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Visual system characterization of the obligate bat ectoparasite *Trichobius frequens* (Diptera: Streblidae)

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ABSTRACT

As an obligate ectoparasite of bats, the bat fly *Trichobius frequens* (Diptera: Streblidae) inhabits the same subterranean environment as their nocturnal bat hosts. In this study, we characterize the macromorphology, optical architecture, rhabdom anatomy, photoreceptor absorbance, and opsin expression of the significantly reduced visual system in *T. frequens* resulting from evolution in the dark. The eyes develop over a 21–22 day pupal developmental period, with pigmentation appearing on pupal day 11. After eclosion as an adult, *T. frequens* eyes consist of on average 8 facets, each overlying a fused rhabdom consisting of anywhere from 11 to 18 estimated retinula cells. The dimensions of the facets and fused rhabdoms are similar to those measured in other nocturnal insects. *T. frequens* eyes are functional as shown by expression of a Rh1 opsin forming a visual pigment with a peak sensitivity to 487 nm, similar to other dipteran Rh1 opsins. Future studies will evaluate how individuals with such reduced capabilities for spatial vision as well as sensitivity still capture enough visual information to use flight to maneuver through dark habitats.

1. Introduction

As obligate hematophagous ectoparasites of bats, the two families of bat flies (Diptera: Schizophora: Hippoboscoidea: Streblidae and Nycteribiidae) have highly modified morphologies associated with unique ecologies. As ectoparasites, bat fly environments are tightly coupled with that of their generally nocturnal bat hosts, including inhabiting many dim to completely dark subterranean habitats (Patterson et al., 2007). Additionally, the diversity of bat fly morphologies is influenced by the variations of host–parasite associations (Patterson et al., 2008; Tello et al., 2008; Hiller et al., 2018). The nycteribiids generally exhibit highly reduced eyes (absent or with at most two ommatidia), are completely wingless, and are “spider-like” in appearance. Moreover, there is generally little variation among the genera and species in overall body plan, though members of this family exhibit wide variation in size (Theodor, 1967; Dick and Patterson, 2006). The streblids, in contrast, exhibit a mélange of body plans with great variation in features such as shape, body compression, leg length, wing size and shape, and degree of eye loss (Wenzel et al., 1966; Dick and Patterson, 2006).

In particular, the variation in degree of eye reduction and loss across the bat flies is noteworthy. The vast majority of schizophoral dipterans (e.g. flies having a ptilinum structure including the louse flies, house flies, blow flies, among others), have large compound eyes (e.g. 700–800 ommatidia in *Drosophila melanogaster*, 3000 in *Musca domestica*, 5000 in *Calliphora*; Hardie, 1985; Land, 1997; Choe and Clandinin, 2005) specialized for motion processing to maneuver through complex aerial environments. Each ommatidium in these large eyes contain the unique neural superposition visual system architecture, where apposition optics that confer higher spatial resolutions are paired with an open rhabdom and a neural wiring network designed for increasing sensitivity (Agi et al., 2014).

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In contrast, all of the species within the two families of bat flies possess significantly reduced visual systems, ranging from eyeless species to one with compound eyes of up to 57 facets per eye in a species that parasitizes a tree-roosting bat (*Trichobius petersoni* - Wenzel, 1975) (Fig. 1). This eye reduction is even more striking given some species use flight to move around their dim habitats. Relative to other Diptera, the Hippoboscoidea, including the bat flies, have a peculiar reproductive strategy - adenotrophic viviparity - where larva hatch from eggs and are nourished internally until ready to pupate. At that point, female bat flies leave the host, move into the surrounding environment (usually the bat's day roost) and deposit the final larval instar into a hardened case where pupal development occurs (Tobe and Langley, 1978; Dittmar et al., 2009). The need to leave the host to find suitable habitats for pupal deposition leads to a diversity of locomotory modes, with some species capable of crawling only short distances away from the host for pupal deposition and some exhibiting complex flight behaviors associated with finding and leaving hosts (Fritz, 1983; Dittmar et al., 2009, 2011). Flight behaviors, driven by the unique bat fly reproductive strategy, may still be guided in part by visual cues, leading to questions about how considerably reduced eyes maintain enough functionality for flying through a dark world.

Despite studies documenting variations in eye reduction among bat flies at the level of eye size, morphology, and facet number (Zeve and Howell, 1962), very little is known about the underlying architecture of bat fly eyes. Because of the variation in facet number and size across species, and the ability to sample discrete time points relative to eye development in the pupal stages, bat flies offer an interesting system in which to study the developmental, structural, molecular, physiological, and anatomical changes associated with eye reduction in dim light environments. However, beyond external descriptions of the number of facets, few studies have provided a detailed examination of bat fly compound eye form and function. In this paper, we characterize the external morphology, photoreceptor structure and peak absorbance, and opsin expression in the eyes of *Trichobius frequens* (Streblidae) as a first step to understanding the evolution of eye loss across the bat flies. We demonstrate that the compound eyes of *T. frequens* are highly reduced in the number of facets, yet the underlying photoreceptors are still physiologically functional and possess receptor modifications associated with dim light environments.

2. Methods

2.1. Specimen collections and developmental series

In *T. frequens*, females leave the bat hosts and fly within dark cave passages to a suitable habitat for pupal deposition (Dittmar et al., 2009, 2011). This host-leaving behavior occurs every evening as bats emerge from cave roosts to hunt, resulting in a swarm of flying *T. frequens* and providing an opportunity for the collection of large numbers of individuals. Adult and pupa of *T. frequens* bat flies were collected inside Cueva de los Culebrones, Puerto Rico (Departmento de Recursos Naturales de Puerto Rico permit DRNA, 2010-IC-030 to K. Dittmar) using two methods: (1) sweep nets during times when female bat flies leave the host to deposit pupae (e.g. sunset), and (2) sticky traps placed near pupal deposition areas that capture adult females from pupal deposition flights and male and female adult flies newly emerged from pupal cases in search of hosts. Flies were also obtained by collecting pupal cases from deposition sites in the cave, transporting them to the Dittmar Lab at SUNY Buffalo, and allowing them to continue development in an incubator set to 29 °C until eclosion. Individual pupal cases freshly deposited in collection tubes by adult flies collected via sweep nets were dissected from their cases to establish a developmental time series from deposition (day 0) to eclosion.

2.2. Scanning electron microscopy

Individual bat flies were dehydrated through a series of increasing concentrations of ethanol with a final three changes of absolute ethanol. Dehydration was followed by critical point drying in a Tousimis SAMDRI-795. The specimens were positioned on stubs to provide the best view of one eye, coated with gold/palladium, and imaged on either a Hitachi S-4800 at the Biological

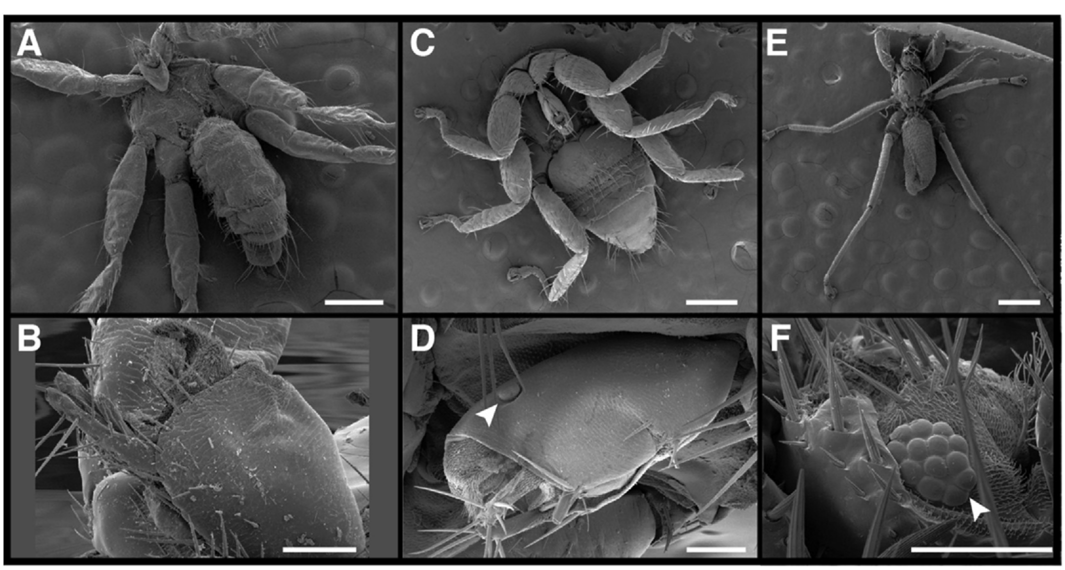

Fig. 1. SEM images of the dorsal view of three bat fly species illustrating the variation in body morphology (A, C, E) and eye reduction in the context of the head (B, D, F). (A, B) *Nycteribia alternada* (Nycteribiidae), 0 facets; (C, D) *Archinycteribia actena* (Nycteribiidae), 1 facet; (E, F) *Neotrichobius stenopterus* (Streblidae), 13 facets. Eyes in panels D and F are indicated with white arrow heads. Scale bar lengths in each panel: A, C, E = 500 μm; B, D, F = 100 μm.

Electron Microscopy Facility, University of Hawai'i at Mānoa, or a Hitachi S-4000 or a Hitachi SU-70 Field Emission SEM at the South Campus Instrument Center in the SUNY Buffalo School of Dental Medicine.

2.3. Photoreceptor morphology

Photoreceptors were imaged using two different methods: staining of paraffin embedded sections and confocal imaging. Using images from both methods, the numbers of retinula cells in individual ommatidia were inferred based on the cellular arrangement of fused rhabdoms with similar flower-shaped rhabdoms from other nocturnal insects (e.g. fireflies - Horridge, 1969; dung beetles - Dacke et al., 2003). For paraffin sections, specimens were placed in 100% methyl benzoate for 18 h to soften the external cuticle and eye lenses, and then infiltrated with periplast paraffin using 1-h incubations at 57 °C of a series of increasing paraffin concentrations in methyl benzoate. Infiltrated specimens were positioned in molds of melted paraffin and allowed to harden at room temperature before storage at 4 °C. Embedded specimens were sectioned at 5 μm on a microtome; sections containing the eyefield were stained using a protocol for staining nervous tissue modified from Klüver and Barrera (1954). Sections were deparaffinized in xylene three times for 5 min, rinsed twice in 100% ethanol for five minutes, then soaked in Luxol Fast Blue (0.1% in ethanol) overnight at 42 °C. The following day sections were rinsed twice in 95% ethanol and twice in distilled water, dipped in 0.005% lithium carbonate, dipped in 70% ethanol, and rinsed again in distilled water two times. The slides were then placed in Cresyl Violet Acetate (0.1% in oxalic acid) solution 30 min; then rinsed twice in distilled water, once in 95% ethanol for 1.5 min, once in 100% ethanol; equilibrated in xylene twice for five minutes; and mounted using Permount media and coverslips.

For confocal imaging, dissected *T. frequens* heads were fixed in freshly made 4% PFA + 0.1% Triton X-100 for 4 h to overnight with gentle rocking, and then dehydrated in 100% ethanol and stored at −20 °C until used for labeling experiments. Tissues were serially rehydrated to PBS, transferred to PBS+0.1% Tween-20, and then stained with DAPI (300 nM in PBS+0.1% Tween-20) for 30 min, washed three times for five minutes in PBS, and mounted with Vectashield (Vector Laboratories). In bat flies, in addition to labeling nuclei DAPI also caused rhabdoms to fluoresce and was therefore used here to visualize *T. frequens* photoreceptor structure. DAPI stained tissues were imaged on a Zeiss LSM 710 confocal microscope with inTune Laser at the University at Buffalo Imaging Facility using a 360 nm excitation laser and a 460 nm emission filter.

2.4. Quantification of screening pigment and photoreceptor absorbance spectra

Recently eclosed adult *T. frequens* reared in the dark were flash frozen and mounted in tissue medium under dim red light. Frozen specimens were sectioned using a cryostat to produce 12−14 μm thick sections that were mounted on coverslips and scanned under dim red light for the presence of photoreceptor structures. Suitable sections were mounted in Ringer's buffer solution between coverslips sealed with a ring of silicone grease and used for microspectrophotometry (MSP) as described by Cronin et al. (1996). Briefly, a linearly polarized scanning beam was placed within a single rhabdom. Scans were made from 400 to 700 nm, with measurements taken at 1-nm steps. Each dark-adapted rhabdom was scanned twice to check for stability. Next the rhabdom was exposed to 2 min of bright white light, followed by a second absorption scan. The rhodopsin absorption spectrum of the measured rhabdom was calculated as the difference between the initial dark-adapted spectrum and the final photobleached spectrum. The wavelength of maximum absorbance (λ_{max}) was estimated for each difference spectrum using a least squares procedure and all photobleach difference spectra were compared with standard rhodopsin templates derived by Govardovskii et al. (2000) and subsequently averaged together those that closely resembled the template. The average spectrum was then fitted to the corresponding template again to determine a λ_{max} value that best represents the spectra of the measured visual pigments. Results from 2 rhabdoms taken from one individual were used to create the average spectrum. For screening pigment characterization, absorbance was characterized by two scans from 400 to 700 nm.

2.5. Molecular characterization of opsins

DNA was extracted from individuals of eight bat fly species (*Eucampsipoda inermis*, *Leptocyclopodia ferrarii*, *Leptocyclopodia* cf *simulans*, *Nycterophilia coxata*, *Nycterophilia natali*, *Penicillidia leptothrinax*, *Strebla mirabilis*, *Trichobius corynorhinus*) using the DNeasy kit (QIAGEN), following manufacturer's instructions. Degenerate primers designed against dipteran Rh1 opsins (opsf1: 5' - TGG WAY CAR TTY CCV SCB ATG - 3'; opsr5: 5' - GAD GTC ARR TTR CCY TCV GGY AC - 3′) were used in a PCR following cycling parameters in Taylor et al. (2005). PCR products of the expected size were confirmed using 1% agarose gel electrophoresis and then sequenced in both directions at the University at Buffalo Genomics and Bioinformatics Core.

For *T. frequens*, the heads from 28 individuals were pooled and RNA was extracted using the NucleoSpin RNA mini kit (Machery Nagel). A 150 bp paired end transcriptome was generated from the pooled RNA sample by MR DNA (Molecular Research LP) and assembled using DNASTAR software with default settings. The assembly was checked for quality using BUSCO (Simão et al., 2015) and QUAST (Gurevich et al., 2013), resulting in a dataset of 10,493,626 bp with an N50 of 1643 bp and a BUSCO score of 57.6%. Putative opsin transcripts were identified from the assembled contigs using Phylogenetically-Informed Annotation (Speiser et al., 2014), and screened for contigs with strong BLAST hits to the six well-characterized *D. melanogaster* visual pigments.

T. frequens contigs matching known *D. melanogaster* opsin transcripts, as well as bat fly opsin DNA sequences generated using PCR with degenerate primers, were translated to amino acids and aligned with a set of opsin sequences representing known visual opsin diversity from brachyceran flies using MAFFT (Katoh et al., 2002, 2019); non-overlapping regions at the start or end of the alignment were trimmed by hand in the program Geneious R10. Outgroup sequences from cephalopods and onychophorans were included to root the tree (Supplemental Data 1). The trimmed alignment was used to reconstruct a maximum likelihood phylogeny of arthropod visual opsins using RAxML (Stamatakis et al., 2008; Kozlov et al., 2019) as implemented on the CIPRES platform (Miller et al., 2010). The phylogeny was visualized using FigTree v1.4.4. All bat fly opsin sequences were deposited to Genbank under accession numbers MW192387-MW192395.

3. Results

3.1. Trichobius frequens visual system development and anatomy

Starting with pupal case deposition (day 0), the development of *T. frequens* to eclosion as an adult takes 21−22 days. Based on the developmental pattern of visual features, pupal eyes develop along a posterior-anterior gradient. In a developmental series captured from day 10 through day 19, the retina turned red on day 11 and adult retinal differentiation was complete by day 19 (Fig. 2).

Fig. 2. Image of a *T. frequens* pupa dissected out of the pupal case on Day 11, where day 0 represents deposition of the third larval instar and day 22 is adult eclosion. The eyes are visible as an assemblage of pale pigmented spots (one for each ommatidium) on the anterior portion of the pupa, as indicated by the black arrowhead. The average pupa length is ~1.1 mm (Dittmar et al., 2009) (photo: S. Morse and K. Dittmar).

At eclosion, adult *T. frequens* exhibit significantly reduced eyes as compared to other schizophoral flies. This significant difference in size is due mainly to the reduction in size of the *T. frequens* eye field (Fig. 3). A single eye consists of eight circular facets with an average facet area of 759 ± 161 μm^2. Noticeably, there are distinct demarcations between facets and the facet arrangement has lost the hexagonal packing and corneal bristles observed in many other schizophoral flies (e.g., *Drosophila*, Stumm-Tegethoff and Dicke, 1974) (Fig. 3). Across 55 individuals, facet numbers in a single eye averaged 8.0 ± 1.0 s.d. facets, but ranged from five to 11 facets, with 57% of specimens having different numbers of facets in the left versus right eye. Among the individual bat flies examined, there was no sexual dimorphism observed in eye morphology, and there was no evidence of any ocelli (data not shown).

Underlying each ommatidial facet, *T. frequens* have photoreceptor modifications distinct from the ubiquitous dipteran neural superposition eye. Longitudinal sections of the eye show that ommatidia are distinct units separated by screening pigments and suggest that *T. frequens* have apposition optics similar to other Diptera (Kunze, 1979; Hardie, 1985; Smith and Butler, 1991) (Fig. 4). Cross sections of the eye, however, show that *T. frequens* ommatidia have fused rhabdoms with teardrop shaped retinular cells arranged in radiating structures (Fig. 5). A survey of 32 fluorescently labeled rhabdoms from four different specimens, where all the retinular cells were clearly visible, showed that the numbers of cells within each ommatidium is variable, with estimates ranging from 11 to 18 cells (mean = 13.6 ± 2.2 s.d.). Additionally, the shape of the rhabdom is not consistent across ommatidia, ranging from round to more elliptical in shape (Fig. 5).

3.2. *Trichobius frequens* visual system molecular and spectral properties

In a survey of four Nycteribiidae (e.g. *E. inermis*, *L. ferrarii*, *Leptocyclopodia* cf *simulans*, *P. leptothrinax*) and four Streblidae species (*N. coxata*, *N. natali*, *S. mirabilis*, *Trichobius corynorhinus*) we characterized a partial sequence (46–147 amino acids spanning transmembrane helices I to IV) of a single dipteran Rh1 opsin from DNA (Fig. 6). We confirmed with a near full length (297 amino acids) Rh1 opsin sequence identified from transcriptome data that the Rh1 gene identified in other bat fly species is expressed in *T. frequens*. All of the characterized bat fly opsin sequences contain typical opsin structural motifs, including the conserved amino acid motif (e.g. aspartic acid (D) - arginine (R) - tyrosine (Y)) in transmembrane-helix 3 that stabilizes the inactive state and, in the expressed *T. frequens* opsin, the lysine residue that forms a protonated Schiff base with the bound retinal chromophore.

Coupled with the opsin expression data, we investigated the function of the *T. frequens* eyes by measuring the absorbance of the screening pigments found between ommatidia (Figs. 4 and 5) and the wavelength of peak absorbance (λ_{max}) of the receptors in each ommatidium using microspectrophotometry (MSP). The measured photoreceptors had a λ_{max} of 487 nm, while the screening pigments had curves characteristic of insect ommochromes (Fig. 7).

4. Discussion

Studies of crepuscular and nocturnal arthropods have catalogued a suite of visual characteristics associated with visual systems optimized for dim light environments. These features include changes at all levels (e.g. optics, receptors, neural processing) and generally represent trade-offs, where increased visual sensitivity is achieved at the expense of decreased spatial resolution (Warrant, 2017). This is evident in the superposition

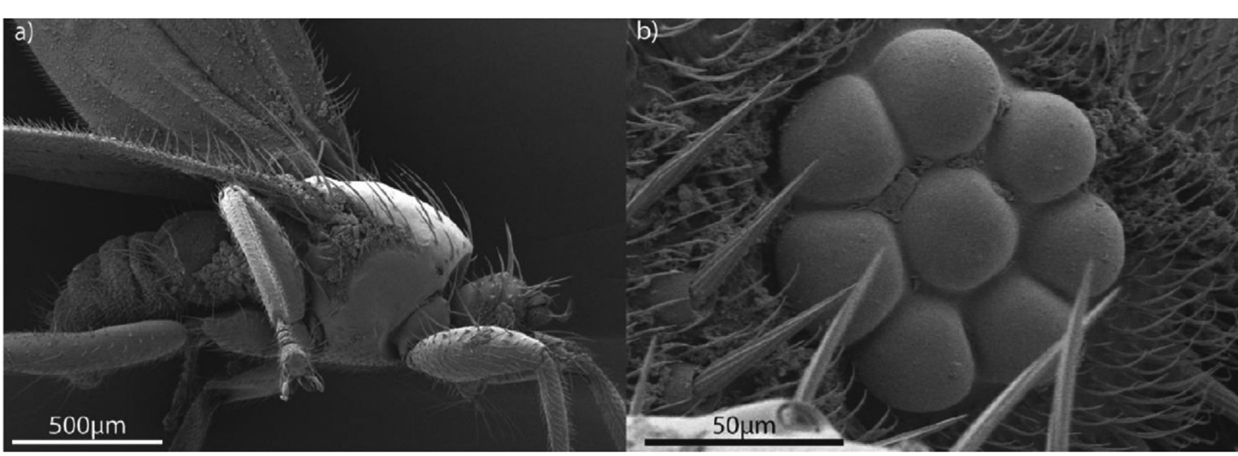

Fig. 3. SEM images of *Trichobius frequens* whole body (A) and eye field with 8 facets (B).

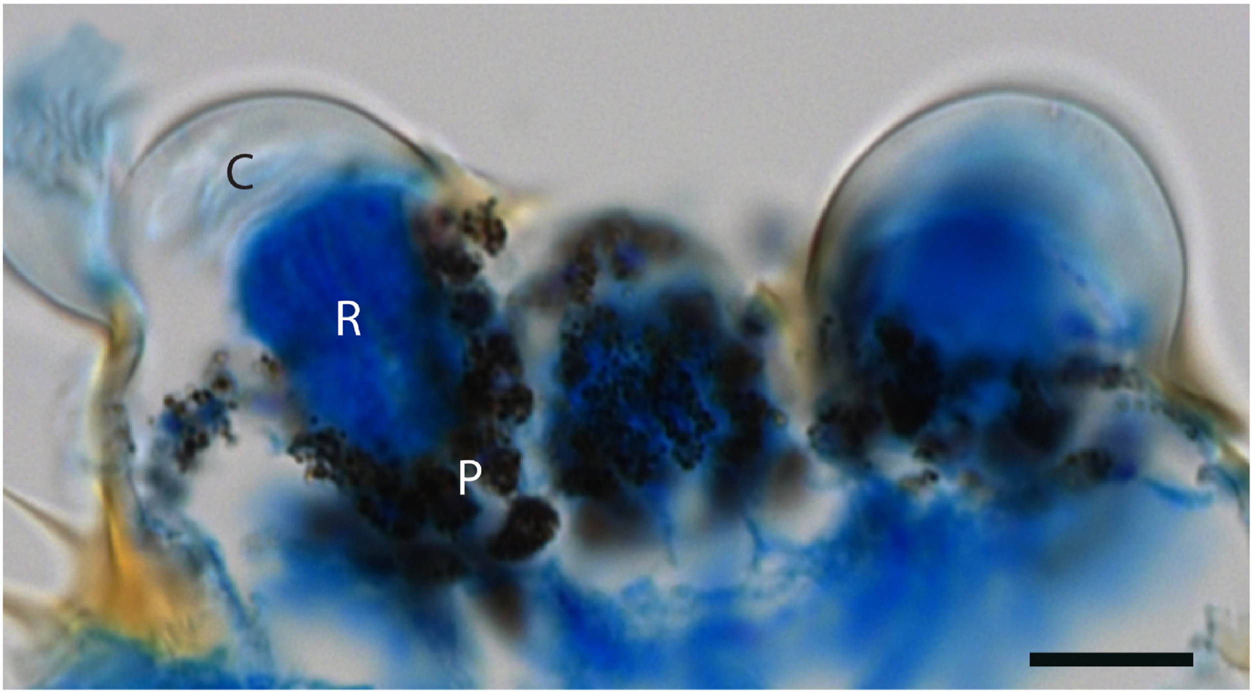

Fig. 4. Longitudinal section of *Trichobius frequens* eyes stained with luxol fast blue. C = crystalline cone; R = rhabdom; P = pigment cells. Scale bar = 10 μm.

compound eyes of many nocturnal species, which optically deliver more photons to each rhabdom at the expense of spatial resolution (e.g., *Deilephila elpenor* - Kelber et al., 2003; dung beetles - Dacke et al., 2013). Although the apposition optical arrangement is typically thought to confer higher resolution at the expense of sensitivity, there are also many examples of nocturnal insects that have apposition optics (Warrant et al., 2004; Hironaka et al., 2008; Warrant and Dacke, 2011). Regardless of optics, a common feature of visual systems specialized for nocturnal habitats are fused rhabdoms (Table 1).

As an ectoparasite of cave-roosting bats, *T. frequens* has a visual system with some of these hallmarks of adaptation to dim environments, including fewer numbers of larger facets. In comparisons of the morphology of the visual system between *T. frequens* and other characterized crepuscular or nocturnal dipteran species, the extreme reduction of bat fly eyes even among species adapted for dim light habitats is evident; despite having similar facet and rhabdom diameters, the number of facets in a *T. frequens* eye is two to three orders of magnitude smaller than any other nocturnal insect yet characterized (Table 1). With only eight facets per eye on average, the *T. frequens* visual system has limited capability for spatial resolution. Optically the *T. frequens* eyes retained the ancestral apposition architecture (Fig. 4); despite the limits to sensitivity imposed by this arrangement, apposition optics may have been retained due to developmental constraints imposed by the ancestral neural superposition eye. Additionally, an eye with so few facets would have limited gains from a superposition optical arrangement as there are not enough facets to provide significant pooling of incoming light for any single receptor. Together, the small numbers of facets coupled with apposition optics suggest that the *T. frequens* visual system has both poor spatial resolution relative to typical brachyceran compound eyes and low sensitivity due to the lack of obvious pooling mechanisms.

It is also of note that the number of facets in an eye varies significantly both among individuals as well as between eyes within a single individual. This has previously been documented in other *Trichobius* species (Zeve and Howell, 1962) and is most likely a common feature of compound eyes. This variation, however, has a much larger influence on the visual field of an organism with so few facets. At the level of reduction observed in bat fly eyes like those of *T. frequens*, the variation in facet numbers observed (e.g. 5–11 facets) represents a two-fold difference in the size of the visual field; whether or not this significantly affects visually-guided behaviors in a dark environment has not been tested, although from the perspective of sensitivity five versus eleven facets is a potentially significant increase in light-capturing ability.

At the level the retinular cell arrangement within each ommatidium, *T. frequens* eyes also have notable differences from other dipteran visual systems. Pancrustacean (e.g. crustacean + insect) visual systems have a conserved number of retinular cells contained within each ommatidium, generally ranging from 7 to 9 cells. Within pancrustacean species, the number and arrangement of retinular cells in each ommatidium tends to be fixed, although these arrangements can vary among species (Melzer et al., 1997; Paulus, 2000). Within a single ommatidium, dipteran neural superposition eyes possess eight retinular cells arranged in a highly ordered pattern, with two of the eight rhabdomeres stacked on top of one another surrounded by the remaining six rhabdomeres arranged in an open configuration with a distinct trapezoidal pattern (Hardie, 1985). Furthermore, the arrangement of the open rhabdoms is highly ordered across the retina, with a mirror-symmetrical arrangement across the equator of the eye in many species (Ready et al., 1976).

In contrast, *T. frequens* have fused rhabdoms with an unusually high and variable number of retinular cells (estimated from 11 to 18); these rhabdoms are also highly variable in shape with no discernable order across the retina (Fig. 5). These retinular cell numbers and variation are similar to the compound eyes of other arthropods, including nocturnal moths, myriapods, and chelicerates. Nocturnal moths typically have fused rhabdoms with nine or more photoreceptor cells, with some species demonstrating

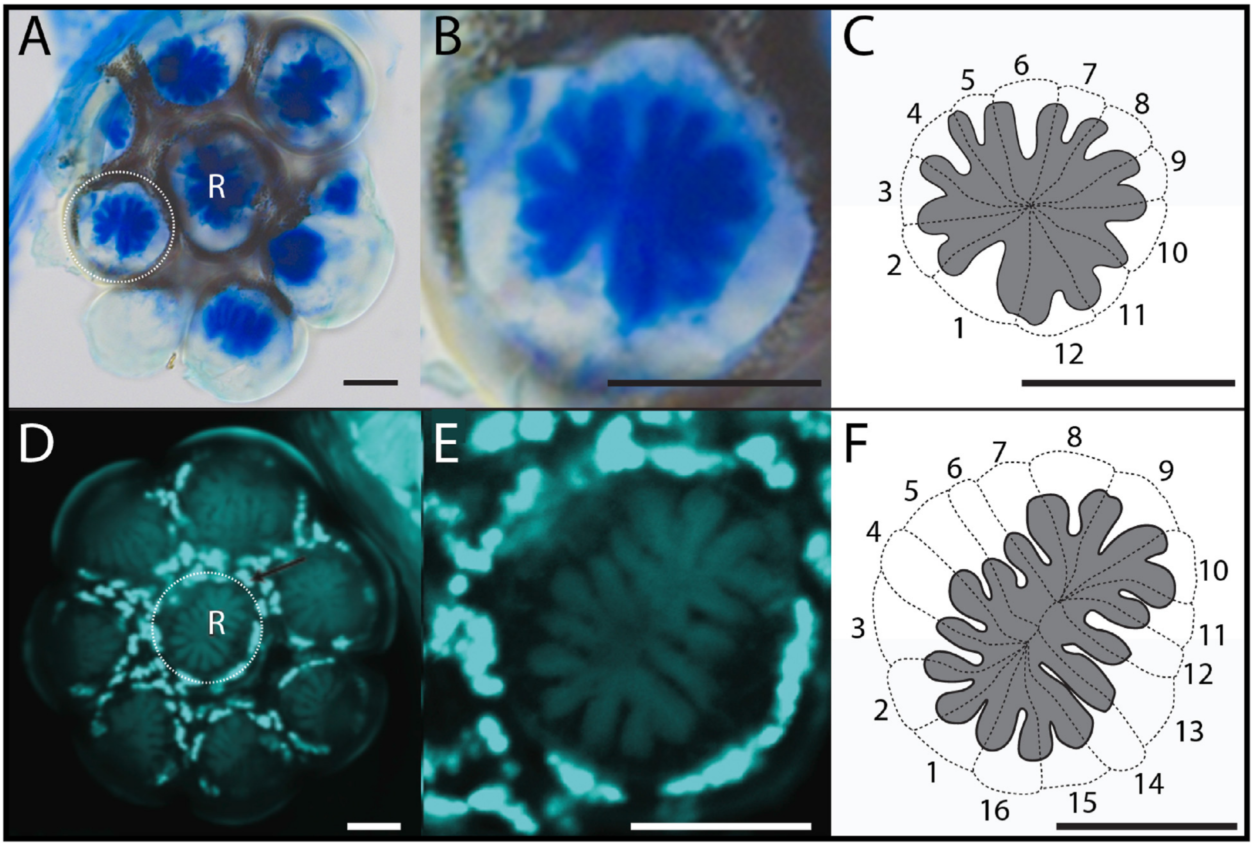

Fig. 5. Cross sections of *Trichobius frequens* eyes showing the variation in the overall shape and the number of cells comprising the flower-like fused rhabdoms. (A) Paraffin section (5 μm) stained with luxol fast blue of an eye with at least nine facets. Rhabdoms - R - in each ommatidium are stained blue and screening pigments between ommatidia are brown. (B) A magnified view of the rhabdom in the dashed circle in panel A. (C) A schematic of the inferred arrangement of 12 photoreceptor cells (dashed lines) contributing to the rhabdom (grey) shown in panel B. (D) An optical section stained with DAPI of an eye with eight facets. Nuclei are bright blue, and rhabdoms (R) are darker blue. (E) A magnified view of the rhabdom in the dashed circle in panel D. (F) A schematic of the inferred arrangement of 16 photoreceptor cells (dashed lines) contributing to the rhabdom (grey) shown in panel E. Scale bars in all panels = 10 μm.

expansions and variations in photoreceptor cell numbers similar to those observed in *T. frequens* (e.g., *Ephestia kuehniella* 9–12 retinula cells – Fischer and Horstmann, 1971; *Galleria mellonella* 12 retinula cells – Stone and Koopowitz 1976; *Ostrinia nubilalis* 9-12 retinula cells – Belušič et al., 2017). These similarities suggest that increased numbers of retinular cells may be an adaptation for dim light vision in order to increase photon capture by increasing the rhabdomeric volume.

Myriapods and chelicerates have even more extreme variation of retinular cell numbers in each ommatidium (Fahrenbach, 1975; Spies 1981; Paulus, 2000). For example, in *Limulus polyphemus* each ommatidium contains anywhere from 4 to 20 retinula cells, with an average of 10–13 cells (Fahrenbach, 1975). Evolutionary models proposed for the large and variable numbers of cells found in myriapod and chelicerate eyes assume that variability is the ancestral state of either chelicerates and myriapods (Nilsson and Kelber, 2007) or all arthropods (Harzsch and Hafner, 2006), or alternatively that retinular cell variability is the result of the fusion of multiple ommatidia (Paulus, 2000). Given that the bat flies clearly evolved from dipteran ancestors with the typical neural superposition eye type, and that the ancestral state of pancrustacean visual systems is ommatidia composed of a small and invariant number of cells (Richter, 2002), one explanation for the high and variable number of retinular cells observed in *T. frequens* ommatidia comes from the fusion of multiple receptor fields under a single facet. The unique developmental cycle of *T. frequens*, with deposition of pupal cases in the environment, will allow for future comparative studies of eye development with well-studied species like *D. melanogaster*. Electron microscopy studies of the rhabdom structure are also needed to better understand the variable arrangement of *T. frequens* retinular cells within and across ommatidia.

To document that the *T. frequens* eye is functional, we characterized the presence of a functional opsin both through RNA expression studies and measurements of photoreceptor absorbance. We documented that *T. frequens* expressed a transcript homologous to dipteran Rh1 opsin sequences (Fig. 6), and that the Rh1 opsin sequence was present in the genome of a selection of other streblid and nycteribiid species. In *D. melanogaster*, the Rh1 opsin protein forms a visual pigment with a peak absorbance of 486 nm that is localized in R1-6 receptor cells found in the open rhabdom structure of the neural superposition arrangement (Stavenga et al., 2017). The measured λ_{max} from *T. frequens* photoreceptors (487 nm; Fig. 7) matches studies from a diversity of brachyceran flies, where most of the photoreceptors containing visual pigments composed of Rh1 opsin proteins have λ_{max} from 480 to 490 nm (Stavenga et al., 2017). Correspondingly, the spectral absorbance of the screening pigment is similar to those measured in other insects (Dontsov et al., 2020). In addition to opsin expression and photoreceptors with a measurable absorbance that matches a standard visual pigment template, previous studies have also demonstrated that *T. frequens* exhibit a startle response to flashes of light (Mayberry, 2014).

An open question in our understanding of the evolution of the *T. frequens* visual system is the underlying neural wiring and

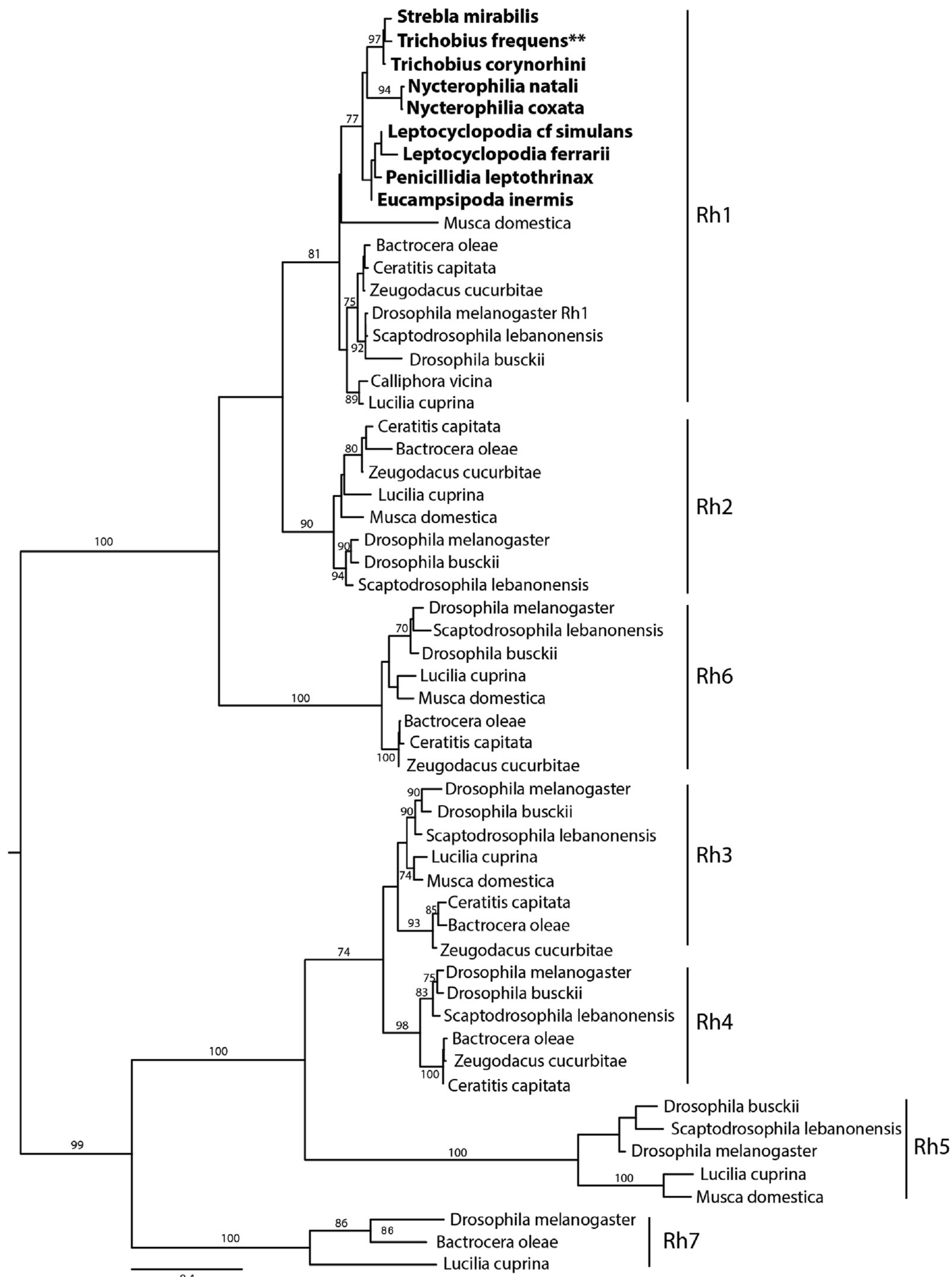

Fig. 6. Maximum likelihood phylogeny of dipteran opsins illustrating the evolutionary placement of bat fly opsins (in bold). Numbers on branches indicate bootstrap support. The clades representing the known dipteran opsins (Rh1−Rh7) are shown. The tree is rooted to onychophoran and cephalopod opsins (not shown). ** opsin from RNA expression data.

information processing. Similar to other nocturnal insects with apposition optics (Table 1), *T. frequens* has fused, enlarged rhabdoms paired with larger facets to maximize the amount of light captured (Land et al., 1999; Greiner et al., 2004). However, the sizes of the *T. frequens* facets and rhabdoms, at least as measured by diameter, are within the range of those measured in other nocturnal insects with many more facets, leading to questions about how individuals in this species achieve a complex visually-guided behavior like flight in a dark habitat. The complex wiring network of the ancestral neural superposition visual system is no longer necessary in an eye with so few ommatidia containing fused rhabdoms. However, because *T. frequens* still uses flight to find suitable pupal deposition areas, summation further downstream in the neural processing of visual information cannot be completely ruled out as a possible mechanism for overcoming the demands for visually-guided flight in very dim light. Preliminary data hints at the possibility that *T. frequens* eyes have retained some neural connections among photoreceptors (Mayberry, 2014). Future studies will address the structure of the neural pathways related to vision in order to understand how

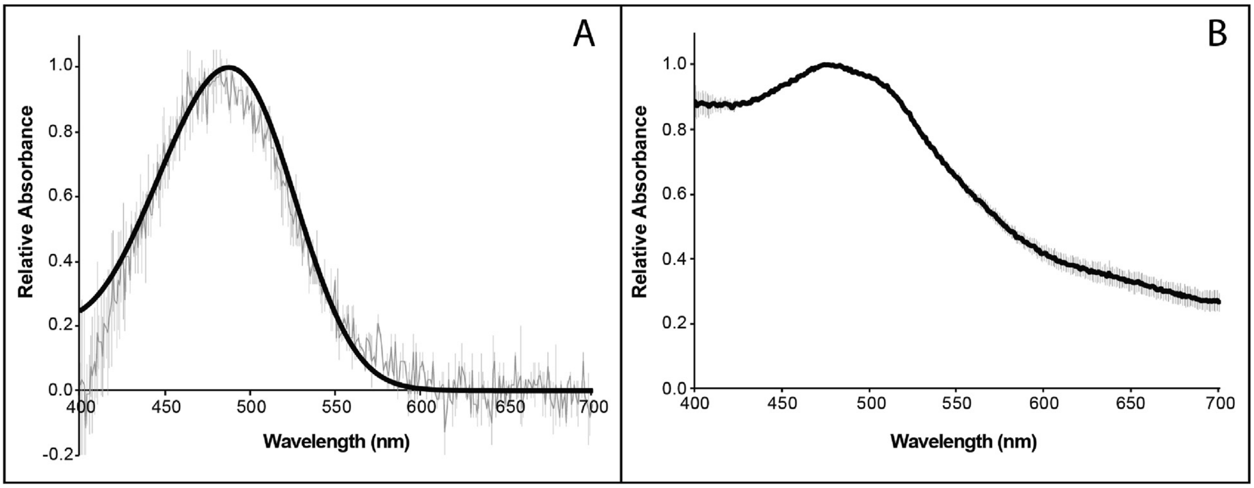

Fig. 7. (A) Average difference spectrum (grey) for photobleaching of rhabdoms (n = 2) in retinal sections of *T. frequens*, with the spectrum of the best-fit rhodopsin template (Govardovskii et al., 2000) in black (peak wavelength = 487 nm). Error bars are standard errors. (B) Average absorbance spectrum (n = 2) for *T. frequens* screening pigments. Error bars are standard deviations.

Table 1
Comparison of visual features of *Trichobius frequens* with other crepuscular and nocturnal insects, including number of facets, average facet diameter, optical arrangement (App = apposition), average rhabdom diameter, and type of rhabdom. Where included, variability is included as standard deviations.

Taxa	Ecology	Facet #	Facet diameter (μm)	Optics	Rh max diameter (μm)	Rhabdom Type	Reference
DIPTERA							
Streblidae:							
Trichobius frequens	ectoparasite	8.0 ± 1.0	30.3 ± 3.8	App	12.8 ± 2.2	fused	this study
Culicidae:							
Aedes detritus	crepuscular	–	23.0	App	8.6	fused	Land et al. (1999)
Aedes punctor	crepuscular	–	25.0	App	6.9	fused	Land et al. (1999)
Anopheles gambiae	nocturnal	–	28.0	App	15.4	fused	Kawada et al., (2006); Land et al., (1999)
Anopheles sinensis	crepuscular	622	20.3	–	8.0	–	Land et al. (1999)
Culiseta litorea	nocturnal	–	30.6	–	13.8	fused	Land et al. (1999)
Culex pipiens	nocturnal	532	23.1–28.0	App	8.5	–	Kawada et al., (2006); Land et al., (1999)
Culex vorax	nocturnal	629	32.4	–	12.5	–	Land et al. (1999)
HYMENOPTERA							
Apoidea:							
Megalopta genalis	crepuscular	4883 ± 10	36	App	8	fused	Greiner et al. (2004)
Megalopta diurnalis	nocturnal	5134	38.4	–	–	–	Kelber et al. (2005)
Rhinetula dentricus	crepuscular	3626	32.5	–	–	–	Kelber et al. (2005)
Xylocopa tranquebarica	nocturnal	18,803	38.7 ± 1.3	App	6	fused	Somanathan et al., (2008); Somanathan et al. (2009)
Formicoidea:							
Myrmecia nigriceps[a]	crepuscular/nocturnal	3210 ± 30	20–30	App	5.6 ± 0.1	fused	Greiner et al. (2007)
Myrmecia pyriformis[a]	crepuscular/nocturnal	3593 ± 66	20–30	App	5.9 ± 0.1	fused	Greiner et al. (2007)
Vespoidea							
Apoica pallens	nocturnal	9176 ± 116	26	App	8	fused	Greiner (2006)

[a] Major worker.

T. frequens is still capable of flight in a dark habitat with highly reduced eyes.

5. Summary

In this first integrated study of bat fly visual systems, we demonstrate that the highly reduced eyes in *T. frequens* show variations among individuals, as well as between eyes in a single individual, in the numbers of facets, the numbers of retinular cells that contribute to the rhabdom, and in the shape of the rhabdom. Although the streblid bat fly *T. frequens* has maintained the apposition optics of the ancestral dipteran neural superposition eye type, the reduction in eye size and facet number have eliminated the possibility of high-resolution vision in this species. Another hallmark of adaptation to dim environments in *T. frequens* eyes is the fused rhabdom; this is one of the first species of brachyceran flies described with a fused rhabdom, demonstrating the remarkable evolutionary plasticity of insect visual systems. Future studies will evaluate how individuals with such reduced capabilities for spatial vision as well as diminished potential for sensitivity still capture enough visual information to use flight to maneuver through dark habitats.

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Author contributions

MLP: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - original draft, review & editing, Visualization, Supervision, Funding acquisition.

TWC: Validation, Formal analysis, Writing - review & editing, Supervision, Funding acquisition.

CD: Validation, Resources, Writing - review & editing.

NS: Investigation, Data Curation.

KD: Conceptualization, Methodology, Investigation, Resources, Writing - review & editing, Supervision, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.asd.2020.101007.

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