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The unusual visual system of the Strepsiptera: external eye and neuropils

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Abstract Adult males of the insect order Strepsiptera are characterized by an unusual visual system that may use design principles from compound as well as simple eyes. The lenses of this eye are unusually large and focus images onto extended retinae. The light-gathering ability of the lens is sufficient to resolve multiple points of an image in each optical unit. We regard each unit as an independent image-forming eye that contributes an inverted partial image. Each partial image is re-inverted by optic chiasmata between the retinae and the lamina, where the complete image could be assembled from the neighboring units. The lamina, medulla and lobula are present, but their organization into cartridges is not clearly discernable. Fluorescent fills, whole-tissue stains, and synaptotagmin immunohistochemistry show that the optic neuropils nevertheless are densely packed, and that several parallel channels within the medulla underlie each of the lenses. The size and shape of the rhabdoms, as well as a relatively slow flicker-fusion frequency could suggest that these eyes evolved through a nocturnal life stage.

Keywords Evolution · Eye-type · Insect · Neuroanatomy · Vision

Abbreviations O object size · U object distance · I image size · f focal length · A lens aperture · D lens diameter · $\Delta\phi$ interommatidial angle · S light sensitivity of optical system

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Introduction

The twisted-wing insects (order Strepsiptera), are one of the most interesting and yet, in terms of neurobiology, least-studied insect orders. Strepsiptera stand out among other insects in that the males of this order are characterized by a peculiar organizational principle for an insect eye (Buschbeck et al. 1999). To contrast a "normal" compound insect eye with that of Strepsiptera, we compare this eye to that of the fruit fly *Drosophila melanogaster*, an insect of similar size with a well-described visual system. Externally, the strepsipteran eye can be seen as having large, strongly convex lenses that are widely spaced, giving the eye a berry-like semblance. The number of lenses is remarkably low and ranges from 12 to 150 among species (Kinzelbach 1971). It is therefore not surprising that the unusual appearance of the male eye has long been noted and debated. Early researchers regarded the eyes based on their internal organization as ocellar complex eyes (Strohm 1910). Rösch (1913) also discussed the similarity of the strepsipteran eyes to ocelli without claiming homology between these structures. Kinzelbach (1971) noted the possibility for a paedomorphic eye based on the similarity to ant-lion larvae, and Paulus (1979) referred to the eyes as stemmataren facetted eyes, emphasizing the general similarity to larval stemmata. Wachmann (1972) pointed out the similarity of the fine structure of the receptors to larval receptors. MacCarthy (1991) finally took a more functionally oriented perspective and suggested that the large lenses of Strepsiptera could be image inverting, thus implying the possibility of image formation within each unit. Despite all these studies on the peripheral morphology and anatomy of the strepsipteran eye a neurobiological evaluation and functional interpretation of the strepsipteran visual system has only been attempted recently (Buschbeck et al. 1999; Pix et al. 2000). *Xenos peckii*, the strepsipteran species on which we focus here (Fig. 1A), has only about 30–50 lenses per eye. This is

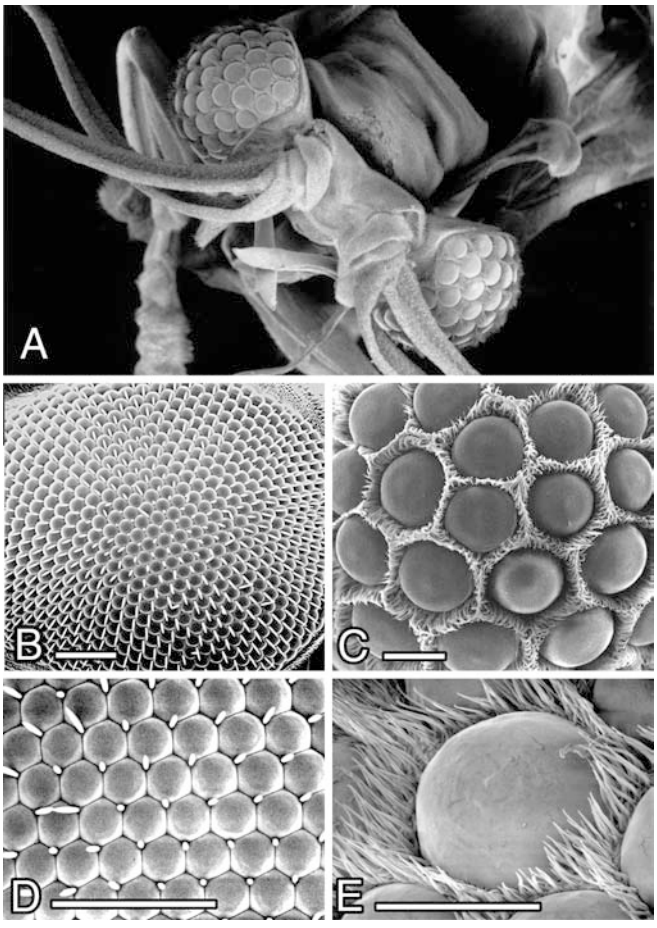

Fig. 1A–E Scanning electron micrographs of the adult strepsipteran *Xenos peckii*. **A** frontal view of the entire head. Note large lenses and prominent, long antennae. **B–E** Comparisons of the external eye and individual units of the fruit fly (**B**, **D**)*Drosophila melanogaster* with those of *X. peckii* (**C**, **E**). **B** and **C**, as well as **D** and **E**, are at the same scale. Scale bars: 50 μm

an order of magnitude fewer than the more than 700 facets of the slightly smaller fly, *D. melanogaster*. However, a typical lens in *X. peckii* is about 65 μm in diameter and covers about the same area as 15 lenses of *D. melanogaster* (compare Fig. 1B and D, which show *Drosophila* lenses with Fig. 1C and E, which depict *Xenos* lenses). The large lenses in *X. peckii* are separated from each other by rows of prominent brush-like microtrichia, another peculiarity of the strepsipteran eye (Fig. 1C, E). The clearest differences, however, are apparent in histological cross-sections. Figure 2A depicts the cross-section of the outer portions of the *Drosophila* eye, which is representative of a typical insect compound eye. While there are many different types of compound eyes such as apposition, superposition, or neural superposition eyes (see Nilsson 1989 for a detailed comparison) they all follow a general blueprint which is common not only to insects, but also to many crustaceans (Paulus 2000). This general organization includes a series of long narrow units, the ommatidia, that are characterized by a peripheral lens, a crystal cone, support cells, and 8–10 retinula cells (Nilsson and Osorio 1997; Paulus 1979, 2000). In osmium-stained tissue, these long channels can be easily visualized (Fig. 2A). A cross-section of a similarly stained *Xenos* preparation gives a completely different picture. Beneath each lens lies a small retina, containing more than 100 photoreceptors surrounded by a pigmented cup (Fig. 2B). We believe that each of these units defines a self-contained functional element that is capable of processing the portion of the visual field projected onto the retina by its overlying lens (Buschbeck et al. 1999). We think that the organization into a series of image-forming simple eyes is distinctly different from any previously described compound eye but we do not want to imply homology with either ocelli or stemmata; therefore, we refer to each of the units as an eyelet. In this paper we investigate further the optical properties of the lens and underlying retina, we examine the physiological properties of the photoreceptors, and extend our anatomical investigations to the optic neuropils.

What are Strepsiptera?

Strepsiptera are an endoparasitic insect order in which the females, in all but the most basal family, never leave their hosts (Kathirithamby 1989). The males that possess the unusual eyes, however, do emerge, but only have a few hours to find a female before they die. In other words, the sole purpose of the male is to find a mate. In this highly adapted parasitic insect unnecessary organ systems are reduced. For example, because the male does not feed during its short life, its mouthparts are greatly reduced and the intestines are abridged to separate pieces (Kinzelbach 1971). The brain is generally small, but has prominent optic lobes. In fact, visual neuropils comprise at least three-quarters of the brain. This alone provides compelling evidence for the importance of vision. While olfactory stimuli have been conjectured to be important for the long-range detection of a female (Perkins 1918), the

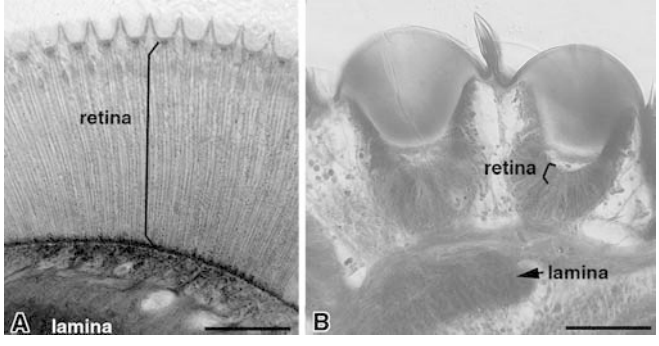

Fig. 2 Comparison of cross sections of **A** *D. heteroneura* and **B** *X. peckii*. While *Drosophila* is characterized by long, narrow facets that are typical of insect compound eyes, in *Xenos* each unit (eyelet) is composed of one large overlying lens and a shallow but extended retina. Scale bars: 50 μm

eyes may be fundamental within the near field, where the olfactory receptors may be saturated or too slow. Furthermore the importance of vision in odor localization has recently been demonstrated for *Drosophila* (Fry et al. 2003). The female of *X. peckii* stays entirely within the abdomen of her host wasp and her anatomy is reduced to a large lumen filled with thousands of eggs. Only her small and rather cryptic cephalothorax protrudes between the segments, and is hardly visible to the human eye.

Materials and methods

Strepsiptera of the species *X. peckii* were either collected as pupae within their wasp hosts near Ithaca, New York, or were reared from primary larvae on their wasp host *Polistes fuscatus* in the laboratory. Because of the short life span of adult males, parasitized hosts were monitored at half hour intervals and emerged males were processed shortly after emergence. Oregon-R strain *D. melanogaster* (Drosophilidae) were kindly provided by the laboratory of Dr. David Deicher at Cornell University. All images of *Drosophila* depict *D. melanogaster* except for Fig. 2A, which depicts *D. heteroneura*, which were kindly provided by Dr. Ken Kaneshiro, University of Hawaii.

Bodian staining

Male *X. peckii* were anesthetized by cooling and small areas of the head cuticle were removed to facilitate penetration by fixatives. Heads were fixed in Gregory's (1980) synthetically aged Bouin's solution, dehydrated, embedded in Paraplast plus (Oxford Labware, St. Louis, Mo., USA), and serially sectioned at 8 μm. Dewaxed sections were incubated for 24 h at 40°C in 1% silver proteinate (Alfa, Ward Hill, Mass., USA) with the addition of 1–4 g pure copper per 150 ml solution (more copper resulted in the staining of larger fibers). Afterwards, sections were conventionally stained after Bodian's (1937) original method with the following small modifications: step 3, only 2% sodium sulfite; step 4a, gold chloride without acetic acid for 10 min; and step 4b, oxalic acid bath for 8 min.

Staining with osmium-ethyl gallate

This procedure is a minor modification of Strausfeld and Seyan (1985). Heads were fixed in phosphate buffer with 3% formaldehyde (EM grade; Electron Microscopy Sciences, Ft. Washington, Pa., USA) and 1.3% sucrose. After several washes in buffer, heads were transferred into buffered 0.5% OsO_4 and kept for 1 h on ice and 1 h at 20°C. Tissue was washed in buffer, then in distilled water, and finally treated with saturated ethyl gallate (2 h at 0°C and 1 h at 20°C). After staining the heads were dehydrated, embedded in Spurrs (Polysciences, Warrington, Pa., USA) and serially sectioned at 8 μm.

Cajal's block silver stain

This procedure is a minor modification from Davenport et al. (1934). The tissue was fixed in Gregory's (1980) synthetically aged Bouin's solution (2–4 h), washed in 70% ethanol and incubated for 24 h in ammoniacal alcohol (2 ml ammonium hydroxide + 98 ml 98% ethanol). After several washes in distilled water tissue was moved into a solution of 4% silver nitrate in distilled water and kept in the dark for 3 days. After a brief wash tissue was developed in aqueous 4% pyrogallol solution for 6 h, washed, dehydrated, embedded in Spurrs (Polysciences) and serially sectioned at 8 μm.

Dextran Texas red and fluorescein backfills

Male *X. peckii* were mounted dorsally on wax. In order to stain photoreceptor bundles an electrolytically sharpened tungsten needle was used to detach single lenses and to injure underlying photoreceptors. A few crystals of dextran beads, conjugated to Texas red or fluorescein (in each case 3,000 MW, lysine-fixable; Molecular Probes, Eugene, Ore., USA) were then applied to the lesioned area. The site was covered by Vaseline and the preparation was left within a moist chamber for 1–2 h. The tissue was fixed in 3% formaldehyde buffered in phosphate buffer (pH = 7.2); 1.3% sucrose was added to maintain adequate osmolarity. Finally the tissue was dehydrated, embedded in Spurrs (Polysciences) and serially sectioned at 8 μm. Finished preparations were viewed on a Biorad 600 Confocal microscope. In order to stain medulla neurons the same basic protocol was followed, except that the tungsten needle was used to lesion the neuropil at the level of the lamina.

Synaptotagmin staining

Synaptotagmin is one of the proteins that are fundamental for synaptic vesicle exocytosis and interacts with components of the SNARE complex (Lin and Scheller 2000). An antibody against synaptotagmin can be used to stain synaptic regions within the neuropils (Meinertzhagen et al. 1998) and thus allows to visualize internal organizations such as the presence of cartridges in the medulla. Heads of male *X. peckii* were fixed in 3% formaldehyde and 1.3% sucrose in phosphate buffer (pH = 7.2) for 16–24 h at 4°C. Tissue was dehydrated, embedded in Paraplast plus (Oxford Labware, St. Louis, Mo., USA), and serially sectioned at 8 μm. After a blocking step dewaxed sections were incubated for 24 h at 4°C in anti-synaptotagmin (raised in rabbit and kindly provided by Dr. Hugo Bellen, Howard Hughes Medical Institute) at a concentration of 1:200. After thoroughly washing, anti-rabbit secondary antibody conjugated to Alexa Fluor 568 was applied for 2 h at room temperature. Finally the tissue was washed and viewed in buffer on a Biorad 600 Confocal microscope.

Transmission electron microscopy

Heads of male *X. peckii* were fixed for 2 h in 2.5% glutaraldehyde in 0.1 mol l^{-1} phosphate buffer (pH = 7.2) and postfixed for 1.5 h in 2% buffered OsO_4. The tissue then was dehydrated, embedded in Spurrs (Polysciences) and sectioned at 100 nm. Sections were mounted onto grids and stained for 20 min with 2% aqueous uranyl acetate and for 6 min in 4% lead acetate and subsequently viewed with a Philips 201 transmission electron microscope.

Scanning electron microscopy

Whole animals were dried, gold coated and viewed with a Philips SEM 505 microscope.

Electroretinograms

The tip of a capillary tube was briefly blunted over a flame, filled with a solution of 0.85% NaCl, and applied to the surface of the eye of a wax-mounted male Strepsiptera. The indifferent electrode, a microelectrode filled with 0.85% NaCl was placed within the thorax of the animal. The field potential of the eye was amplified with an A-M Systems 1600 amplifier and recorded by a computer. The visual stimulus for longer time exposures was applied from a fiber optic lamp with a hand-held shutter, and for critical flicker fusion frequency measurements by a strobe light (1540-P4 Strobolume) at a maximum light level of 1.3 mW m^{-2}. The actual light stimulus was monitored and recorded with a photocell.

Results

Eyelet organization and optical parameter

In this section we summarize measurements and calculations that describe the optical system of individual eyelets. While we have briefly mentioned some of these parameters in a preceding paper (primarily within the footnotes of Buschbeck et al. 1999), we now present a summary of important factors and how they were derived in Table 1.

Staining with osmium/ethyl gallate was used to visualize the size and shape of the lens and the spatial relationship between the lens and the underlying retina (Fig. 3A). The lens of each eyelet is quite thick; in fact it is almost as deep as it is wide. Directly underneath the lens a small number of relatively big and fairly transparent cells are visible which, in contrast to compound eyes, do not correspond to crystal cones, but to corneal cells (Kinzelbach 1990). Proximal to those cells lies an extended cup-shaped retina.

The rhabdoms are organized into a somewhat irregular mesh, which can be visualized by fluorescent staining as the dye is particularly well adsorbed by the rhabdoms of each receptor cell (Fig. 3B). The rhabdoms of neighboring cells are adjacent to each other, together forming an irregular, roughly hexagonal continuous mesh that extends throughout the entire extent of the retina. This organization is different from *Stylops*, where the rhabdoms, at least at the most distal end, form a ring that surrounds the granular extracellular matrix (Wachman 1971). The irregular nature of the *Xenos* rhabdoms is best examined in transmission electron micrographs (Fig. 4). A section along the long axis of a photoreceptor reveals that the rhabdoms which are only about 11 μm long are widest at the most distal part of each receptor cell and narrow somewhat in the more proximal region (Fig. 4A). In cross-section the close physical proximity of neighboring segments of rhabdoms becomes apparent (Fig. 4B). The rhabdomeric meshwork is irregular with different patches that are contributed from different cells. Microvilli of adjacent patches of rhabdoms generally are oriented differently, resulting in a clear border between them (arrow).

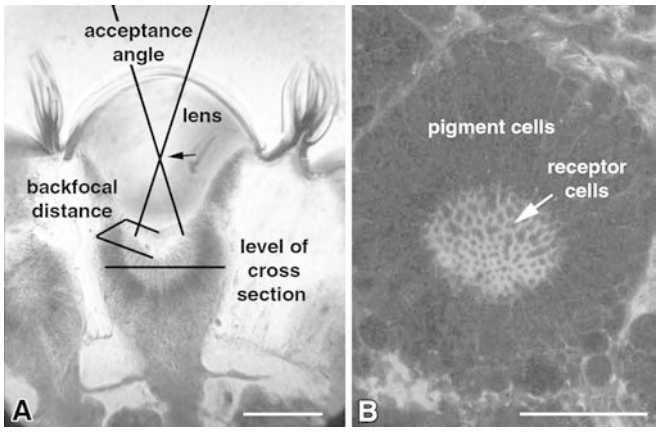

Fig. 3 **A** Cross-section of one eyelet indicating some of the optical parameters that are discussed in Table 1. The *arrow* indicates the nodal point which is situated close to the middle of the very thick lens. **B** Cross-section of the retina, with the network of rhabdoms of the retinula cells of a single eyelet stained red with fluorescent dye. The level of the cross-sections is indicated in **A**. Scale bars: 25 μm

Table 1 Summary of optical measurements including standard deviations and calculations based on individual eyelets of the strepsipteran *Xenos peckii*

Parameter	Value	Method
Back focal distance	12.4 ± 1.5 μm	Hanging drop method, using a cover-slip corrected oil immersion lens and correcting values for the refractive index of the immersion oil (see Wilson 1978)
Histological distance between the back of the lens and the retina	12.1 ± 3.5 μm	Measured from osmium-stained cross-sections These values are not corrected for shrinkage, which would be less than 3% given the fixation (Brunschwig and Salt 1997). $n = 10$
Focal length (f)	44 ± 5 μm	The hanging drop method was used to measure the image magnification. Because $O/U = I/f$ (O object size, U object distance, I image size; see Land 1981) f can be calculated. $n = 21$
F-number	0.68	Calculated from f/A where A, the aperture = 65 μm, which is the average diameter of a lens
Acceptance angle	33 ± 6°	Calculated from the geometrical constraints of the retina and the focal length. $n = 10$
Inter-eyelet angle	27 ± 6°	Measured from osmium and Bodian-stained cross-sections. $n = 10$
Eye parameter (p)	31 μm	Calculated as $D\Delta\phi$ (see Fordyce and Cronin 1989, 1993)
P adjusted for individual receptors	2.8 μm	31 μm/11 receptors (which is the number of histologically discernable receptors on a cross-section)
Receptor diameter	1.5 μm	Estimated from osmium-stained and TEM sections
Receptor length	11 ± 2 μm	Measured from osmium-stained sections. $n = 10$
Light sensitivity of optical system (s)	0.22 μm2	Calculated after Land (1981): $S = (\pi/4)^2(A/f)^2 \times d^2 \times (1 - e^{-kx})$; d receptor diameter (1.5 μm, estimated), x receptor length (11 μm, measured) k absorption coefficient (we used the estimate of 0.0067 mm^{-1} based on measurements by Bruno et al. (1977)

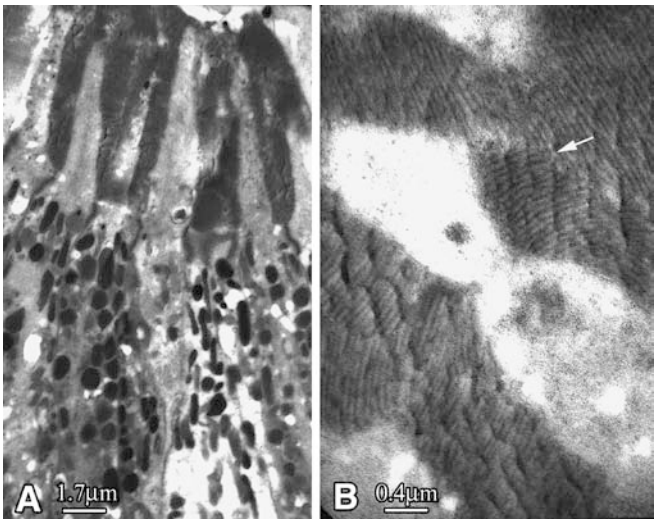

Fig. 4A,B Transmission electron micrographs of retinula cells of a single eyelet. **A** Section along the long axis of some receptor cells. The rhabdoms are visible as *dark areas* in the upper part of the image, on top of the pigment cup. **B** Cross-section of individual photoreceptors. The rhabdoms of neighboring cells show microvilli that are oriented differently but are immediately adjacent to each other (*arrow*)

The irregular nature of the retinal organization makes it difficult to estimate the number of contributing photoreceptors. From light-microscopic cross-sections of many individual eyelets, we estimate that on average 11 receptors contribute to each cross-section. This suggests that a total of nearly 100 ($r = 5.5$ photoreceptors; $\pi r^2 = 95$) receptor cells together contribute to each retinal cup. While these light-microscope-based estimates might be most informative in terms of the functional organization of the optical system, the actual number of receptor cells could be considerably higher. It has been suggested from electron-microscopic serial sections in *X. vesparum,* that the retina might be tiered (Pix et al. 2000), which could greatly increase the number of retinula cells that contribute to each rhabdom. The retina itself is surrounded by pigmented cells that optically separate neighboring eyelets. However no screening pigment is found within the retina itself (Wachmann 1972; Pix et al. 2000), which could lead to the loss of resolution due to the lateral spread of light. Lateral spread would reduce the spatial resolution, but would nevertheless allow for some image resolution within each eyelet. Interestingly the rhabdoms are rather short, only about 11 µm long, which has a minimizing effect on lateral spread. Image resolution can be achieved within each eyelet as long as the image of an object in the visual field is in sharp focus.

Is the image focused at the level of the retina so that the eyelet can function as an image-forming eye? While the answer may seem obvious, there are, in fact, cases of insect eyes in which this is not true. For example the large median ocelli of many insects are considered to be underfocused (Schuppe and Hengstenberg 1993), though recently it has been reported that there is some image resolution within the median ocellus in the dragonfly (Stange et al. 2002). The back focal distance (the distance from the back of the lens to the focus) can be determined by projecting an image through the lens (Wilson 1978). These and other optical parameters are summarized in Table 1. According to our measurements the image is indeed focused at the level of the retina (Buschbeck et al. 1999). Using the same general set-up in which an image is projected through an isolated strepsipteran lens it is also possible to measure the magnification of the optical system. Because the ratio of object size to object distance equals the ratio of image size to focal length ($O/U = I/f$; see Land 1981), we calculated the focal length to be on average 44 µm (Table 1). This puts the nodal point of the lens system into the middle of the lens (Fig. 3A, arrow). Once the focal length is known, it is easy to calculate the *F*-number, which is a value that has been well described for visual systems in other organisms and is also used in photography. The *F*-number simply is f/A, where A is the aperture of the lens. Our calculation is based on the simplification that in the strepsipteran eye, which lacks any kind of iris, the aperture should approximately equal the lens diameter. It is, however, difficult to assess the precise aperture simply from the shape of the lens, and the lens diameter describes the highest possible value. In other words, our calculation of the *F*-number could be a low estimate.

The focal length and the position of the nodal point are also useful parameters for estimating the acceptance angle. Figure 3A illustrates how the nodal point and the extent of the retina together form a triangle, the acute angle of which is equivalent to the acceptance angle. We have calculated this to be approximately 33°, which is slightly higher than the purely geometrically determined angle between the individual eyelets (about 27°). This means that the field of view of neighboring eyelets could overlap. Naturally the existence and magnitude of such an overlap greatly influences the functional organization of the eye as a whole and will have to be investigated further. We hope to empirically measure the acceptance angle in future by using ophthalmoscopy.

The eye parameter (p), which describes the product of the lens diameter (D) and the interommatidial angle ($\Delta\phi$) has previously been used in compound eyes to describe the light gathering ability of each facet. In the Strepsiptera, when calculated for each eyelet this parameter results in much higher values than would be expected for optimal compound eye design in daylight. Similar results have been obtained for schizochroal trilobites, implying that schizochroal trilobites may have possessed a similar eye organization as we suggest for Strepsiptera (Fordyce and Cronin 1989, 1993), but see Horváth et al. (1997). Although the value for p is rather high for the entire eyelet, it is comparable to other insects (Land 1981) if we adjust the value for the high number of receptor cells that are found in the strepsipteran retina (p/number of receptors = 2.8).

While the eye parameter gives and indication of the light gathering ability of the lens and eyelet as a whole,

the sensitivity function, S, directly calculates light levels that can be captured by individual receptors considering their size and shape. As for the eye parameter p when applied to individual photoreceptors, our value for S (0.22 μm^2) compares well to other diurnal insects (Land 1981).

At this point one might want to ask if male *X. peckii* do indeed lead a diurnal life style. To this end we have collected data regarding the time of the day when adult males emerge from their puparium. Interestingly, with very few exceptions Strepsiptera always emerged in the early afternoon, with a peak emergence time between 2:00 and 4:00 p.m. ($n = 69$). This way they spend most of their short lives during the prime foraging hours of their wasp hosts.

Electroretinograms

In order to measure physiological properties of photoreceptors we performed electroretinogram recordings. Figure 5A, B illustrates a comparison of the general shape of the response of most of one eye of *Drosophila* (Fig. 5A) and *Xenos* (Fig. 5B). While we measured a comparable drop in field potential in both species, which is caused by the light response of the receptor cells themselves (Goldsmith 1960; Leutscher-Hazelhofe and Kuiper 1964; Loew 1975), there is an interesting difference between the transients at the beginning and end of the light stimulus. At the onset, *Drosophila* is characterized by a short strong rise in the extracellular potential, presumably a current that arises from synaptic responses at the level of the lamina (Heisenberg 1971). A similar electroretinogram and its components also has been described for other flies (Loew 1975). In *Xenos*, on the other hand, the transient at the onset is of opposite polarity, reflecting a sudden drop in the extracellular trace. There is also a transient current at the offset of the visual signal in *Drosophila*. At this point it remains unclear if such a current exists in *Xenos*, as we have observed it only in two out of six individuals. It is possible that this transient current indeed exists, but for some reasons degenerates more rapidly.

We have also used electroretinograms to measure the critical flicker fusion frequency. Figure 5D is an example of the response of the photoreceptors to an increasing frequency of light flashes. While the photoreceptors follow accurately to relatively low frequencies (left half), recovery gradually decreases at higher frequencies and the receptors fail to follow frequencies that exceed 35 Hz on average ($n = 3$). At higher frequencies the receptors are unable to follow individual light flashes, but their response is more similar to that of a long, sustained light stimulus with several transients which, however, skip most of the light flashes. The value of 35 Hz is lower than for many other insects (Autrum 1958), and in our own measurements the critical flicker fusion frequency of *Drosophila* exceeds at least 57 Hz (this is the highest frequency which we could achieve with equal stimulus intensity to that used for *Xenos*).

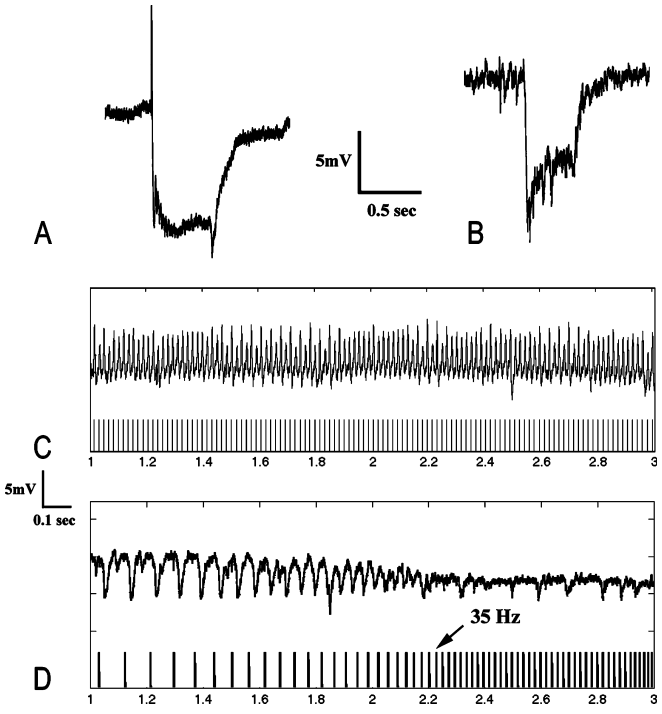

Fig. 5A–D Electroretinograms. A, **B** Comparison of the shape of the retinal response to a single light pulse in *D. melanogaster* (**A**) and *X. peckii* (**B**). While the general response of the photoreceptors is comparable in the two species, the presence and polarity of transients at the beginning and the end of the stimulus differ greatly. **C**, **D** Example traces for the critical flicker fusion frequency of *D. melanogaster* (**C**) and *X. peckii* (**D**). In each case the *upper trace* depicts the response of the photoreceptors of most of one eye, and the *lower trace* illustrates actual light pulses recorded with a photocell. While the photoreceptors of *Drosophila* follow frequencies beyond 50 Hz (**C**), the photoreceptors of *X. peckii* only follow the stimulus to frequencies of about 35 Hz (**D**). The latter is illustrated by a light stimulus that gradually increases in frequency

Neural organization

Lamina

Photoreceptors of each eyelet leave the retina in a bundle that twists around its own axis (Fig. 6A, D) and projects to the lamina, where it widens to an oval, fan-shaped projection (Fig. 6A–C). As we previously suggested, the twist may provide image continuity across the entire eye (Buschbeck et al. 1999). Because each lens projects an inverted image onto the retina, the physical twist of the receptor bundle becomes necessary to reorient the visual inputs of neighboring eyelets next to each other at the level of the lamina.

The fan-shaped nature of the receptor projections within the lamina is surprising. This is different form the organization of other insects (Strausfeld 1976; Meinertzhagen and Hanson 1993; Fischbach and Dittrich 1989), where receptor projections always enter the neuropil perpendicular to its surface. Furthermore, in other insects additional cells in the lamina (monopolar cells) are also oriented perpendicular to the lamina surface and have lateral processes that are strictly parallel to the

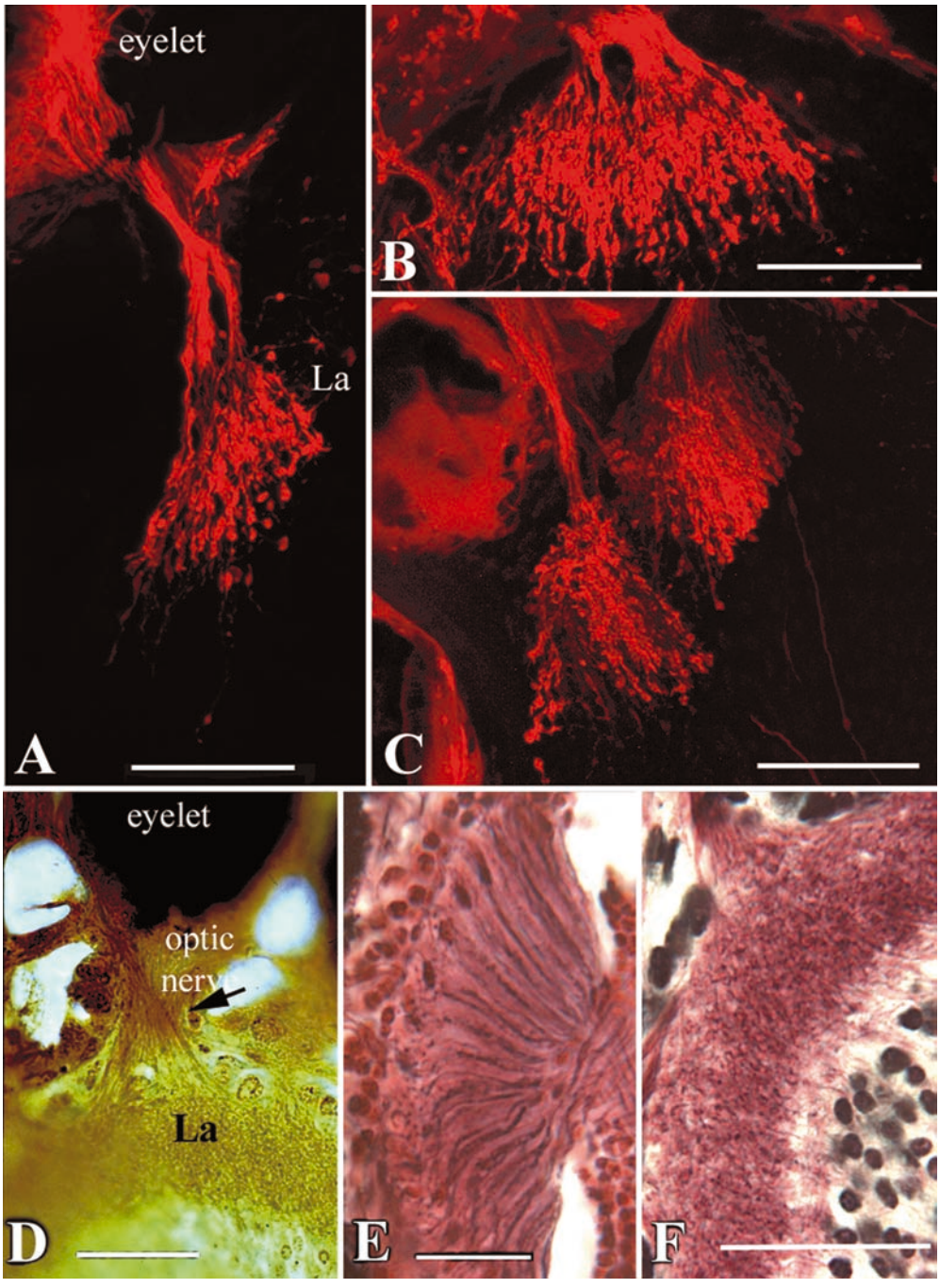

Fig. 6A–F Organization of the *Xenos* lamina, with comparison to *Drosophila*. **A**–**C** Examples of photoreceptor projections into the lamina. **A** Projection from the eyelet to the lamina (*La*) along its narrow axis. **B** In a second example the wide axis of the oval lamina projection is depicted. **C** In horizontal sections two projections of neighboring units appear to project into neighboring areas at the level of the lamina. **D** Block silver staining of the optic nerve, which twists along its axis (*arrow*) before entering the lamina. **E**, **F** Comparison of Bodian-stained laminae of *D. melanogaster* (**D**) and *X. peckii* (**E**). While cartridges are clearly recognizable in *Drosophila* they appear absent in *Xenos*, which may be due to the unusual shape of the incoming photoreceptor bundles. Scale bar: 25 μm in all figures except **E**, where it represents 10 μm

neuropil surface. This crystalline organization is clear in Bodian-stained *Drosophila* (Fig. 6E) but is absent in the equivalently stained *Xenos* lamina (Fig. 6F). The homogeneous appearance of the lamina in *Xenos* may largely be due to the unusual fiber arrangement. The cross-sections of the terminating receptor bundles are not round in the lamina. Rather, a cross-section of the fan-shaped bundle appears as an oval that extends almost twice as far along the wider axis than the narrow axis. Figure 6A and C depict horizontal sections, whereas Fig. 6B shows an example of a projection bundle from a frontal section, illustrating the difference in shape from those two directions. Looking primarily at horizontal sections we have observed several examples of neighboring projections that appear to terminate in neighboring areas of the lamina (Fig. 6C) apparently lacking spatial overlap. However, we also came across one example of a frontal section, where two neighboring bundles showed some overlap. The pattern of projections may differ in the horizontal and frontal plane and could also vary according to the precise location of the respective bundle. A more detailed analysis will be necessary to establish receptor terminal organizations within different parts of the eye.

In our backfills, all photoreceptor projections terminate in the lamina. This too is unusual, because in many insects chromatic receptors (Hardie 1979) cross the lamina and terminate at two discrete levels of the medulla (Strausfeld 1976; Strausfeld and Lee 1991).

Medulla

As in other insects, projections from the lamina traverse to the medulla through an optic chiasma (Fig. 7A, arrow). As in the lamina, the medulla fiber architecture appears more precisely organized in other insects such as *Drosophila* (Fig. 7B) than it is in *Xenos* (Fig. 7C). Individual projections, presumably monopolar cells, were visualized by applying fluorescent dye to injured cells at the level of the lamina. This way small bundles of cells were stained which reveal several different cell types. In a number of cases two neighboring cells of the same type were stained (Fig. 7D–F). Presumably those cells project into neighboring domains within the medulla and thus are informative in regards to the spacing and density of adjacent channels (see Fig. 7D). Although Bodian staining does not reveal a clear cartridge organization in the medulla (Fig. 7C), staining of individual neurons illustrates that, in contrast to the retinula cells of the lamina, many cells indeed enter the medulla perpendicular to its surface, as is common in other insects. Massive staining of a bundle of medulla fibers (Fig. 7G) illustrates a high density of projections and shows that some of the cells have lateral processes that leave the main axon at an angle. Such cells

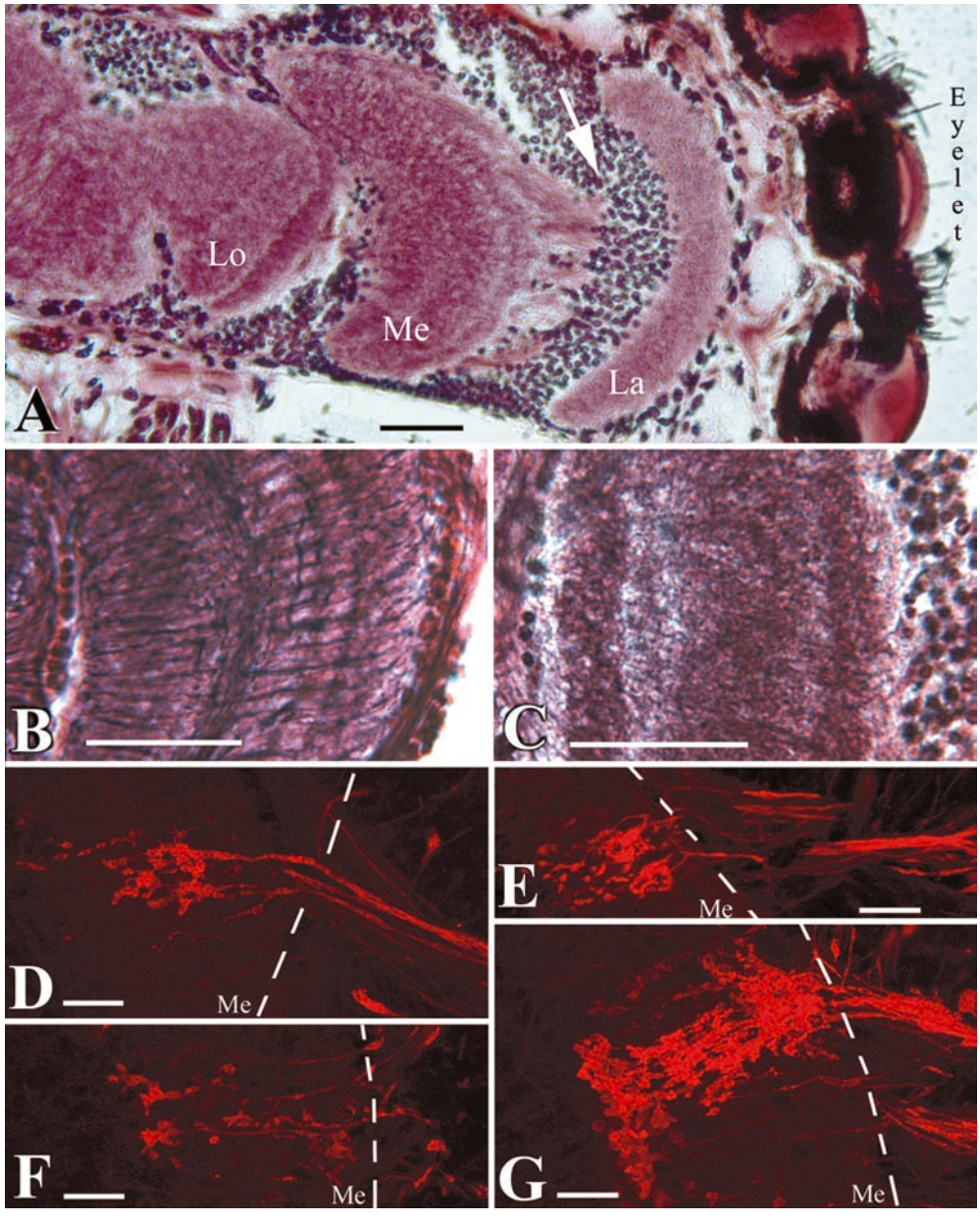

Fig. 7A–G Organization of the *Xenos* medulla with comparison to *Drosophila*. **A** Overview of the optic neuropils of *Xenos* in Bodian staining (*La* lamina; *Me* medulla; *Lo* lobula). Note that no clear cartridges are visible in the medulla. This is also illustrated in comparison to *Drosophila* (**B**, **C**). In this larger magnification in *Drosophila* (**B**) cartridges are clearly visible, but they are absent in *Xenos* (**C**). **F**, **G** Individual cells or a bundle of cells projecting into the medulla that have been filled with fluorescent dye. Several examples of similar cells that project next to each other are suggestive of periodicity patterns within the medulla. Scale bars: 25 μm

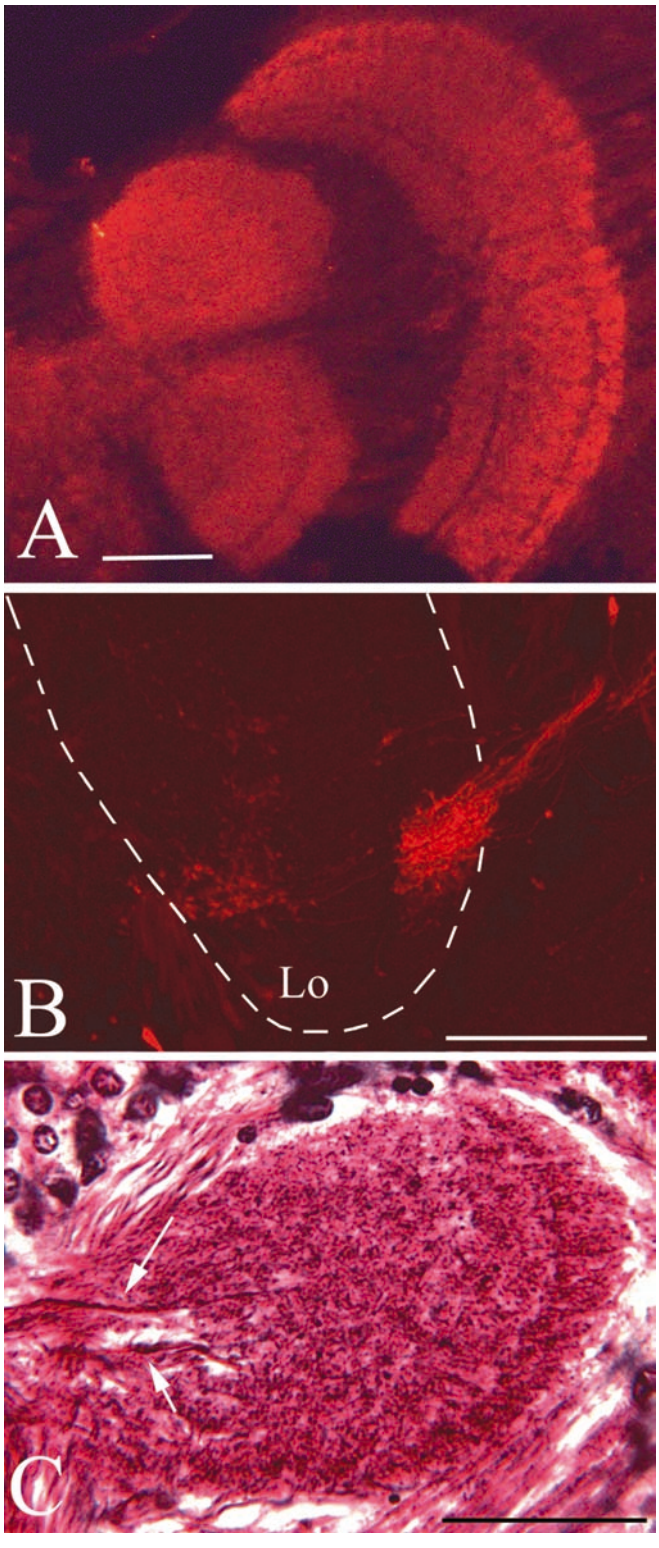

Fig. 8A–C Organization of the *Xenos* lobula. **A** Staining against the synaptic vesicle protein Synaptotagmin illustrates the general shape of the medulla and lobula. In this frontal section a strong division of the lobula into a dorsal and a ventral section is visible. Fluorescent staining of a bundle of individual cells (**B**) illustrate terminals in the outermost region of the lobula. A segregation of that outer region is also visible in **A**, and in Bodian staining (**C**). In **C**, which shows an example of a horizontal section, some larger tangential fibers are visible in the proximal part of the neuropil (*arrows*). Scale bars: 25 μm

presumably contribute to the peculiar organization of the Strepsiptera in which no clear medulla cartridges are discernable.

Lobula

Like the lamina and medulla, the lobula does not reveal stringent fiber architecture, as is apparent from antibody staining against synaptotagmin (Fig. 8A) as well as Bodian staining (Fig. 8C). However, the outermost layer is clearly separated from deeper neuropil regions, as can be seen in the same two images. This separation results from medulla projections that terminate within the outer layer of the lobula (Fig. 8B). The lobula furthermore stands out in its shape. In frontal sections, a strong division into a dorsal and a ventral portion is visible in the posterior region of the neuropil (Fig. 8A). The most anterior part of the lobula is continuous. The division is formed by a physical separation that results from a fiber tract originating in the medulla. It appears as though the lobula wraps around this projection. At the base of the dorsal lobula large fibers are discernable (Fig. 8C), indicating the presence of some larger cells, possibly tangential cells. Furthermore, and unusual when compared to other insects, the lobula in the Strepsiptera is not sharply separated from the proximal part of the brain, but rather partially fuses with medial neuropil regions.

Periodicity within the neuropil

In insects and crustaceans the optic neuropils, especially the lamina and medulla, generally are characterized by strict, regular patterns. These patterns result from a precise retinotopic organization, in which neighboring points in space are processed in parallel within the neuropils. In flies for example, a set of uniquely identifiable neurons in the lamina can be found for every lens in the periphery (Strausfeld 1970, 1984). Likewise, for each lens in the periphery there is a set of identifiable neurons in the medulla which forms a cartridge. This precise one-to-one organization of insect visual neuropils allows for predictions regarding the visual sampling array, simply by measuring the density of cartridges within the neuropil. Because one of the most prominent questions regarding the strepsipteran visual system is its effective visual resolution, a periodic organization within the neuropils could give insights into the density of the visual sampling array. Unfortunately, as we have previously pointed out, several general staining techniques such as Bodian or ethyl gallate staining, which reveal periodicities very well in other insects, fail to show similar patterns in *Xenos*. This may be due to the lack of precisely orthogonally organized fiber architecture.

Nevertheless, in virtually all visual systems neighboring visual sampling points are processed in parallel next to each other, and all our anatomical observations thus far suggest that this is also the case in *Xenos*. If we were able to visualize periodicities within the lamina and/or medulla we could predict to what extent visual resolution is maintained within the strepsipteran visual neuropils.

One way to look at such periodicities is to analyze the size and spacing of neighboring, individually stained medulla cells such as those illustrated in Fig. 7D–F. In each of the three figures a pair of similar cells are illustrated that spaced between 10 and 20 μm from each other. If those cells are representatives of visual neurons from neighboring parallel pathways, then this spacing can be used for a rough estimate of the periodicity within individual medulla sections, at least in regards to those cell types. Based on these and other cells there are anywhere between 15 and 30 parallel channels on cross sections in which only 5 or 6 eyelets are visible.

A second approach towards revealing periodicities within the strepsipteran neuropils has been to visualize internal organizations by staining for synaptic regions by using an antibody against synaptotagmin (Fig. 9). In *Drosophila* staining with an antibody against synaptotagmin reveals the cartridge organization within the lamina and most of the layered medulla (Fig. 9A). The same staining protocol also shows periodicities in the medulla of *Xenos*, at least within the outermost region of the neuropil (Fig. 9B, C). The spacing between neighboring units is comparable to that of the individually identified cell pairs (see above). A rough estimate of the number of channels that can be visualized within a single cross section lies between 20 and 30 on cross sections where only 5 or 6 eyelets are visible. Although at this point such estimates remain tentative and rather rough, it becomes clear that the number of potential parallel channels far exceeds the number of eyelets on any given cross section. In some sections staining with synaptotagmin (Fig. 9D) also reveals periodicity in the lamina.

In addition to the cartridge organization, staining against synaptotagmin also shows specific layering of the medulla in both species. In *Drosophila* three major layers are visible: the outer medulla, the serpentine layer and the inner medulla. Interestingly in *Xenos* as well three major layers are visible. However, in contrast to *Drosophila*, in *Xenos* most of the medulla is part of the middle layer, with only a small outer and inner layer. In *Drosophila* the middle layer of the medulla is formed primarily by large tangential cells that project directly from the medulla to the middle of the brain. While this projection does not show up in the synaptotagmin stain, it is clearly visible in Bodian staining. In *Xenos* no such projection has been observed (Fig. 7A), which makes it unlikely that the middle layers of the two species are equivalent.

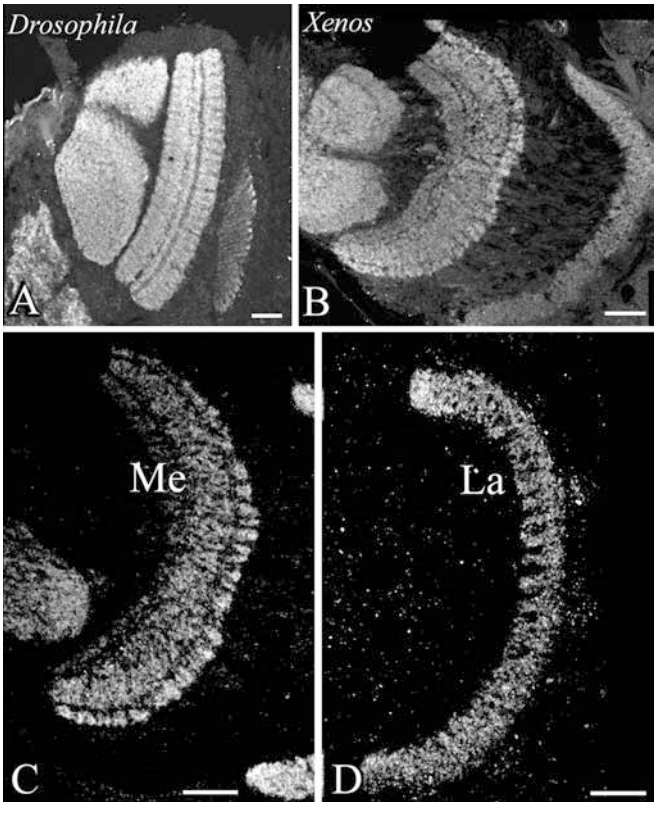

Fig. 9 Comparison of lamina and medulla periodicities in *Drosophila* (**A**) and *Xenos* (**B**) revealed by staining for the synaptic vesicle fusion protein synaptotagmin. In both species periodic patterns are visible at least in the outermost layer of the medulla, and the medulla shows several discrete layers. Regularities within the strepsipteran medulla and lamina are also illustrated at a larger scale (**C**, **D**). *Me* medulla, *La* lamina. Scale bars: 25 μm

Discussion

In this account we have examined the eye of the male strepsipteran *Xenos peckii*, including its optics, receptor physiology and neuropil organization in order to deepen our functional understanding of this peculiar eye. By comparison to the much studied *Drosophila* eye, which serves as a canonical example of an insect compound eye, we have established several ways in which the strepsipteran eye differs, confirming our previous claim that Strepsiptera do not possess a compound eye, but rather their own unique eye type which reflects an intermediate form between a compound eye and image forming single eyes (Buschbeck et al. 1999).

The eyelets

Several of our optical measurements and calculations such as the back focal distance, the acceptance angle, the eye parameter, p, and the light sensitivity, S, of the optical system suggest a potential for relatively high spatial resolution within each functional unit, the eyelet of the strepsipteran eye. At the light-microscope level there are about 100 receptor cells visible per eyelet, which would represent the highest level of resolution that potentially could be achieved. However, there are several parameters and organizational principles which are not

compatible with such high resolution, and it is likely that the actual spatial resolution of the system is considerably lower than the number of receptor cells. For example the *F*-number of the lens is relatively low, comparable to image forming lens eyes with a low resolution, such as those of the nocturnal spider *Dinopsis* (Land 1981). A certain amount of pooling of the visual content of several receptor cells would not be surprising, as even in normal compound eyes eight to ten photoreceptors work together to analyze a single image point (Nilsson 1989). The ultrastructural organization of retinula cells is also consistent with some degree of overlap of visual information between neighboring cells. The rhabdoms of neighboring cells are frequently adjacent to each other, together forming a continuous network rather than discrete, separated units. Furthermore, no screening pigment is found within the retina itself. On the other hand the retina is so thin (only about 11 μm) that screening pigment is not necessary to maintain some visual resolution.

Our measurements of the shape of the electroretinogram is interesting in relation to systematic considerations. One of the most compelling questions that remain to be addressed is that of the evolution of this peculiar eye, and of Strepsiptera themselves. Unfortunately, the systematic position of this parasitic insect order still remains unclear. Most recent findings are based on molecular data and suggest a close phylogenetic relationship to flies (Diptera; Whiting et al. 1997; Whiting 1998; Wheeler et al. 2001). However, the validity of this placement has been vigorously debated (Huelsenbeck 1998, 2001) because of potential problems in phylogenetic reconstruction algorithms (Felsenstein 1978; Huelsenbeck and Hillis 1993). We find it interesting that the shape of the strepsipteran electroretinogram (especially the reversal of the initial transient) is very different from that of flies, but is more similar to those of other insects such as beetles, grasshoppers (Fouchard and Carricaburu 1972) or thrips (Matteson et al. 1992).

We have found that the critical flicker fusion frequency of *X. peckii* lies only around 35 Hz. This value is considerably lower than has been reported for other diurnal rapidly flying insects, such as flies or dragonflies (Autrum 1958), but is similar to slower eyed nocturnal insects (Autrum 1958; Campan et al. 1965) or to the visual system of tiger beetle larvae (Mizutani and Toh 1995). A low flicker-fusion frequency is consistent with *Xenos*' flight style, which appears relatively slow when compared to fast flying insects. On the other hand, the longer integration times that result from a low frequency contribute to increased light capture.

The optic neuropils

Along with the outer optical system of the eye, the optic neuropils of *Xenos* also appear to be organized in an unusual fashion. The neuropils of insects and crustacea generally are organized in a very precise and retinotopic way, which can easily be visualized by almost any general staining technique. The strepsipteran optic neuropils, on the other hand, lack such a clear organization. So far, it remains unclear why the strepsipteran neuropils appear more diffuse than those of other insects. Wachmann (1971) concludes that the strepsipteran eye really is most similar to the eyes of insect larvae, with each unit being homologous to a stemma rather than an insect ommatidium. If his theory is correct, then the neuropil organization itself also could be more comparable to that of insect larvae than to adults, and an immature organization of the Strepsiptera could be the result of a fairly extreme case of paedomorphy. While the general appearance of the strepsipteran neuropils indeed is reminiscent of some insect larvae (i.e., tiger beetle: Toh and Mizutani 1994a, 1994b), larval insects are usually characterized by only two neuropils that underlie each stemma, the lamina and the medulla, whereas Strepsiptera are characterized by lamina, medulla and lobula. However, if such a comparison is made, then the strepsipteran lobula with its large tangential neurons would have to be compared to the tiger beetle medulla. In tiger beetle larvae, which do not possess a lobula, it is the medulla in which large field neurons have been demonstrated to respond selectively to moving objects (Mizutani and Toh 1995) and even can discriminate distances (Okamura and Toh 2001). Another major difference in the Strepsiptera is that in each of the two eyes there is only one set of neuropils that subserves all eyelets, in contrast to tiger beetle larvae which possesses a set of neuropils for each of the stemmata.

A further aspect that would profoundly affect the organization of the strepsipteran visual neuropils is the potential for visual overlap of neighboring eyelets. According to our calculations the visual image of neighboring units may indeed overlap to a certain extent. Evaluating the organization of neighboring projections of photoreceptor terminals at the level of the lamina provides conflicting results, but at least in some regions and orientations there could be overlap at the anatomical level. If there is indeed some overlap, redundancy in visual information has to be recognized and pooled, which could easily lead to strong modifications in the general fiber architecture of the neuropils. In fact the presence of fibers that cross between parallel neighboring visual pathways would be expected. The most parsimonious place for such reorganization would be at the level of the outermost optic neuropil, the lamina, but other regions could be affected as well.

Despite all these deviations from the virtually universal insect plan, the strepsipteran optic lobes are characterized by a clearly demarcated lamina, medulla and lobula, similar to those found in other insects (Cajal and Sánchez 1915; Strausfeld 1976). Photoreceptors from the retina project in a twisting bundle to the lamina. This twist in the bundle reverses the partial images of individual eyelets so that they are in register at the

first stage of visual processing. In our backfills all receptors were found to terminate at the outermost level, the lamina. This is different from some other insects, such as flies (Strausfeld 1976), where chromatic receptors cross through the lamina and terminate within the medulla. This raises the question of the spectral wavelengths that are processed by the *Xenos* photoreceptors. In flies all fibers that terminate in the lamina are blue-green receptors with a broad absorption curve that peaks around 490 nm (Hardie 1979). Those fibers that project into the medulla are typically associated with chromatic photoreceptors. It thus could be possible that the absence of such deep fibers in *Xenos* reflects the lack of chromatic vision. The lamina terminals of the *Xenos* retinula cells also are unusual in that they fan out within the neuropil, rather than conform to architecture that is perpendicular to the lamina surface. This slanting organization may be one of the reasons that the level of periodicity is difficult to assess within the lamina.

By looking at individual cells, and by using an antibody against the synaptic protein synaptotagmin, we nevertheless managed to get some insights into periodic repetitions within at least the medulla. By assessing the number of such repetitions within a cross-section, and by comparing it to the number of eyelets in the periphery, it becomes possible to give some vague estimates of how many parallel channels (or cartridge equivalents) could underlie a single eyelet. Here we avoid the name cartridges, because we do not know how these repetitions develop and if they are homologous to cartridges in other insect visual systems. In addition, it is possible that there are repeating units at different frequencies, such as on the level of the smallest receptive unit as well as on the level of individual eyelets. The regular structure in "normal" compound eyes is due to strictly repeating neuronal units of a single periodicity. The presence of units with a different periodicity could contribute to a less ordered appearance. Nevertheless we think that the periodicity, or clustering, of visual fibers is of functional significance, capturing the spatial resolution that is maintained by particular pathways of the visual system. Based on our staining of pairs of identifiable cells, and of synaptotagmin staining of the outermost layer of the medulla, there could be anywhere between 6 and 35 parallel channels underlying each eyelet. While this describes a rather wide range, it is important to note that independent of the accuracy of the resolution, all of our observations are consistent with the fact that image formation within eyelets is maintained at the neuropil level. Other anatomical observations, such as the size and density of the optic neuropils are also consistent with this notion. A much more detailed anatomical study and more extensive physiological measurements will be required to determine the precise level or levels of resolution. In principle there could be multiple levels, as there is no clear functional reason to suppose that different visual pathways have to follow the same spatial resolution. In fact it is common in both vertebrate (DeYoe and Van Essen 1988) and invertebrate visual systems (Strausfeld and Lee 1991) that different visual modalities are processed separately and in parallel. It is thus conceivable that some of these pathways operate at a higher spatial resolution than others. Because, in insects, different visual modalities are processed at different levels of the medulla (Strausfeld and Lee 1991) it could be particularly fruitful to study periodicities at different medulla levels separately.

The most remarkable features regarding the gross anatomy of the lobula are its division into a dorsal and a ventral portion in the posterior part of the neuropil, and its intimate connection to more centrally situated brain regions. While a complete separation is of the visual system into a dorsal and ventral half has previously been described for insects with divided eyes (see Bibionidae; Zeil 1983; Simuliidae; Buschbeck and Strausfeld 1997), the partial division of the lobula in the Strepsiptera appears to result from the presence of a large fiber tract, similar to some Lepidoptera (B. Ehmer, personal observation).

General considerations

All of our findings, including periodic patterns within the neuropil organization, are compatible with our previous suggestion that the strepsipteran eye is a composite eye that is built from about 50 small, image forming eyelets (Buschbeck et al. 1999). While the precise level of resolution within each eyelet still remains subject to further investigation, we have brought forth evidence, based on the histology, that the resolution could be anywhere between 6 and 35 points per eyelet. These findings are in contrast to the analysis of the optomotor behavior by Pix et al. (2000), which suggests that the motion vision system of *X. vesparum* could operate at the level of the individual eyelet (or facet) units rather than at a higher level of visual resolution. As Pix et al. (2000) mention, their results only refer to the motion pathway and do not exclude a higher level of image resolution.

Also consistent with a higher level of resolution is the high number of retinula cells, which are among the most energetically costly cells of an insect (Laughlin et al. 1998). For example in *Drosophila*, which has only 8 receptor cells/facet, the energetic consumption of the retina alone amounts to 8% of the resting level of the entire animal. It therefore is hard to imagine that a strepsipteran would maintain 100 cells for a function that can be achieved with just a few. Along the same line of argument it is worth mentioning that the visual neuropils are large and densely packed. Together they comprise about 75% of the entire male *Xenos* brain. Again, it is hard to conceive why this abundance should occur, if it was not needed for visual processing.

One of the biggest mysteries regarding the strepsipteran eye remains its origin. Systematists are still uncertain where to place this peculiar order in the insect phylogenetic tree (Rokas et al. 1999; Huelsenbeck 2001),

with the most common placements being close to flies (Diptera: Whiting et al. 1997; Whiting 1998; Wheeler et al. 2001) or beetles (Coleoptera: Paulus 1979; Kinzelbach 1990). Whichever the correct systematic placement, it appears safe to assume that the strepsipteran eye evolved from a normal compound eye ancestor. But how can an eye as complex as the compound lens eye of the Strepsiptera evolve from a fully functional sophisticated compound eye? Because these eye types operate under such different organizational principles it seems that the transition would require some stages of rather poor vision, or at least of poor visual resolution. One possible scenario for this to happen could be a transition through a nocturnal life stage. One would expect that a compound eye that is moved to lower light levels would evolve larger lenses, a higher number of receptor cells that pool visual information, a decrease in the length of the rhabdom and a relatively slow flicker fusion frequency. Interestingly our investigation shows that all these conditions are, at least to some extent, met in *X. peckii*. If *X. peckii* indeed is incapable of color vision, which may be suggested by the absence of long receptor fibers, this also would be consistent with a nocturnal state. We note that the most ancestral group of Strepsiptera, the Mengenillidae, appear to be a nocturnal group, as the short-lived adult males can be found in light traps (Meixner 1936; Kathirithamby 1989) and are parasites of night-active silverfish (Lepismatidae: Kathirithamby 1992). Unfortunately, thus far there are no data available on the functional organization of the eye of that group. If this evolutionary scenario turns out to be correct, some of the more derived lines of Strepsiptera could have regained a higher level of resolution secondarily by improving the resolution within each unit rather than by adding more units. Much future work will be necessary to determine if this indeed is how the marvelous eyes of *X. peckii* evolved.

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