and evolutionary aspects. It is a challenge for the future to further validate the conclusions reached on the basis of the research reported in this thesis and also to test if further minimal limiting factors can be identified and described in detail.

Furthermore, work on vision may be envisioned as the beginning of future investigations that include other sensory mechanisms, so that an increasingly detailed picture of how small species perceive their environments can be drawn and questions like "Are the restrictions imposed on one sensory system as a consequence of miniaturization compensated by other senses?" can be answered. By including other sensory mechanisms, a qualified functional interpretation of the total information gained from the environment in small species is possible and the portion and importance of single senses, like vision, can be considered and discussed on a wider data basis and broader context. This more comprehensive approach can therefore increase our overall knowledge and understanding of interrelated sensory information channels and impacts on sensory mechanisms caused by miniaturization. However, without a study of the neuronal circuits that transmit the gathered information to visual association centres, which themselves have to be subjects of miniaturization in small species, our understanding remains incomplete. Ultimately the question will be how far simplifications and miniaturization are possible, so that an animal is still capable of processing the information its sense organs have been able to gather. In summary: how small can you go?
References


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