THROUGH THE EYES OF BAT FLIES:
BEHAVIORAL, PHYLOGENETIC, AND HISTOLOGICAL ANALYSES OF COMPOUND EYE REDUCTION IN BAT FLIES (STREBLIDAE) PROVIDE EVIDENCE FOR POSITIVE SELECTION

by

Jason Robert Mayberry
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Doctor of Philosophy

Department of Biology

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 - 1346

Dedication

This work is dedicated to my children, who I hope will ever strive for knowledge and excellence in the fulfillment of their dreams. Whatever you look at, look closely.

It is further dedicated to my wife. Without her patience and support, completing this would never have been possible.

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Abstract

It is often presumed that evolutionary reduction is tantamount to deconstruction, or even destruction, because relaxed selective forces have been insufficient to maintain the organ in its original state. However, studies on reduction are often limited by a lack of diversity, both of related species exhibiting reduction and of the reduced form itself. There have also been very few studies on the reduction of compound eyes, despite the fact that their near ubiquity among arthropods alone makes them perhaps the most common type of eye. Bat flies (Streblidae and Nycteribiidae) are a group of dipterans that exhibit variable degrees of compound eye reduction, and therefore provide the opportunity to study reduction of this organ in a phylogenetic context. The first chapter of this work reports on behavioral experiments demonstrating that the eyes of one bat fly species, *Trichobius frequens*, are functional, and that they neither exhibit phototaxis typical of other dipteran species, nor move toward a light source. The second chapter uses molecular phylogenetics to identify a correlation between eye and wing morphology. The results also suggest that secondary to their eye reduction, bat flies (at least in the case of New World specie, including *Trichobius spp*.) have secondarily experienced a shift in the structure of their facets that is convergent with other insects whose eyes have been selected for increased sensitivity. In the final chapter, histological and optical analyses of *T. frequens* eyes are used to reveal significant structural changes to the microstructure of its ommatidia that increase sensitivity at the expense of acuity. Many of these changes are also convergent with similar adaptations that have been demonstrated to increase sensitivity in organisms that function in reduced light environments. The results of these analyses suggest that reduction in *T. frequens* eyes may have been part of an active remodeling process resulting from a shift in the relative importance of sensitivity and acuity. As this is a process of reduction not generally considered, the findings here turn our attention to alternative hypotheses that should be considered when studying evolutionary reduction of any organ.

Introduction

Eyes and Evolution

From the penning of the *Origin of Species*, to modern studies of molecular phylogenetics and the evolution of developmental biology, the eye has served as a paragon of evolutionary theory. Darwin set the stage for the role eyes would play when he confessed that on first blush it seemed "*absurd in the highest degree*" to think that an organ such as the eye, with all its "*inimitable contrivances…could have been formed by natural selection.*" Thus pre-empting the potential criticism, he followed up with a masterful review of the diversity of eye morphologies found in the animal kingdom, finally concluding: "*when we reflect on these facts…with respect to the wide, diversified, and graduated range of structure in the eyes of lower animals…the difficulty ceases to be very great*" (Darwin, 1859).

Far from being a difficulty for evolutionary theory, the study of eyes has at once provided convincing evidence for evolution, while illuminating its fundamental principles. One would be hard pressed to find a richer example of descent with modification than in the finding that from an ancestral metazoan, which perhaps was equipped with only a simple eye spot, all eyed animals today have inherited their opsin photopigments, and the master eye regulatory gene, Pax6 (Arendt, 2003; Callaerts et al., 2006). Furthermore, despite the fact that eyes have hence evolved independently as the animal phyla have diverged, they have nonetheless converged repeatedly on the two primary eye forms, simple and compound (Land and Nilsson, 2002; Goldsmith, 1990; Land and Fernald, 1992); yet, in independent example of these forms we find distinct developmental mechanisms for producing eyes (Friedrich, 2003), different mechanical solutions for capturing and focusing light (Land and Nilsson, 2002; Nilsson, 1989), and examples of what is often considered intermediate forms between early eyes and derived forms with more sophisticated optics (Land and Nilsson, 2002).

Adding both to the diversity of eyes, and to the evolutionary principles the study of eyes illuminates, are the eyes of many species that are evolutionarily reduced (Buschbeck and Friedrich, 2008). It is in this regard that the studies presented herein find their place by addressing the function, evolution, and structure of the compound eyes of bat flies (order Diptera; Hennig, 1941; Wenzel et al., 1966). All species in this parasitic group of insects possess reduced eyes, but exhibit variable degrees of reduction and loss. Considered together, these three studies suggest a unique role selection can play in the process of reduction.

Reduction

When approaching the study of a reduced organ, one of the immediate questions that is always asked is what factors have played a role in bringing about its reduction. While there are multiple factors that can be considered, this usually focuses on the relative role for selection. The two major alternatives often considered are: 1) that the relaxation or removal of purifying selection alone has been sufficient to allow neutral processes to bring about reduction by drift, and 2) selection against the development and/or maintenance of larger forms has hastened reduction. Darwin recognized the former possibility when he commented that the "rudimentary" size of subterranean animals was "probably due to gradual reduction from disuse"; but, recognizing that selection could also play a role in bringing about reduction, he added that the reduction could be "aided perhaps by natural selection" (Darwin, 1859). We now understand that the rate and degree of reduction may be affected by genetic pleitropy and developmental canalization, as well as continued (perhaps less obvious) selective pressure to maintain the trait (Lahti et al., 2009). As Darwin also noted, it ought not be thought that the evolutionary options for such traits are binary: maintenance or reduction, as traits which have lost their usefulness for one purpose "might be easily modified [by natural selection] and used for another purpose" (Darwin, 1859).

Our understanding of reduction in general has come a long way through studies of the reduced eyes of a few organisms. Most famously, the Mexican cave fish (*Astyanax mexicanus*) has proven to be an excellent model for studying the genetics and development of eye reduction (Jeffery et al., 2003; Jeffery, 2005; Protas et al., 2007; Sadoglu, 1967). Additionally, subterranean mammals such as the mole rats and the talpid moles, which are represented by multiple species that have converged on eye reduction, exhibit reduction that has maintained a degree of eye function (Cernuda-Cernuda et al., 2003; Cooper et al., 1993; Kott et al., 2010). However, as with these examples, the bulk of the literature on eye reduction is focused on simple eyes. Despite the prevalence of compound eyes, and a number of examples with reduced forms, including fleas (Crum et al., 1974; Osbrink and Rust, 1985; Rust and Dryden, 1997; Taylor et al., 2005), bee lice(Nowogrodzki and Morse, 1990; Wiegmann et al., 2011), and scale insects (Morse and Normark, 2006) (to name just a few), reduction of compound eyes has been the subject of very few studies (e.g. see the references for fleas). Moreover, the organisms whose reduced eyes have been studied (both with simple and compound eyes) possess limited diversity in their reduced forms.

The study of reduced eyes in bat flies therefore offers an opportunity to examine reduction in compound eyes. Moreover, the diversity of eye phenotypes found among bat flies provides the opportunity to examine their phylogeny for patterns that reveal, among other things, factors that may have influenced the relative role for selection in shaping the eyes along different lineages. The clear connection between form and function in eye structure also allows the relatively easy exploration of the functional significance of changes resulting from their evolutionary reduction.

Bat Flies

Bat flies comprise two families within Diptera, Streblidae (Dick and Graciolli, 2006) and Nycteribiidae (Graciolli and Dick, 2006). All bat flies are obligate, hematophagous parasites of tropical bats worldwide.

Because different species of their bat hosts occupy a variety roosts, ranging from dark caves to tree foliage, bat flies also occupy a variety of habitats. As is common with other ectoparasites, the eyes of all bat fly species are significantly reduced in terms of size, and their numbers of facets. Nonetheless, as mentioned above, the eyes of different bat fly species exhibit an array of phenotypes. Some flies retain compound eyes with a up to three dozen well-formed facets, others have eyes that appear to be vestigial, and still other species have lost their eyes altogether (see Chapter 2, Table 1). Similarly, even though many bat fly species have well developed wings and are relatively good fliers, the wings of many others are reduced in size, and in some cases are absent.

In contrast to the majority of insects, which are oviparous and deposit large numbers of fertilized eggs each reproductive cycle, bat flies are viviparous and produce a single offspring each reproductive cycle (Dittmar et al., 2011; Hennig, 1941; Wenzel et al., 1966). After fertilization, the single egg is retained within the female where it develops inside a uterus and is nourished by a milk gland until it has completed all larval stages. Upon completion of the larval development, the female leaves the host to find a suitable substrate to deposit her young, complete with a soft pupal case. Deposition may take place within the bat roost (Fritz, 1983; Overal, 1980), but at other times it can be at some distance, requiring bat flies to fly to and from the deposition site (Dittmar and Mayberry, 2010; Dittmar et al., 2009).

While these life history traits make bat flies a difficult model to work with (for instance, there is currently no way to maintain a population in the lab), their study has the potential to contribute significantly to our understanding of the process of reduction, specifically as they pertain to compound eyes.

Research Questions and Hypotheses

As with other studies of reduction, one of the general questions that influenced the research presented here was what role selection has played in shaping the reduction of bat fly eyes. Because nothing was known at the outset of this research about bat fly eye evolution or function, except for the fact that they were reduced in overall size, and that there was some variability, this larger question was assessed while exploring several more focused questions regarding the function, evolution , and structure of bat fly eyes.

In chapter 1 the question asked is simply whether or not the eyes of one species, *Trichobius frequens*, are functional. This is an important preliminary question because if they are not functional, it would be an indication that purifying selection for their function (at least for this species) has been removed. On the other hand, if they are functional, this opens up the possibility that selection may be acting for their maintenance. *T. frequens* was chosen primarily because they can be captured in relatively large numbers from a population in Cueva de los Culebrones (Chapater 1 Figure 2) at the Mata de Platano field station near Arecibo, Puerto Rico. Their host bats often roost approximately 180 or more meters within this cave, well within the aphotic zone, yet they only deposit their pupae nearer the entrance. Every night when the bats exit the cave to forage, females can be found flying in swarms nearer the entrance where they deposit their pupae on the walls of the cave (Dittmar and Mayberry, 2010; Dittmar et al., 2009; Dittmar et al., 2011). This provides the opportunity to collect them in relatively large numbers using sweep nets. This species was also an interesting candidate for study because flying near the entrance of the cave could lead to the possibility of individuals being exposed to light from the outside if they move towards the entrance. Though being exposed to light itself is not sufficient for eye functionality, it is a necessary pre-requisite for selection to favor eye function. *T. frequens* also possess a small number facets (8 on average; see chapter 2) whose external symmetry suggested that they might

not be vestigial. Because of this it was hypothesized that their eyes are functional and that this could be demonstrated by identifying a behavioral response elicited by light stimulus. Because the adults of all related dipteran species (with the exception of *Braulidae coeca,* Kaschef, 1959) exhibit positive phototoaxis (technically photo-tropo-taxis, see Chapter 1), it was further hypothesized that *T. frequens* would also exhibit this same behavior. Experimental observations of *T. frequens* behavioral response to different light conditions reveal that their eyes are functional, but the modality of their behavior is unique among Dipterans.

In chapter 2 the diversity of eyes exhibited within Streblidae is utilized in a phylogenetic context to address questions regarding their evolution. The first question asked is whether or not reduction has been an ongoing process in all bat fly lineages. It was hypothesized that if it has, then no measures of eye size in extant taxa should be significantly larger than reconstructed values for the same measures at ancestral nodes. It was further hypothesized that values falling outside the 95% confidence intervals for reconstructed nodes could suggest hastened evolution due to the influence of selection. Additionally, to assess whether differential selective pressures have played a role in different bat fly lineages, it was asked whether measures of eye size could be correlated with factors related to bat fly biology and ecology. Specifically, because exposure to light is a pre-requisite for eye function, it was hypothesized that measures of eye size in different bat fly species would be correlated with the light conditions of their hosts' roosts. Additionally, because vision is often thought to be necessary for flight, it was hypothesized that measures of eye size would also correlate with wing morphology. The findings in this chapter provide compelling evidence that selection has acted differentially between different bat fly species to explain much of the observed variation.

The final chapter returns again to *T. frequens* to assess how the ultrastructure of its eyes has been affected by its reduction. The first question addressed is which type of compound eye they possess. Compound eye are found in two major forms: optical superposition, in which light is focused by the many lenses on a common retina; and apposition, in which each lens is associated with a discrete unit of the retina called a rhabdom, each such unit being called an ommatidium. All dipterans closely related to bat flies possess a special type of apposition eye called neural superposition (Hardie, 1986; Nilsson and Kelber, 2007; Land and Nilsson, 2002; Stavenga, 1975), in which the rhabdom of each ommatidium is divided into discrete light sensitive units (rhabdomerse) with a unique field of view that is shared by one such rhabdomere of a neighboring ommatidium. Because this is the type of eye found in all related families of Diptera, it was hypothesized that *T. frequens* would also exhibit this form, and that their eyes have been reduced by simply decreasing the number of ommatidia. Nonetheless, inasmuch as differences were to be observed in the structure of the ommatidia, it was hypothesized that these differences would diminish function of the eye overall. Histological and optical analysis of the structure of *T. frequens* eyes reveals that, contrary to these expectations, their eyes do not exhibit neural superposition, and that they have been modified for enhanced sensitivity of each remaining ommatidium. These findings highlight a novel role for selection in shaping the structure of a reduced organ.

Thus, though the work presented here represents only an initial attempt to understand the evolution of bat fly eyes, it provides another example eyes being used as a model for understanding evolutionary principles.

Chapter 1: The Responses of a Bat Fly, *T. frequens* (Diptera, Streblidae), to Light

Abstract

Bat flies are obligate, blood-feeding ectoparasites of bats. As with other obligate ectoparasites, the eyes of all species have been either lost or significantly reduced in size. In contrast to insects' eyes with hundreds or thousands of facets, visual function in organisms with reduced eyes has not been the subject of much research. Therefore, the purpose of this study was to determine if the eight-faceted eyes of one bat fly species, *Trichobius frequens* are functional. The possibility that they could retain eye function was suggested because their eyes are symmetrical in shape, and they live in conditions where exposure to light may have provided the opportunity for eye function to be advantageous. This study demonstrates that *T. frequens* eyes are functional, but that the behaviors associated with their response are novel among dipterans. While all adult dipterans previously studied respond to a light gradient by orienting their movements relative to its direction of origin (tropotaxis), the main feature of *T. frequens* behavior is an increase in activity without obviously orienting their movements relative to the source. This increase in activity ceases with diminished light intensity, leading to their aggregation in the dark.

Introduction

Bat flies are a diverse group of dipterans (Streblidae and Nycteribiidae) that live as obligate, hematophagous ectoparasites of bats, and exhibit marked eye reduction, and in some cases, total eye loss. That their eyes are reduced can be inferred from the fact that (with few exceptions) species in both their sister taxa (Hippoboscidae), and taxa related by ancestral nodes (Glossinidae and others) have large well developed eyes comprised of facets numbering in the hundreds, or sometimes thousands (Buschbeck and Friedrich, 2008; Hardie, 1986). The observation that bat fly eyes are reduced begs the question of their function.

When purifying selective pressures for an organ's function are removed, deleterious mutations are expected, after some time, to interrupt its development, and destroy its functionality (Darwin, 1859; Lahti et al., 2009). Organs thus affected are generally reduced in size and become vestigial, or are lost entirely; examples of this are seen in the eyes of many cave dwelling organisms, including the Mexican cavefish, *Astyanax mexicanus* (Jeffery, 2005; Jeffery et al., 2003; Sadoglu, 1967). However, if selective pressures are merely relaxed, the organ may be reduced in size and retain diminished function. Such is the case for the eyes of mole-rats (Kott et al., 2010) and fleas (Benton and Lee, 1965; Crum et al., 1974; Osbrink and Rust, 1985; Rust and Dryden, 1997; Taylor et al., 2005). Thus when species are inferred to possess an organ that is reduced, as with the eyes of bat flies, the question can be posed as to whether or not the organ is functional. If it is not, the organ can be inferred to be vestigial. If the organ is functional, it may have been maintained by weak selection. It is also possible that either sufficient time has not elapsed for function to be lost, or that it is a byproduct of selection on other traits, as many genes in the visual cascade are pleiotropic (Lahti et al., 2009)

Some bat fly species have lost their eyes entirely (e.g. *Ascodipteron* spp., *Metelasmus* spp.) (Hastriter and Bush, 2006; and personal observation), suggesting that selective pressures for their eye function were lost. Because the optics of eye function requires symmetrically shaped structures, the eyes of other species with irregularly shaped eye facets (e.g. *Nycterophilia* and *Strebla spp*.; personal observation) may either be vestigial, or have limited, non-image forming, function. However, there are a number of species that retain a smaller number of symmetrically shaped facets that may be functional. One such species, *Trichobius frequens* (Figure 1), which has an average of 8 symmetrical facets, was the subject of this study.

In addition to the fact that they retain symmetrically shaped eye facets, the possibility that *T. frequens* eyes may be functional is further suggested by a previous study on the host-finding behavior of the congener species *T. major*. Though not focused on ascertaining the behavioral reaction to light, it was noted that light may have had an inhibitory effect on geotaxis (Overal, 1980). However, a later study using the same species reported that light had no effect on attraction to other stimuli (Caire et al., 1985). If *T. major* has retained functional eyes, this increases the likelihood that *T. frequens* has also retained functional eyes because of their shared evolutionary history.

If visual responses are observed in *T. frequens*, they might be understood in relation to other organisms with reduced eyes, or which have lost their eyes. Reduction in both total eye size and in the number of facets is seen convergently in other dipteran ectoparasites such as bee lice (*Braulia coeca, B*raulidae) (Kaschef, 1959; Nowogrodzki and Morse, 1990; Wiegmann et al., 2011), and sheep keds (Hippoboscoidea) (Mullen et al., 2009), as well as in ectoparasites from other insect orders such as fleas (order Siphonaptera) (Benton and Lee, 1965; Crum et al., 1974; Osbrink and Rust, 1985; Rust and Dryden, 1997; Taylor et al., 2005), lice (order Phthiraptera) (Patterson et al., 2007), and scale insects (order Hemiptera) (Morse and Normark, 2006). Among these organisms (including some bat fly species) there are many that have lost eyes altogether and are likely to rely on non-visual senses. But there are a few species that retain eyes in a reduced state for which visual responsiveness has been demonstrated. *B. coeca*, for instance, exhibits negative phototaxis (Kaschef, 1959), the only adult dipteran species known so far to do so. Cat fleas (*Ctenocephalides felis*), on the other hand, orient positively to a light source, as well as a moving dark object on a light background (Dryden and Broce, 1993; Osbrink and Rust, 1985). Because these latter species spend a part of their life history off of their host, and light during this time can play a role in influencing their behaviors, it is presumed that this aspect of their

biology may provide the selective pressures responsible for retention of eye function (Patterson et al., 2007).

As with bee lice and fleas, bat flies also spend a part of their life away from their host. All bat fly species are adenotrophic viviparous, depositing a single pupa at a time on a suitable substrate away from their host. While some species have been observed depositing pupae in or near the roost of their host (Fritz, 1983; Overal, 1980) other species, such as *T. frequens* (the subject of this study), have been observed depositing pupae at a considerable distance from the roost, often near the entrance of a cave (Dittmar et al., 2009; Dittmar and Mayberry, 2010). This requires them to fly to and from the deposition site, and provides the opportunity for light to play a role in their behavior.

The visual behavior of bat flies, if observed, may also be understood in relation to the visual behavior of other Diptera. In all previously reported cases for non-parasitic Diptera, larvae exhibit negative phototaxis (movement away from a light source) [e.g. fruit flies (Ballinger and Benzer, 1988; Lilly and Carlson, 1990), mosquitoes (Simonet et al., 1978) , wheat bulb flies (Marriott and Evans, 2003), and tsetse flies (Mullen et al., 2009)], but shift to positive phototaxis (movement towards a light source) as adults; this includes diurnal species such as fruit flies (Benzer, 1967; Choe and Clandinin, 2005; Hadler, 1964a; Hadler, 1964b; Hirsch and Boudreau, 1958), tsetse flies (*Glossina spp*.; Green, 1985; Green and Cosens, 1983), sciarid flies (Jess and Bingham, 2004), and cabbage root flies (Kostal, 1991), as well as crepuscular and nocturnal species such as mosquitoes (Davies, 1975) and sandflies (Scorza, 1972).

Vision is heavily relied upon by these flies for activities such as flight navigation, foraging, mate seeking, and predator avoidance. In the case of fruit flies, negative phototaxis contributes to a larva's behavior of burrowing into its food source; during the late larval stages there is a permanent switch to positive

phototaxis (Hu and Stark, 1980) associated with the late 3rd instar climbing to the surface, and often up the sides of its container, where it pupates (Bainbridge and Bownes, 1981; Walther and Pichaud, 2007). In the case of tsetse flies, the female deposits a late 3rd instar larva whose negative phototactic response contributes to its burrowing into the soil, where pupation takes place; when metamorphosis is complete and the adult fly emerges from the ground, it exhibits positive phototaxis for the remainder of its life (Leak, 1999). Fruit flies have also been observed exhibiting a reflexive "startle" response when exposed to a dark "looming" stimulus (Fotowat et al., 2009; Gibson, 1986), or a dark flash (a flicker in ambient lighting) (Allen et al., 2006; Hammond and O'Shea, 2007; Tanouye and King, 1983; Zhang et al., 2007).

While a number of publications have focused on various aspects of bat fly biology, including their phylogeny (Dittmar et al., 2006; Petersen et al., 2007), sex ratios (Dittmar et al., 2011), and ecology (Dick, 2005; Dittmar et al., 2009; Dittmar and Mayberry, 2010; Patterson et al., 2007; Ter Hofstede et al., 2004), none thus far have dealt with the function of their eyes and related behaviors. The primary objective of this study was to determine whether or not *T. frequens* eyes are functional, and if they are, what behaviors are associated with visual stimuli. It was hypothesized that if their eyes are functional, that this could be demonstrated by identifying a behavioral response elicited by a light stimulus. Because the only dipteran studied to date that exhibits negative phototaxis as an adult is the parasitic *B. coeca*, and all others that have been studied exhibit positive phototaxis as adults, it was further hypothesized that *T. frequens* would also orient their movements relative to a light gradient. To this end experiments were conducted similar to those used to test for phototaxis in *Drosophila melanogaster* and other insects (Benzer, 1967; Choe and Clandinin, 2005; Hadler, 1964a; Hadler, 1964b; Hirsch and Boudreau, 1958; Meyer, 1976; Simon et al., 2006). The results of these experiments indicate that *T.*

frequens does indeed respond to light, and that their behavioral response to light differs significantly from what has been reported for other dipteran species.

Materials and Methods

Collection

Because adult bat flies do not live for more than a few hours off of their host, and cannot easily be maintained in a lab, collecting sufficient numbers for behavioral testing under controlled conditions that are amenable to statistical analysis can prove difficult. In addition to the evidence suggesting that their eyes may be functional, *T. frequens* is also an ideal candidate for this study because they can be easily collected in relatively large numbers from a population in Cueva de los Culebrones (Figure 2), Mata de Platano field station near Arecibo, Puerto Rico. The entrance to this hot cave descends many meters into a large entrance room and some smaller side chambers (orange in Figure 2). *T. frequens*'s primary hosts, *Erophylla sezekorni*, *Brachyphylla cavernarum,* and *Monophyllus redmani* (Dittmar et al., 2011), roost both in the large entrance room and side chambers, and deeper within the cave where it is significantly warmer (see Figure 2) (Dittmar and Mayberry, 2010). *T. frequens* pupae can be found scattered throughout the entrance room, but are never found further back in the hotter regions of the cave, even though bat flies have been captured there both flying in the air and on roosting bats (Dittmar et al., 2011). Near the time of sunset, when bats exit the cave to forage, pregnant female bat flies can be found in large numbers flying in the chambers just beyond the main entrance room. Immediately upon moving into the cooler main entrance room, the majority of females deposit their pupa on the cave wall, forming a large and dense pupal deposition field (red circle in Figure 2) (Dittmar et al., 2011). During these times when they can be found in sizeable groups in one location, it possible to collect them in numbers large enough for testing.

Adult bat flies were collected in Cueva de los Culebrones using a sweep net that was modified to hold an open 50ml conical vial at the end. Flies were collected by sweeping in the chamber following the entrance room between 7 and 9 pm. Immediately after sweeping, the conical vial was removed and capped to capture the flies. Because adult bat flies only live for a few hours away from their host, they cannot be easily maintained under laboratory conditions. Therefore, all experiments were performed at the Mata de Platano field station, immediately after collecting the flies. Tenerals (newly emerged, unfed adults) were obtained by collecting pupae from the deposition site in the cave and allowing them to continue development until they eclosed in the field station.

Because the behavioral response of *D. melanogaster* is well characterized, they were used as controls for all experiments. Wild type *D. melanogaster* were collected either from 2-5 days post eclosure, or more than 5 days post eclosure in order to match the age of the bat flies used for various experiments (tenerals and adults respectively).

Response to Flashes of Light

The response to short duration pulses of high intensity light stimulus was tested on 54 teneral bat flies, and 116 fruit flies between 2 and 5 days past eclosure. Illumination was produced using a Nikon SB-800 Speedlight camera flash at a distance of either 36 or 72 cm. The distance of 72 cm was selected after preliminary experiments indicated that beyond this distance no flies responded regardless of the intensity of the flash, suggesting that the flashes at this distance were near the threshold required to elicit a response. The levels of light exposure were varied using the built-in flash output level compensation at setting of +3, +2, +1, 0, -1, -2, and -3. As a control for other factors that might be associated with the flash, the flash was also triggered while shielding the flies from the light output. Background illumination was provided by ambient day light, in a shaded room, with no overhead lights.

All flies were dark adapted by keeping them in a light-tight container from the time of collection until experimentation began (1-3 hours). An average of 6 flies was tested at a time inside a 15 cm square, transparent, plastic container. The number of flies observed changing their location immediately following the flash stimulus was recorded for analysis. Because initial experiments indicated that the response of bat flies to the flash was not diminished under the conditions used by repeated exposure, all flies were exposed to all testing conditions. Experiments were recorded by video and at least two independent observers counted the number of flies responding to each flash.

Alternating Continuous Light Stimuli

To test the response to a continuous light stimulus of lower intensity, adult bat flies collected with sweep nets were kept in the dark in the same 50ml conical vials in which they were collected; fruit flies were transferred from the rearing bottle to a clean 50ml vial, and allowed to acclimate for 45 minutes in the dark. Flies were then tested by exposing them to two alternating light sources for one minute each, for a total of 6 minutes. Illuminance of the testing chambers was measured for each of the testing conditions using an Extech EA33 EasyView Light Meter; 4 independent measurements were taken to ensure consistency and accuracy of the readings. The first light source was a dim red light from a 3 lumens red LED placed 36 cm away from the side of the tube producing an average of 1.39 lux (stdev = 0.021) measured at the surface of the container; the second light sources was a GE Reveal 25 watt bulb placed 15 cm away from the side of the tube producing an average of 332 lux (stdev = 1.26). The responses to the alternating light sources were recorded by video for subsequent analysis.

Orienting Responses

Orienting responses when exposed to a light gradient were tested using the conditions illustrated in Figure 5. A clear plastic tube 40cm long was illuminated using the same light sources as for the

continuous light stimuli, and from the same respective distances, but used simultaneously to illuminate opposite ends of the tube. The GE Reveal 25 watt bulb was placed 15 cm away from one end of the tube to produce a white-light gradient along the length of the tube. White light was prevented from illuminating the opposite half of the tube by a piece of cardboard placed over the tube at its midpoint. To make it possible to see the flies on the dark side of the tube, the red LED was placed 30 cm from the side of this end of the tube. Illuminance of the end of the tube closest to the white light was as above, as was illuminance of the dark half under the red LED; illuminance of the tube at the light side of the barrier averaged 94.8 lux (stdev = 0.263). After collection, flies were transferred under dim red light to one end of the testing chamber, alternating the starting end each trial so that each group of flies began the experiment in either the light or the dark end. To control for possible inconsistencies in the tube, the tube itself was intermittently rotated 180^{o} to randomize which end was exposed to white light. Flies were prevented from moving past the midpoint before the experiment began by a removable barrier placed in a narrow slit cut in the middle of the tube (Figure 5). Each experiment began when the light was turned on and the barrier was simultaneously removed. Flies were then allowed to move freely for 3 minutes, at which point the barrier was replaced, preventing further movement between the two halves of the tube. Flies found in each end of the tube at the end of the 3 minutes were counted two times by two different individuals to ensure accuracy. Each group of flies was only tested once.

Statistical Analyses

All statistical analyses were carried out using the R environment for statistical computing (R Development Core Team 2008).

Because small numbers of flies were used in each flash experiment (6 on average), each fly was assumed to act independently, responding to the flash itself and not the movement of other flies. Treating each

fly as the unit of analysis, logistic regression was used to determine if the number of flies reacting to the flash correlated with increasing intensity at each of the two distances, using flash intensity as the predictor variable. To determine if the distance of the flash had a significant effect on the proportion of flies that responded, logit-transformed proportion of flies responding under each condition was used to conduct a paired student's t-test comparing the proportions that responded at the two distances. Additionally, to determine if the proportions responding at each distance were significantly different from zero (which was the number of fruit flies which responded), a one sample t-test was conducted using the same logit-transformed proportions from each distance.

Because a larger number of flies were used for the orientation experiments, and becase the testing chamber was significantly smaller compared to the flash experiments, group effects acting on the flies could not be ignored. Therefore, the proportion of flies moving to the opposite side of the tube was treated as the unit of analysis. Welch two sample t-test was used on logit-transformed proportions to determine if there was a difference in the proportion of flies moving from the light to the dark when compared to the proportion moving from the dark to the light.

Results

Response to Flashes of Light and Alternating Light Stimuli

Flashes of light and continuous light stimuli were used to test the hypothesis that *T. frequens* eyes are functional. In both of these experiments, a marked response was observed by *T. frequens*, which differed from that of *D. melanogaster*.

In response to the flash stimulus, *T. frequens* responded by suddenly flying away from the spot where they were previous to the flash, and quickly landing in a new location (see supplemental video "Bat Fly

Flash Response"); however none of the bat flies exhibited a response when shielded from the light. In contrast, none of the *D. melanogaster* responded to any of the flashes. For bat flies, it was found that their response was affected both by the intensity and the distance of the flash. At a distance of 36 cm (Figure 3), an average of 93.7% of the bat flies reacted while at a distance of 72 cm (Figure 4), an average of only 8.5% responded; the difference in the proportion of bat flies reacting at each distance was significant (t = 19.869, df = 6, p-value = 1.054e-06). Additionally, the proportion reacting was also significantly different from zero both at 36 cm (t = 17.6874, df = 6, p-value = 2.097e-06) and 72 cm (t = -10.8864, df = 6, p-value = 3.562e-05), indicating that the bat fly response was significantly different from that of the fruit flies, which never reacted to the flashes.

Logistic regression using intensity of the flash as a predictor of the number of bat flies responding found no significant correlation at a distance of 36 cm (p-value = 0.673); however, the correlation of intensity with the number of flies responding was significant at a distance of 72 cm (p-value = 0.00894) with an average of 3.7% responding at the lowest intensity and 14.8% at the highest intensity.

The reaction of fruit flies and bat flies also differed considerably in response to the continuous light stimulus. As would be expected given previous studies on the response of fruit flies to light, they exhibited phototaxis towards both light sources used (dim red and white). However, the response of bat flies varied with the light source. Under dim red lighting, the vast majority of the bat flies remained in place. When the white light was turned on, their activity, which consisted mostly of flying and some walking in all directions within the container, increased suddenly and dramatically. When the white light was turned off and the dim red light back on, most of the flies returned immediately to their inactive state. This behavior switching with the lighting conditions was exhibited throughout the length of the experiment. Because the numbers of bat flies moving or remaining still during the experiment could not

be assessed, a video record of their response can be downloaded with the supplemental materials ("Alternating Light Stimuli").

Orienting Responses

Experiments using light gradients were conducted to determine whether or not *T. frequens* orients their movements relative to a light gradient, as has been observed in other dipterans. The response of fruit flies to the orienting experiment was in agreement with the long history of published experiments on phototaxis in *Drosophila* species (Benzer, 1967; Choe and Clandinin, 2005; Hadler, 1964a; Hadler, 1964b; Hirsch and Boudreau, 1958). When fruit flies began the experiment in the dark end of the tube, they immediately oriented their movements in the direction of the white light and walked en masse in a direct line towards the source, without increasing their overall rate of movement (Figure 6). When they began in the dark side of the tube, an average of 81.4% moved into the light side. On the other hand, when they began in the light side of the tube, only 7.2% moved into the dark; this later movement was attributed to crowding of the flies in the tube. The difference in the proportion of flies moving in the two directions was significant (t = -11.0585, df =9.047, p-value = 1.474e-06; see Table 1).

On the other hand, the response of bat flies was essentially the opposite in every way. As with the light switching experiment, bat flies in the light side of the tube increased their activity by flying and sometimes walking around vigorously when in the light, with no discernible preferred direction of movement (Figure 6), but became still if landing in the dark side of the tube. Thus, when the bat flies began the experiment in the light side of the tube, they tended to collect in the dark side. On average 67.7% of the flies starting in the light were in the dark side of the tube at the end of the trial time. When bat flies began the experiment in the dark side of the tube, their limited activity resulted in only 19.9%

moving into the light side of the tube. The difference between these proportions was also highly significant (t = 0.53304, df = 12.33; p-value = 1.634e-04; see Table 2).

It was also noted in both the experiments with constant light and with a light gradient, that when *T. frequens* walked, they often tended to walk sideways, while *D. melanogaster* always walked in the forward direction. This behavior has also been in bat flies on the membranes of bat wings.

Discussion

T. frequens Eyes are Functional

As with all bat flies, the eyes of *T. frequens* are dramatically reduced relative to their dipteran relatives. Their reduced state suggests that selective pressures for their function have been relaxed and that they may have lost their function as a part of their reduction; however, because the external facets of *T. frequens* are symmetrically shaped, it was hypothesized that they may retain some function. Each of the experiments conducted clearly indicate that *T. frequens* reacts to a variety of light stimuli, which suggests that their eyes are likely to be functional, at least in the sense that they initiate a response to light intensity. The experiments conducted do not allow conclusions to be made about their image forming capabilities.

The first indication that their eyes are functional came from observing their reaction to flash stimulus. That none of the flies responded when they were shielded from the light confirms that the response was elicited by light, not by another stimulus that could be associated with the flash, such as sound. This is further confirmed by the significant positive correlation between the flash intensity setting and the number of bat flies responding when at 72 cm.

The functionality of *T. frequens* eyes is also suggested both by the results of the light switching and orientation experiments. In both of these experiments the bat flies responded to a constant light stimulus with a marked, sustained increase in activity compared to their relative calm when in darkness.

Even though these experiments suggest that *T. frequens* eyes are functional, there are distinct differences in the nature of *T. frequens's* response compared to *D. melanogaster* and other dipterans.

Analysis of Flash Response

Because *D. melanogaster* is clearly highly visual, it seemed surprising at first that they failed to respond to the flash stimulus, while *T. frequens* reacted in an obvious way. Even though white-eyed Drosophila mutants were not tested, it has long been known that they respond to a sudden "lights-off" stimulus (a flicker in the ambient lighting) with a reflexive and characteristic escape response (Allen et al., 2006; Hammond and O'Shea, 2007; Zhang et al., 2007). Wild type (red eyed) Drosophila species do not readily respond to this same stimulus, but they do exhibit a similar, distinct reflexive response to a "looming" stimulus (i.e. an expanding shadow produced by an approaching object such as a would-be predator (Fotowat et al., 2009; Gibson, 1986). It is thought that the escape behavior in response to the looming stimulus represents the primary escape mechanism by Drosophila species, and that the response to the "lights-off" stimulus, which relies on a distinct neural circuit, may only be activated in wild type flies when combined with appropriate mechano-sensory inputs (Fotowat et al., 2009). In light of this, it is possible that *D. melanogaster* does not exhibit the same escape reflex in response to a bright flash of light because this stimulus is not associated with a natural predator.

While the response of *T. frequens* to bright flashes superficially resembles those of *D. melanogaster* (though to the opposite stimulus) it is unclear in what way, if any, their behavior may be advantageous.

Furthermore, while *T. frequens's* response to bright flashes may be a reflexive response, it is also possible that it could simply be an increase in activity similar to that seen with the sustained light experiments, though shorter in duration, or even a pain response do to the sudden intensity of the light. Regardless, *T. frequens*'s response to sudden flashes of light is the first of its kind described for any dipteran species, as is *D. melanogaster's* lack of a response.

Analysis of Sustained Lighting and Orienting Behaviors

The response of *T. frequens* to the orienting experiments resulted in net movement away from the light source, while *D. melanogaster's* response resulted in net movement toward the light source; however, as illustrated in figure 6, the behavior of *T. frequens* is not simply a reverse in the direction of movement relative to that of *D. melanogaster*. Three classes of behavioral responses can result in net movement relative to a stimulus: Two of these are different forms of taxis: tropotaxis and klinotaxis. The third is a special case of kinesis known as differential klinokinesis; other classes of kinesis have also been described but do not by themselves result in net directional movement (Figure 7) (Codling et al., 2008).

Taxis in general is used to describe behaviors when individuals determine the orientation of a stimulus gradient such as light, chemicals, and humidity, and move relative thereto; taxis alone does not involve a change in the rate of movement. Tropotaxis results when individuals possess sensors of sufficient spatial resolution to determine the directionality of the gradient *instantaneously*, without having to move within the gradient. Klinotaxis is exhibited when the spatial resolution of sensors is insufficient to determine the orientation of the gradient, so individuals must first make movements within the gradient (either moving their whole body, or using swaying movements of the head), in effect sampling different regions of the gradient to determine its orientation (Codling et al., 2008).

This contrasts with kinesis, where the orientation of the gradient is never determined. Instead, individuals respond either to the absolute intensity of the stimulus at any one location (absolute kinesis) or to the relative change in intensity as they move through the gradient (differential kinesis). If the response involves a change in the rate of turning movements (where the direction turned is random), it is further classified as klinokinesis, but if the response involves a change in the speed, or rate, of movement it is considered orthokinesis. The combinations of these possibilities results in 4 distinct forms of kinesis (Figure 7). Interestingly, even though the direction of turning is random and the orientation of the gradient is never determined, differential klinokinesis is capable of producing net movement relative to the stimulus gradient, resulting in aggregation of conspecifics in preferred microenvironments; experiments with the other forms of kinesis (absolute klinokinesis, differential orthokinesis, and absolute orthokinesis) have been incapable of generating this result (Codling et al., 2008; Codling et al., 2010; Benhamou and Bovet, 1989; Doucet and Wilschut, 1987; Doucet and Drost, 1985).

The behavior of *D. melanogaster* typifies positive photo-tropotaxis because they immediately alter their movements to walk directly towards a light source, without altering their rate of movement.

In the case of *T. frequens*, however, their response to light includes both increased movement (manifest by the increased number of flies walking and flying within the chamber), and moving in multiple directions within the testing chamber. Neither the increased activity nor the directionality of the individual movements can be explained by tropotaxis. However, if switching from remaining stationary to moving around within the chamber is considered an increase in their speed of movement, then this aspect of their behavior can be interpreted as orthokinetic. Under the circumstances of the experiment, it is possible that this alone could be responsible for an aggregation of flies in the dark end of the tube: If

encountering one of the walls or ends of the tube resulted in a forced turning movement, then any such movement which resulted in flies subsequently landing in the dark side of the tube where they became stationary, would result in an aggregation of flies in the dark. This same phenomenon could result in an aggregation of flies in the dark in their natural environment, albeit with less efficiency because they will less frequently encounter the walls of the cave than they did the walls in the tube. Nevertheless, this does not rule out the possibility that the movement of individuals in various directions could be due to one of the other components of their behavior, either klinotaxis or klinokinesis.

If movement of flies in all directions within the tube when exposed to light results from individual efforts to determine the directionality of the gradient, then the behavior would be klinotaxis. If this is the case, then the observed tendency for *T. frequens* to walk sideways could contribute to their ability to detect the gradient, because this would maximize the difference in light intensities sampled by each eye when moving in the direction of the gradient. This same sideways-walk could play a similar role in detecting the orientation of other stimuli gradients such as odors, temperature, or carbon dioxide. Inasmuch as reduction in both the overall size and number of receptors of *T. frequens* eyes has led to a decrease in spatial resolution, and if the behavior is indeed klinotaxis, then klinotaxis may have come about as a means of compensating for this loss in resolution.

If, on the other hand, *T. frequens* is responding to the light without detecting the gradient directly, or orienting their movement's relative thereto, then the turning movements could be viewed as differential klinokinesis. In this case, the movements of *T. frequens* in all directions within the tube could be interpreted as an increase in path sinuosity due to the stimulus intensity, where the direction of turning for each fly was random. Because differential klinokinesis is capable of moving individuals

towards preferred environments, this could cause flies in the natural environment to move efficiently away from the entrance of the cave.

Mathematical models based on random walks have been developed which make it possible to identify taxic (non-random) components in an organism's movement through an environment. In principle these could be applied to distinguish between taxis and kinesis in *T. frequens*; however, the experiments conducted here do not lend themselves to this type of scrutiny because they require an analysis of the paths traversed during a subject's response to the stimulus (Benhamou, 2006; Benhamou and Bovet, 1992; Codling et al., 2008). Because flies were enclosed in narrow tubes (a highly artificial environment), their paths were frequently interrupted and therefore altered by the borders of the tube themselves. These forced changes in direction are likely to mask any non-random component of their natural behavior. This not only precludes a rigorous analysis of the resultant paths, but would likely make the conclusions of any such analysis incorrect. It has also been demonstrated that highly tortuous movement alone can preclude such an analysis, even if they were not confounded by interactions with the borders of the testing chamber (Codling et al., 2008).

Furthermore, the data do not make it possible to distinguish between absolute or relative kinesis because the intensity of the stimulus was not altered in separate experiments. At this time then, the available data make tentative any conclusion as to whether or not the net movement of *T. frequens*'s in response to light is due to klinotaxis, differential klinokinesis, or simply orthokinesis. In either case, it is clear that the behavior exhibited by *T. frequens* is distinct from that of *D. melanogaster* and other Diptera that have been studied.

Considerations for Bat Fly Evolution

Among the adult dipteran species for which behavioral response to light has been reported, only *B. coeca (Braulidae),* (Kaschef, 1959) and *T. frequens* (Streblideae) respond negatively. Phylogenetically, each of these species is more closely related to others that have been demonstrated to exhibit positive phototaxis (Figure 8) (Dittmar et al., 2006; Wiegmann et al., 2011). These relationships suggest that each has independently switched the sign (and for *T. frequens*, the mode) of their behavior from ancestors that exhibited positive photo-tropo-taxis as adults.

From a developmental perspective, a potential means for switching the sign of the response stems from the fact that even though adult Diptera typically exhibit positive phototaxis, they exhibit negative phototaxis as larvae. Because *T. frequens* females deposit a fully formed pupa inside a soft pupal case, larvae do not have the opportunity to orient relative to light; however, it is possible that the developmental switch to a positively oriented response common to other dipteran species (see Introduction) simply doesn't take place, resulting in an adult fly that responds negatively to light. This does not, however, speak to the shift away from tropotaxis to another mode of movement.

As already noted, the fact that *T. frequens* deposit their pupae near the entrance of the cave where they can be exposed to light, provides the opportunity for their reaction to affect their fitness. Specifically, it is possible that dim light from the cave entrance could act as a warning that they are heading in the wrong direction, eliciting behaviors that cause them to move deeper into the cave, where they are more likely to encounter a host. Once there, other sensory inputs such as mechanical stimuli, heat, and carbon dioxide levels might take over in guiding them to a specific host (Caire et al., 1985). Nevertheless, given that pupae are sometimes observed near enough to the entrance to be exposed to light, this is not to say that there are no other factors that contribute to selection of pupal deposition sites.

T. frequens pupae are only observed in the cooler main entrance rooms of Cueva de los Culebrones, but never in the deeper, hotter parts of the cave, and congeners in Cueva de Cucaracha in Puerto Rico (personal observations), and in a cave in Tamaulipas Mexico (Dittmar et al., 2009), both of which are hot caves, also preferentially deposit pupae near the cave entrance. This suggests there may be some shared aspect of their biology that prevents them from depositing pupae in the hotter parts of the cave. One possibility is that Trichobius species may possess a developmental constraint necessitating them to deposit pupa in cooler parts of the cave, where both pupa depositing females and newly emerged tenerals may be exposed to light from the cave entrance. Thus, if this hypothesized developmental constraint exists, it could be responsible for forcing ancestors of *T. frequens* (and congeners) into locations where they could be exposed to light from the entrance of the cave. Thus being exposed to light, the potential exists that selection could favor individuals in the population that exhibit an advantageous response thereto, such as initiating movement to where hosts are more likely to be found.

Interestingly species of a related genus, Nycterophilia, in both Cueva de los Culebrones and Cueva de Cucaracha have been observed depositing pupae on the wall in the hottest parts of the cave (personal observation). This suggests that Nycterophilia species may not have the same developmental constraint hypothesized for Trichobius species. Because Nycterophilia are able to deposit their pupae in the deepest and hottest parts of these caves, the chances for members of this genus to be exposed to light are very low. Consequently, they may have experienced little or no purifying selection for eye function, and, as might be expected, their eyes are reduced in all cases to what appears to be a small, vestigial fold of tissue (personal observation).

Inasmuch as *Nycterophilia* and other bat fly species possess what appears to be vestigial eyes, and still other bat fly species have no discernible eye at all, this suggests that sufficient time may have transpired for deleterious mutations to destroy the function of *T. frequens* eyes, if selective pressure were not acting to maintain them. Additionally, because eyes are developmentally and functionally expensive (Lahti et al., 2009; Buschbeck and Friedrich, 2008), they may be less likely to evolve under neutral processes. Though tentative, these consideration suggests that the evolutionary reduction of *T. frequens*'s eyes, and the changes in their behavior, may have taken place under weak but persistent purifying selection.

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Chapter 1 Figure Captions

Figure 1: SEM Showing the Reduced Eye of Trichobius frequens

SEMs of *T. frequens* collected from Cuevo de los Culebrones. The scale bar on the left is 500µm and on the right is 50µm.

Figure 2: Depiction of Cueva de los Culebrones, near Arecibo, Puerto Rico

T. frequens pupa can be found throughout the entrance room to the cave (orange outline), but are generally sparse except at the primary pupal deposition field (red circle) where pupae densely cover the cave wall. Even though *T. frequens* is found beyond the entrance room, pupal deposition only takes place inside the main entrance. The cave outline was extracted from the current Culebrones cave map (Sociedad Espeleologica de Puerto Rico, Suunto and nylon tape survey, 2007). See text for details.

Figure 3: Response to Flash Stimulus from 36 cm Away.

Percentage of flies exhibiting a response to flash stimuli of increasing intensity from 36 cm away: Under no circumstances did any of the fruit flies respond to a flash stimulus. Bat flies responded on average 93.7% of the time, with logistic regression revealing no difference between different flash intensities from this distance ($p=0.673$). Each fly was tested at all flash intensities.

Figure 4: Response to Flash Stimulus from 72 cm Away.

Percentage of flies exhibiting a response to flash stimuli of increasing intensity from 72 cm away: Under no circumstances did any of the fruit flies respond to a flash stimulus. Bat flies responded on average 8.5% of the time, but logistic regression found a significant correlation with flash intensity and the percent of flies responding, which ranged from 3.7% at the lowest intensity to 14.8% at the highest. Each fly was tested at all flash intensities.

Figure 5: Experimental Setup Used to Test for an Oriented Response to Light

Experimental setup used to test for an oriented response in *T. frequens* and *D. melanogaster*. See text for a description of the experiments.

Figure 6: Representative Responses of Fruit Flies and Bat Flies to a Light Gradient

Fruit flies respond to the presentation of a light gradient by turning and walking in a straight line towards the direction of the light. Bat flies respond to the presentation of a light gradient by increasing their activity – flying, jumping, and walking around – with no apparent preference for a given direction.

Figure 7: Summary of Behavioral Responses to a Stimulus Gradient

Summary of the types of possible behaviors that an organism can exhibit in response to a stimulus gradient (Codling et al., 2008).

Figure 8: Abbreviated dipteran Phylogeny

Phylogenetic relationships of select dipteran species supporting independent changes in the response to light of bee lice and bat flies (red branches). Based on Wiegmann et al., 2011 and Dittmar et al., 2006.

Chapter 1 Figures

Figure 1: SEM Showing the Reduced Eye of Trichobius frequens

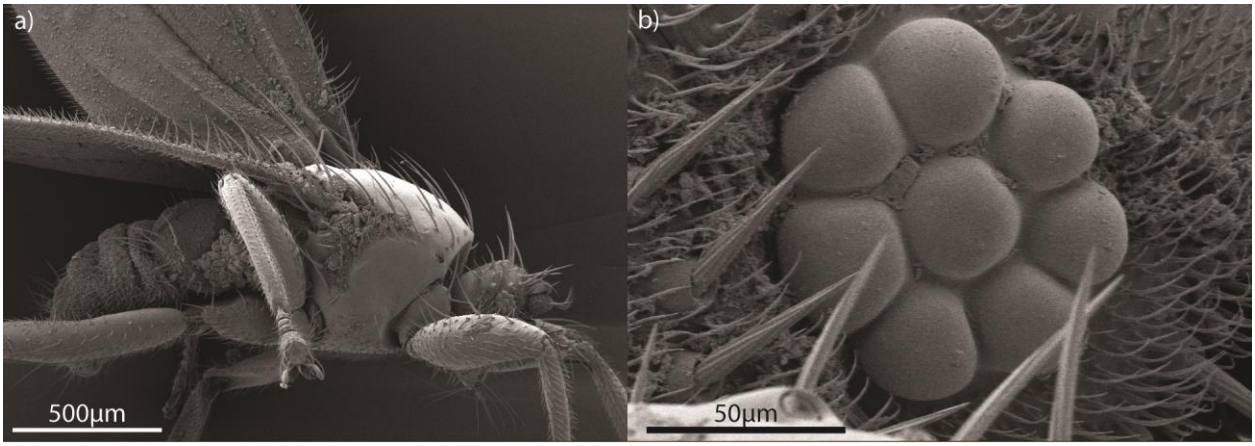


Figure 2: Depiction of Cueva de los Culebrones, near Arecibo, Puerto Rico

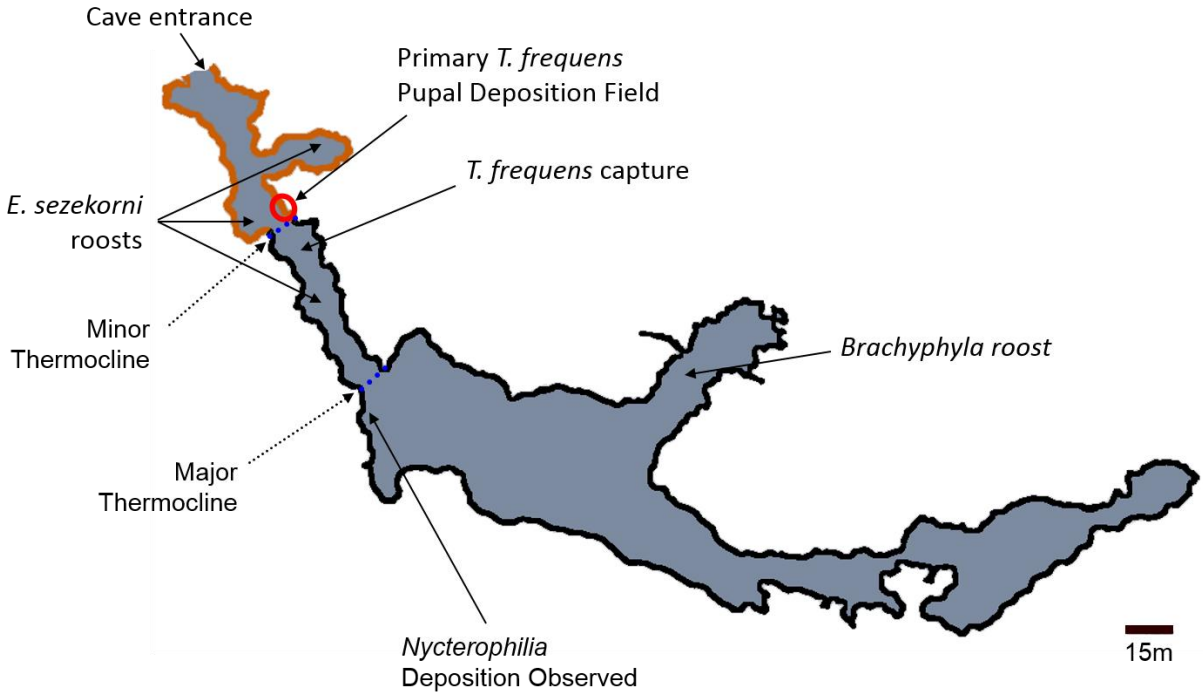


Figure 3: Response to Flash Stimulus from 36 cm Away.

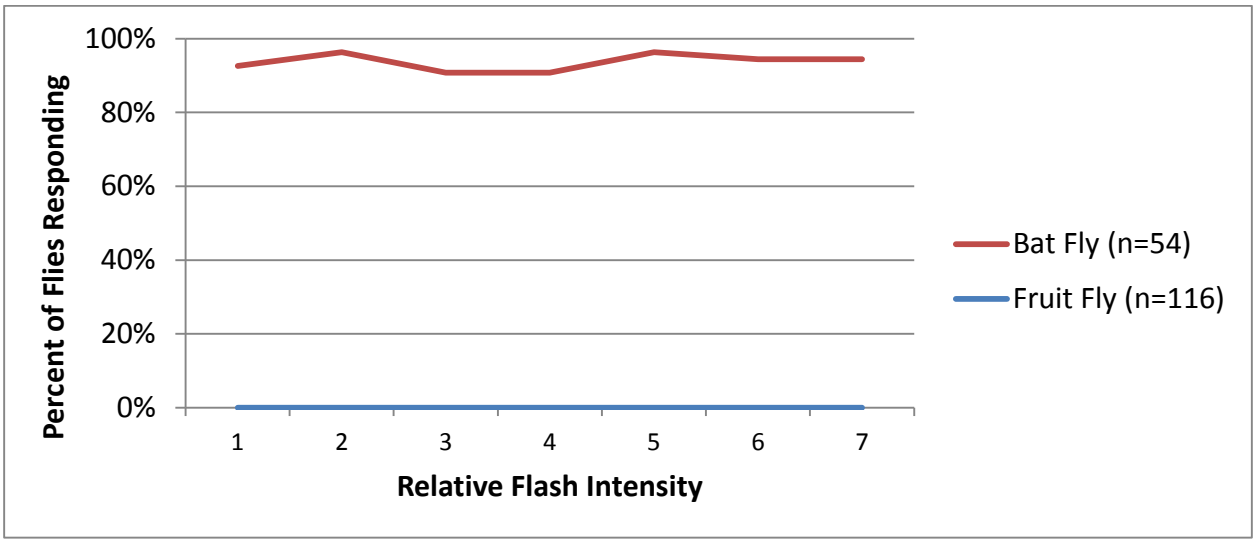


Figure 4: Response to Flash Stimulus from 72 cm Away.

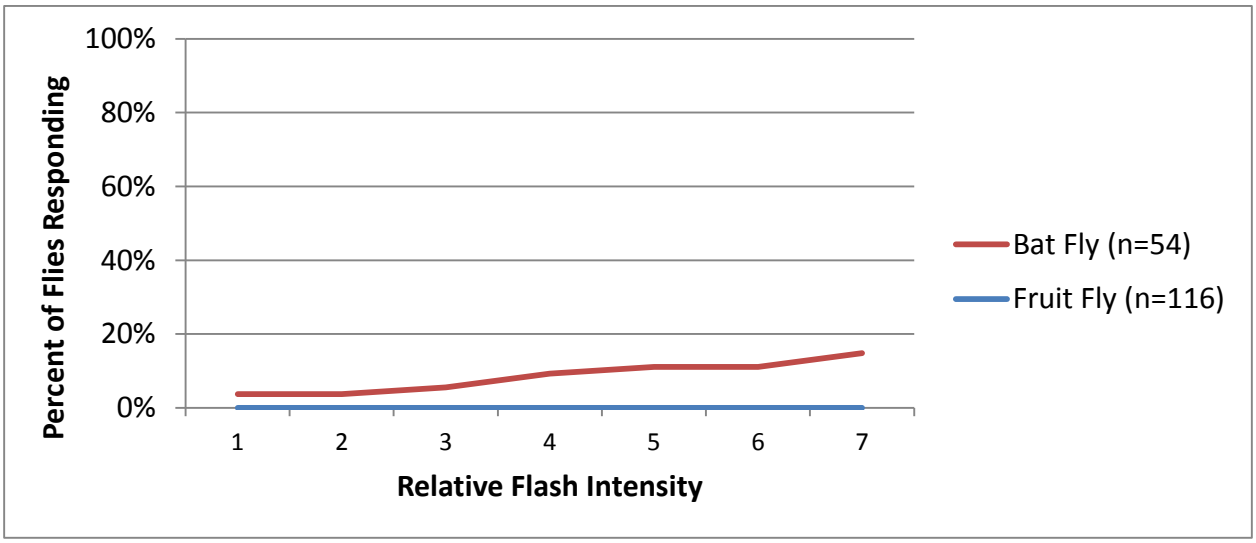


Figure 5: Experimental Setup Used to Test for an Oriented Response to Light

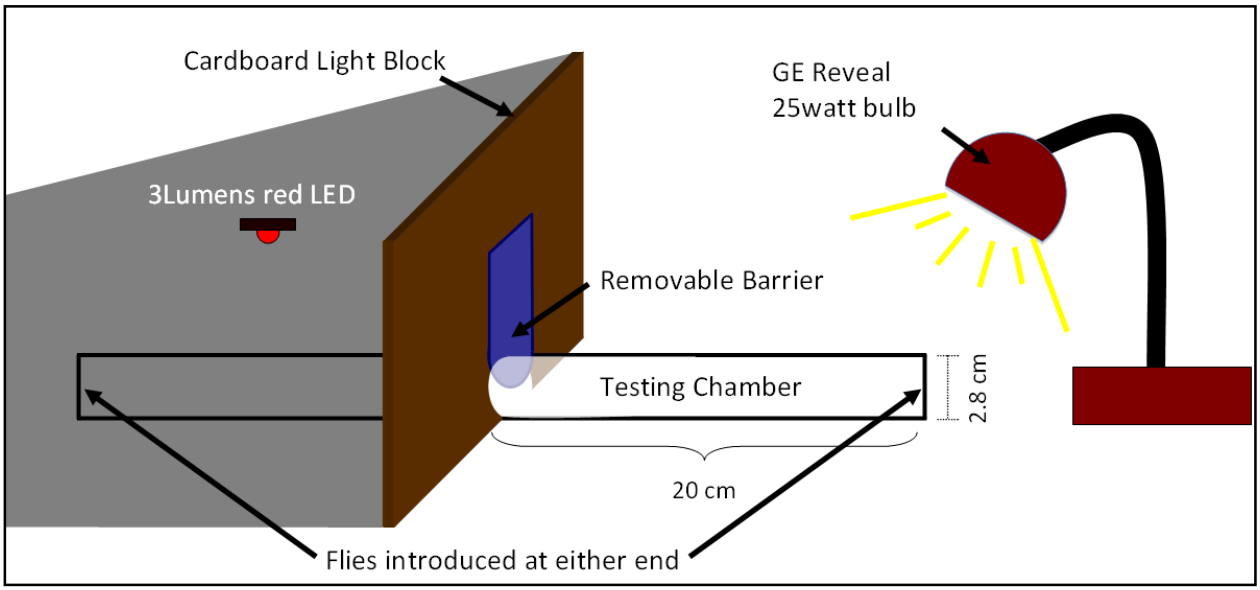


Figure 6: Representative Responses of Fruit Flies and Bat Flies to a Light Gradient

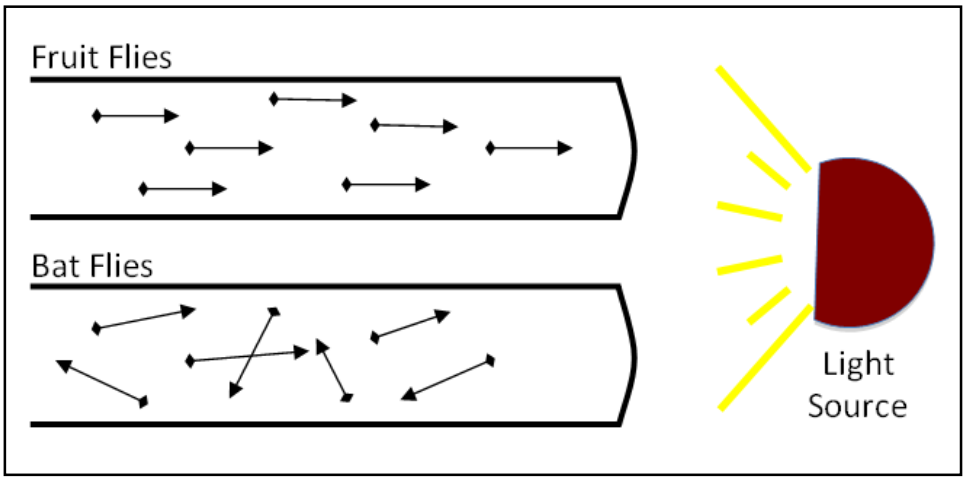


Figure 7: Summary of Behavioral Responses to a Stimulus Gradient

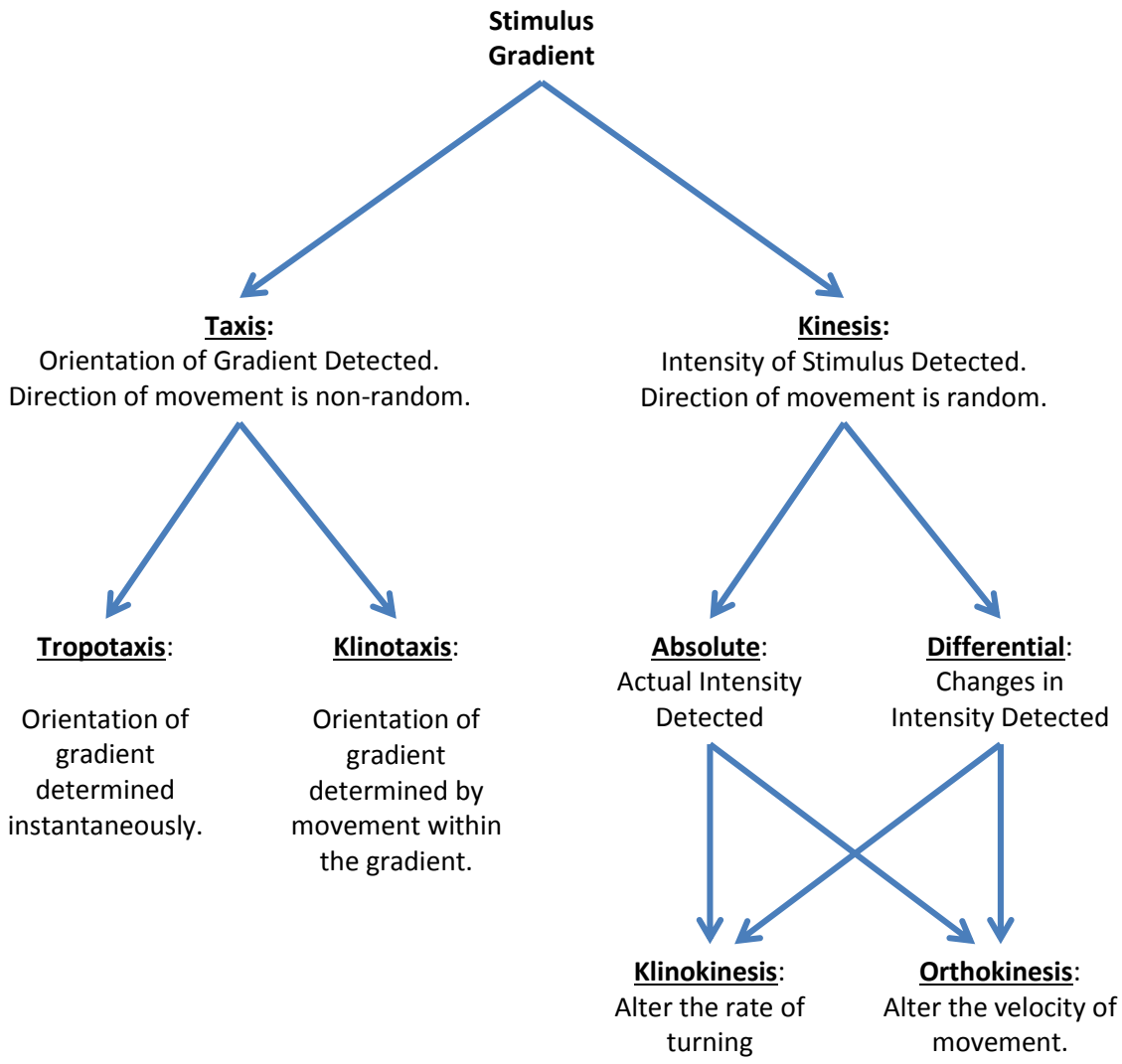
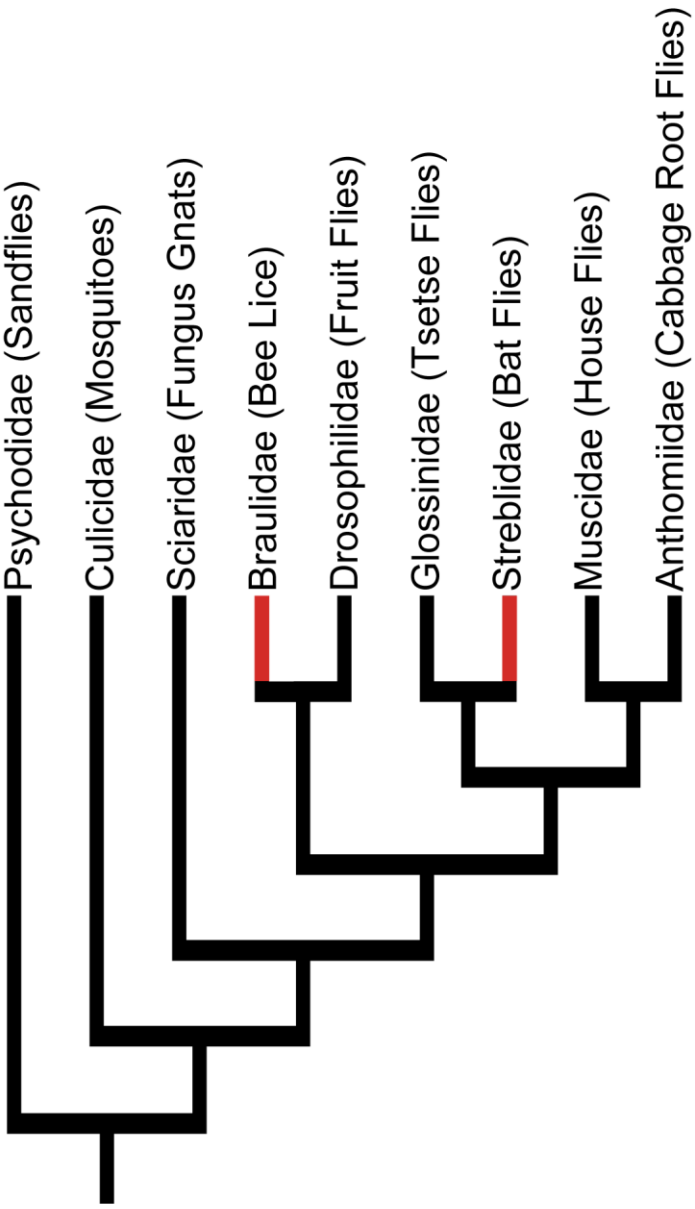


Figure 8: Abbreviated dipteran Phylogeny



Chapter 1 Table Captions

Table 1: Results from Light Orienting Experiments with Fruit Flies

Results from the light orienting experiments conducted with fruit flies (*D. melanogaster*). The table on the left reports the results from 8 independent trials where fruit flies began in the dark. Across these trials an average of 81.42% moved into the light. The table on the right reports the results from 8 independent trials where fruit flies began in the light. Across these trials an average of 7.18% moved into the dark.

Table 2: Results from Light Orienting Experiments with Bat Flies

Results from the light orienting experiments conducted with bat flies (*T. frequens*). The table on the left reports the results from 7 independent trials where bat flies began in the dark. Across these trials an average of 19.91% moved into the light. The table on the right reports the results from 9 independent trials where bat flies began in the light. Across these trials an average of 67.65% moved into the dark.

Chapter 1 Tables

Table 1: Results from Light Orienting Experiments with Fruit Flies

Fruit Flies Starting in Dark		Fruit Flies Starting in Light	
Number of Flies in Trial	Percent Moving to Light	Number of Flies in Trial	Percent Moving to Dark
22	77.27%	35	14.29%
36	80.56%	23	8.70%
21	71.43%	23	4.35%
31	80.65%	38	13.16%
21	85.71%	32	1.56%
35	85.71%	34	11.76%
42	90.48%	42	1.18%
49	79.59%	41	2.44%
Average	**81.42%**	**Average**	**7.18%**

Welch Two Sample t-test of the Difference in the Percent Moving Between the Two Starting Conditions
t = 11.0585; degrees of freedom = 9.047; p-value = 0.000001474

Table 2: Results from Light Orienting Experiments with Bat Flies

Bat Flies Starting in Dark		Bat Flies Starting in Light	
Number of Flies in Trial	Percent Moving to Light	Number of Flies in Trial	Percent Moving to Dark
6	33.33%	12	66.67%
12	14.29%	7	71.43%
12	33.33%	19	73.68%
16	15.79%	31	67.74%
20	20.00%	14	28.57%
17	10.53%	16	43.75%
29	12.12%	12	100.00%
		15	86.67%
		27	70.37%
Average	**19.91%**	**Average**	**67.65%**

Welch Two Sample t-test of the Difference in the Percent Moving Between the Two Starting Conditions

t = -5.3304; degrees of freedem = 12.33; p-value = 0.0001634

Chapter 2: Phylogenetic Patterns of Eye Reduction in Bat Flies (Streblidae)

Abstract

The dipteran family Streblidae is comprised of a speciose group of bat flies that all exhibit either eye loss or significant reduction in the number of facets. Variability between species makes this group ideal for studying the patterns of evolutionary reduction in a phylogenetic context. Analyses presented hear reveal that selection has acted both on different aspects of eye structure and differentially in separate bat fly lineages. Evidence for this includes a correlation of parameters measuring eye size with wing morphology, independent instances of secondary expansion in the number of facets, and restructuring the lenses to make them more hemispherical.

Introduction

Theory of Reduction

Numerous examples of evolutionary reduction demonstrate the many factors that can influence both the rate of reduction, and the phenotypic effects that occur during the process (Lahti et al., 2009). Among the issues in question are potential roles for selection (both positive and negative with respect to reduction), genetic and functional correlation with other traits (including developmental and pleitropic effects), and neutral effects.

The challenge of determining which factors are involved in instances of reduction is often magnified by the limited variation of reduced forms that typically exists among related species. Using eye reduction as an example, species such as the Mexican cave fish (Jeffery, 2005; Jeffery et al., 2003; Sadoglu, 1967) and subterranean mammals (Cooper et al., 1993; Kott et al., 2010; Cernuda-Cernuda et al., 2003) have offered fertile ground for characterizing instances of reduction, but because these cases are confined to

a small number of species (or populations) with little variation, they provide only a snapshot of a potentially lengthy and complicated process. Consequently, they offer a limited perspective on the process of reduction, and the factors that affect it. Other, more speciose groups, such as fleas (Order Siphonaptera) exhibit general eye reduction (Crum et al., 1974; Osbrink and Rust, 1985; Taylor et al., 2005), but their eyes also exhibit minimal diversity.

More rarely, a diversity of reduced forms among closely related organisms has made it possible for phylogenetics to provide insights into the processes affecting reduction. Instances include variable degrees of armor loss in stickleback populations (Bell et al., 2004), and variable degrees of oil gland loss in Ceratandra orchids (Steiner, 1998).

Bat flies offer an excellent opportunity to similarly apply phylogenetics to study evolutionary reduction. Bat flies consist of two families of hematophagous dipterans, Streblidae and Nycteribiidae, that exhibit both eye reduction and diversity in eye form. This diversity is seen primarily in the number of facets (the externally visible units of a compound eye) that comprise the eyes of a species, but is also seen in the structure of the facets themselves, being smooth and symmetrical in some species, and irregularly shaped in others (see below).

Bat flies are closely related to two other families of hematophagous dipterans: Glossinidae (e.g. Tsetse flies) and Hippoboscidae (e.g. ked and louse flies) (Dittmar et al., 2006; Peterson et al. 2007; Wiegmann et al., 2011). Together, these families comprise the superfamily Hippoboscoidea. Reduction in the number of facets of bat fly species stands in contrast to the large multi-faceted eyes of Glossinidae, Hippoboscidae (with the exception of a few species, such as *Melophagus ovinus*, that have independently experienced eye reduction), and most other dipterans.

Of the two bat fly families, Nycteribiidae [274 species; (Graciolli and Dick, 2006)] may be considered the more highly derived, but has less intrafamilial eye variation than Streblidae [230 species (Dick and Graciolli, 2006)]. While Nycteribiidae eyes range from being absent to a maximum of only two facets per eye (Maa, 1962; Bertola et al., 2005; and personal observation), eyes within Streblidae range from being absent to having around three dozen facets per eye in a few species (Table 1). Observation of the behavioral responses to light in one Streblidae species (*Trichobius frequens*), which has well-formed facets, indicate that their eyes are likely to be functional (Chapter 1); however, the irregularly shaped facets of other Streblidae species are more likely to be vestigial with respect to light detection and/or image formation (e.g. *Nycterophilia spp*, and *Strebla spp.*; personal observation). This variability within Streblidae provides the opportunity to study the process of reduction in what may be either different stages of reduction, or separate but related evolutionary paths that have experienced different evolutionary pressures, and exhibit different outcomes. Streblidae were therefore chosen as the subject for analysis

Biological Factors That May Affect Eye Evolution And Objectives

Even though eye variability within Streblidae is evident from descriptions in the literature of individual species (Table 1), their diversity has not been studied in a phylogenetic context. Therefore, the first aim of this study was to analyze the evolution of Streblidae eye morphology in an effort to illuminate any patterns that may exist. To this end, a molecular based phylogeny is produced that is used to examine the evolutionary relationships within Streblidae, as this is essential to understanding their eye evolution. Because detailed descriptions of eye morphology are also lacking, data on eye morphology to be used in this analysis were also generated.

It has been noted for a number of insect species, that their eye morphology appears to correlate with ecological and behavioral factors (Greiner, 2006; Greiner et al., 2007; Kawada et al., 2006; Land et al., 1999; Land et al., 1997). If the variation in eye morphology seen within Streblidae is due to differential selective pressures, then their eye variation is expected to exhibit phylogenetic correlation with aspects of their biology that are a part of the selective regime affecting eye evolution, including potential for exposure to light and utility of eye function for foraging and mating (Felsenstein, 1985; Pagel, 1999). The factors of bat fly biology that may correlate with their eye diversity can be identified by surveying the life history of bat flies.

As with other members of their superfamily, bat flies are adenotrophic viviparous, meaning that females produce a single offspring at a time. After fertilization, the developing offspring is nourished internally through the larval stages of development. While Glossinidae and Hippoboscidae females deposit a late 3rd instar larva (Hutson and others, 1984; Tobe and Langley, 1978), female bat flies deposit a fully formed pupa inside a soft pupal case that hardens shortly after deposition (personal observation). Pupae are deposited on a suitable substrate away from the host, such as the walls of the cave where the bat hosts roost. This requires females and tenerals (newly emerged adults) to employ means to locate their hosts. If the roost location is in places where bat flies can be exposed to light, such as in the foliage of trees, then the ability to respond to light may have provided sufficient advantage to cause eyes to be retained; on the other hand, if the roost is in a location where the potential for light exposure is negligible, such as deep within a dark (i.e. aphotic) cave, then this could lead to a complete loss of selective pressures acting for eye retention.

Recognizing this, Maa (1971) has suggested that the reduced eyes of Brachytarsina (Figure 5D) was "*obviously an adaptation to the conditions of the roosting sites of its specific host Notopteris, which*

roosts usually in deep dark caves", particularly when contrasted to the large eyes of *Megastrebla* (Figure 5C) and the "*partially illuminated roosting sites*" of their hosts. The second aim of this study therefore was to test the hypothesis that variation in Streblidae eye morphology will show phylogenetic correlation with the degree to which their host's preferred roosting locations give them the potential to be exposed to light.

It is often assumed that insect flight is dependent on visual capabilities for navigation (Buschbeck and Friedrich, 2008; Kalmus, 1945; Kerfoot, 1967), and thus the capacity for flight, and thus wing morphology may correlate with eye morphology. In some instances, as with *T. frequens* and other Trichobius species (Dittmar et al., 2009; Dittmar and Mayberry, 2010), flight is used to move some distance out of the roost to a preferred location for depositing pupae, and then again to locate a host. Flight might also be used for activities such as mating or predator avoidance. If it is true that flight navigation generally requires vision (or at least benefits from it), then bat flies that possess well-formed wings may experience selective pressures for maintaining functional eyes. However, even if bat flies are able to navigate using other senses, flight capable flies may be more likely to be exposed to light, even if the roost is in a dark location, because they can easily move out of the roost to locations where they can be exposed to light. Under such circumstances, the ability to respond to visual cues may provide an advantage sufficient to select for eye retention. In either case (whether vision is required for navigation or simply makes light exposure more likely), eye and wing morphology would be expected to show a phylogenetic correlation. Thus the third objective of this study is to test this hypothesis.

Materials and Methods

Taxon Sampling

The phylogenetic analysis included a total of 55 Streblidae and 6 other dipteran species as outgroups. The outgroups consisted of *Drosophila melanogaster*, *Musca domestica*, *Stomoxys calcitrans*, *Belvosia* sp., and one individual from each of the other two families of Hippoboscoidea: *Glossina palpalis (*Glossinidae*)*, and *Lipoptena cervi* (Hippoboscidae). The 55 Streblidae include 24 representatives of Trichobius, 8 representatives of Strebla, 2 representatives of Speiseria, and 1 representative for each of 22 other genera. The data for twelve of the in-group species were from previous analyses (Dittmar et al., 2006), and the remaining are new specimen collected in 96% EtOH and stored at -80˚C in the Dittmar lab at SUNY, Buffalo NY.

DNA extraction, Sequencing, and Alignments

DNA was extracted from whole specimens using Qiagen DNeasy (Valencia, CA, USA) protocol for animal tissues. Samples were cut open to facilitate extraction, but exoskeletons were saved and slide mounted for identification and preservation; voucher DNA samples and mounted specimens are stored in the Dittmar Lab. The 9 DNA loci targeted for amplification and sequencing included 4 nuclear genes: 28S rDNA, 18S rDNA, carbamyl-P synthetase/aspartate transcarbamylase/dihydroorotase (CAD), and Histone 3 (H3); and 5 mitochondrial genes: cytochrome oxidase 1 (CO1), cytochrome oxidase 2 (CO2), 16S rDNA, 12S rDNA, and cytochrome B (CytB). A list of primers used and references, including protocols used for amplification are found in Table 2.

Each PCR reaction was run with a total volume of 25 µl made up of 13 µl ddH_20, 2 µl 50mM $MgCl_2$, 3 µl 10X Buffer, 2.5 µl of 10mM dNTP's, 2.5 µl loading dye, 1 µl each primer (10 pmol/µl), 0.1 µl Taq polymerase and 1 µl of genomic DNA. Each PCR was run with negative controls to detect false positives

resulting from contamination. Reactions were confirmed successful using agarose gel electrophoresis and sent to the High Throughput Genomics Unit (Seattle, WA) for cleaning and DNA sequencing.

Sequences were edited, assembled, and proofread in Sequencher 4.2 (Gene Codes Corporation) and Geneious Pro 5.0.4 (Drummond et al., 2010). Sequences were initially aligned using default parameters on MAFFT (Katoh et al., 2009) under one of two strategies. For ribosomal genes (28S, 18S, 16S, and 12S) the E-INS-i strategy was used, which is optimized for sequences with multiple conserved domains and long gaps. Ribosomal sequences were then edited by eye with portions extracted when homology was in question. For protein coding genes (CAD, CO1, CO2, CytB, and H3) the G-INS-i strategy was used, which is optimized for global homology. Alignments were edited to ensure that codons were aligned and any gaps were in multiples of three. Data for individual genes were concatenated in Geneious Pro, with distinct coding used for missing data (?) and gaps (–).

Phylogenetic Analysis

Phylogenetic trees were constructed using Maximum Likelihood as implemented in RAxML 7.0.0 (Stamatakis, 2006) and Bayesian inference as implemented in MrBayes 3.1 (Altekar et al., 2004; Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003). In both cases each individual gene and the concatenated data set were analyzed separately, with individual protein coding genes partitioned by codon position, and the concatenated data set partitioned by gene, with coding genes further partitioned by codon position. All analyses were run using the desktop, command-line versions of the software. In RAxML the option for rapid bootstrap analysis was used in conjunction with GTR+GAMMA model of nucleotide substitution to generate 2000 bootstrap replicates, and independently search for the best-scoring maximum likelihood (ML) tree (i.e. the –f a option). jModelTest (Guindon and Gascuel, 2003; Posada, 2008) was used to select models of nucleotide substitution for implementation in

MrBayes for each partition individually, using the Akaike information criteria. Two separate MrBayes analyses were run, each with 8 chains and 10 swaps attempted every generation, sampling trees every 1000 generations. The likelihood values generated by each run were periodically graphed side by side to ensure that the values had reached an asymptote at the same value before stopping the analysis. Once the analysis was stopped, the burnin value was determined visually by graphing the likelihood values in Microsoft Excel and the last 2000 trees were selected from one of the runs for further analysis. Consensus trees representing the bootstrap consensus tree (BS) from the likelihood analysis and a posterior probability tree (PP) from the Bayesian analysis were generated in Mesquite 2.75 (Maddison and Maddison, 2011) from the trees resulting from each analysis respectively.

Trait Data

Eye Measurements: Scanning electron micrographs were taken of the eyes of 21 of the 55 in-group species, and 4 of the outgroup species (*Drosophila melanogaster*, *Musca domestica, Glossina sp.,* and *Lipoptena sp.)*. In 14 cases, specimens used for SEM were only identified to the genus level. All SEMs were completed at the South Campus Instrument Center in the SUNY Buffalo School of Dental Medicine, or at Western Kentucky University on a Hitachi S-4000 or a Hitachi SU-70 Field Emission SEM. Samples were sputter coated with gold and positioned to provide the best view of one eye of each specimen.

Measurements of each eye were completed by four individual raters, each of whom received the same training on how to use ImageJ (Abràmoff et al., 2004) to make the measurements. Because the angle at which facets were imaged differed slightly both within an eye and between samples, the maximum observable length across each facet was measured, regardless of the angle over which the measurement was made (see Figure 2 for an example). Facets that were obscured to the point that a reasonably accurate measurement of its width was not possible were removed from future analyses, as was the

area for the associated eyes. The average facet length of each eye was used for analysis. The area was measured using ImageJ by tracing a polygon around the circumference of the eye. Because area is an exponential term, the natural logarithm (ln) of the measurement was used for all analyses. For species which were either observed to lack eyes, or were noted as such in the keys used (see Facet Counts below), values of 0 were used for all eye measurements.

Facet Counts: The number of facets found in the eye of each streblid species in the analysis was determined by counting the number of facets in the SEMs of specimen when available. If specimen were not available to get direct counts, data were gathered from the literature, including several taxonomic keys, which are available for streblid bat flies (Barbous, 1910; Jobling, 1929; Wenzel and Tipton, 1966; Maa, 1971; Wenzel, 1976). If a range was given (as was sometimes true for a group of related species), the intermediate value was recorded.

Because multiple specimens of *T. frequens* were available, facet counts from 55 eyes of this species were recorded as a rough estimate of the amount of variability that could be expected for all streblid species. To account for the lack of variance due to the low sample size of other species (most of which were only represented by a single specimen), facet counts were binned conservatively by adding 1 to the natural log of the count, and rounding to the nearest whole number; species without external facets were placed in the 0 bin.

Wings: Data on the general wing morphology were determined using taxonomic keys for streblid flies (Wenzel and Tipton, 1966; Wenzel, 1976). Species without wings were coded with a 0. Species with reduced wings were coded with a 1, and species with fully developed wings were coded with a 2. For examples, see Figure 1.

Host Roost: Data on the preferred host of each species in the analysis were obtained using the bat fly database at the Chicago Field Museum (The Field Museum), or in a few cases from specific publications (see Table 1). Information on the preferred roost of each host was then obtained primarily from one of two online databases: Animal Diversity Web (Myers et al., 2011) at animaldiversity.org, and the IUCN Red List of Threatened Species at iucn.org (Table 1). Based on the information from these sources, the preferred roost of each host species was categorized as, tree or foliage, shallow cave or cave like, dark cave, and generalist. For the purpose of this study, bat flies found on hosts that roost in trees or foliage were considered to have the potential to be exposed to the most light, while those found on bats that roost in shallow caves or cave like structures (such as bridges), or are generalist, would have the potential to be exposed to intermediate levels of light, and those that parasitize bats that roost in dark caves would have the potential to be exposed to the least amount of light; roosts were coded as 3, 2, or 1 respectively.

Statistical Analyses

Correlated Trait Evolution: Tests for correlated evolution of each of the eye measurements (binned facet counts, average facet diameter, and Ln eye area) with wing morphology and host roost were conducted using the method of Independent Contrasts as implemented by PDAP (Garland Jr et al., 2005) in Mesquite. All contrasts were evaluated using both the tree from the Bayesian analysis with the highest likelihood and the maximum likelihood tree found using RAxML. Outgroup taxa and taxa for each analysis for which data were not available were pruned from the tree before each analysis. When testing the assumptions for this analysis, facet diameter was found to be significantly correlated with the square root of sum of corrected branch lengths; these measurements were natural-log transformed to remove this correlation for all analyses. Furthermore, host roost was found to violate the same

assumption for some of the topologies analyzed, so for analyses involving this parameter, branch lengths were transformed using Grafen's rho = 0.25 (Grafen, 1989).

Ancestral State Reconstructions: Ancestral states were reconstructed using both trees for each of the eye measurements using the squared change parsimony analysis as implemented in Mesquite 2.75, with confidence intervals calculated using root node reconstruction in PDAP (Maddison, 1991; Garland Jr and Ives, 2000; Garland Jr et al., 2005; Garland et al., 1993) for Mesquite.

SH Tests: Three taxa within the New World streblids (*Metelasmus pseudopterus*, *Exastinion clovisi*, *Mastoptera guimaraesi*) appear to represent independent instances of complete loss of ommatidia; similarly, three different taxa within New World streblids (*Trichobius petersoni*, *Noctiliostrebla traubi*, *Paratrichobius longicrus*), appear to represent independent instances of expansion in ommatidia number relative to the predicted state for ancestral nodes (see results, Figure 6). In each of these cases, an SH test (Goldman et al., 2000; Shimodaira and Hasegawa, 1999) was used to confirm that the taxa exhibiting loss or expansion respectively are independent (*i.e.* not monophyletic). The analysis compared 6 trees. The first tree was the ML tree discovered using RAxML, and the second was the tree from the Bayesian analysis with the highest likelihood; these two representing the hypothesis of independence. The remaining trees were found employing the same methods described above, but in each case the three taxa of interest were constrained to be monophyletic. This resulted in two trees (one from RAxML and one from Mr. Bayes) used to confirm the independence of New World taxa without eyes, and two trees (from the same programs) used to confirm the independence of New World taxa that appear to exhibit an expansion in the number of eye facets. The SH test was run in PAUP* 4.0 (Win 32/DOS Beta Version 10) (Swofford, 2003) using 1000 bootstrap replicates and the RELL approximation (Kishino et al., 1990). The likelihood model of evolution for the analysis was specified for 6 types of nucleotide

substitutions with rates drawn from 4 gamma categories, and allowing PAUP to use likelihood estimates of all other parameters (base frequencies, percent invariable sites, the rate matrix, and the shape of the gamma distribution).

Results

Sequence Alignments and Model fitting

A DNA sequence alignment was generated so it could be used to construct a phylogeny of the bat fly taxa sampled. The final DNA alignments consisted of 386 base pairs (bp) for 12s, 530 bp for 16s, 1794 bp for 18s,754 bp for 28s, 755 bp for CAD, 776 bp for CO1, 678 bp for CO2, 389 bp for CytB, and 354 bp for H3, resulting in a total of 6416 bp in the concatenated alignment. The second codon position of H3 was found to be almost completely invariable; results from jModelTest therefore suggested a model using 1 type of substitution for this partition. For all other partitions a model with 6 types of substitutions was implemented, with the rates parameter set either to *invgamma* (12S, 162, 18S, the first position nucleotides of CO2, and the second position nucleotides for CAD, CO1, CO2, and CytB), or *gamma* (28S, the first position nucleotides for CAD, CO1, CytB, and H3, the third position nucleotides for CAD, CO1, CO2, CytB, and H3).

Phylogenetic Analysis

Phylograms were constructed both to analyze the evolutionary relationships of the bat fly species sampled, and for further hypothesis testing. A phylogram of the best tree found by MrBayes is depicted in Figure 3. The BS and the PP trees are compared in Figure 4. Major clades in the trees have been numbered to facilitate discussion: OG1 is the outgroup containing the more distantly related dipterans, OG2 is the outgroup containing other members of the superfamily Hippoboscoidea. Streblidae clades are numbered S1 through S13. All trees are numbered with the same 15 clades.

For the PP tree, nodes were considered to have statistically significant support if the posterior probability was 95% or above; for the BS tree, nodes were considered to have statistically significant support if the bootstrap value was 70% or greater (Hillis and Bull, 1993). All non-significant nodes were collapsed in the BS tree unless they were found to be significant in the PP tree, and vice versa (e.g. clade S13 in the PP tree and clade S7 in the BS tree). In an effort to avoid a biased interpretation depending on how the branches are arranged, the phylogram in Figure 3 has been ladderized so that clades with fewer taxa are found to the top relative to each node. Within each of the numbered clades, the nodes have been rotated so that when taxa could be ordered without altering their relationships, the taxa with the largest number of facets are found on the top, with eye area breaking a tie when applicable. Nodes have been rotated in both the PP and BS tree to match the ordering of taxa in Figure 3; that this was possible speaks to the considerable agreement between the two trees.

The differences between the two trees was relatively few in number. In both trees, all bat flies in the analysis formed a monophyletic group. Within the bat fly clade, *Megastrebla parvior* and *Brachyotheca lobulata* (both Old World Streblidae)(S1) were in a sister relationship to all bat fly species, including the only other Old World bat fly in this analysis, *Ascodipteron sp.*, found in clade S2. The node supporting the sister group to *M. parvior* and *B. lobulata* was strongly supported in the PP tree (100%), but falls below of the determined level of significance for the BS tree (64.7%). In both trees all New World bat flies except one formed a monophyletic clade joining S3 through S13; the exception being *Nycterophilia parnelli*, which forms a clade (S2) with the *Ascodipteron sp.*.

Within the New World clade (S3-S13), the PP tree differed from the BS tree by the inclusion of three additional nodes: Whereas clades S4-13 formed a large polytomy in the BS tree, the PP tree showed

statistically significant support for a grouping of clades S8-S13 (a polytomy), S7-13, and S5-13 (S5, S6, and S7-13 forming a polytomy). The trees also differed slightly in the arrangement of the tips within clades S3 and S5.

Also within the larger New World clade, *Trichobius species* were scattered throughout the New World phylogeny in a polyphyletic manner, interspersed with multiple groupings of and with other genera, including *Strebla spp.*. Strebla also formed a paraphyletic clade due to the inclusion of *Paraeuctenodes sp*. and *Metalasmus pseudopterus*.

Trait Data

Data on eye morphology, wing morphology, and ecological factors that could be used to examine evolutionary patterns, and test hypotheses of phylogenetic correlation were generated using SEM, and by gathering data available from the literature. To provide a sense of how bat fly eye diversity is dispersed across their phylogeny, and to facilitate their analysis, micrograph images of selected taxa have been displayed relative to their phylogeny (Figure 5).

Measurements of eye area revealed a large range of values for streblid bat flies, spanning from largest eye of *M. parvior* at 23,208µm^2 to 852µm^2 for *T. parasiticus*, the streblid fly with the smallest area; there are also a number of streblid species that lack eyes altogether (0µm^2) (Table 1 and Figure 6). All of the streblid eyes are markedly smaller than the dipteran outgroups, which ranged from 89,661µm^2 for *D. melanogaster* to 1,557,740µm^2 for *M. domestica* (Table 1 and Figure 6). Note that measurements of eye areas for the outgroup flies are underestimates because they were calculated from a single image without taking into account the curvature of the eye as it wraps around the head.

As an approximation of the amount of variability that could be expected in the number of facets for each species, the number of facets in 55 *T. frequens* eyes were enumerated. The majority of eyes possessed 8 facets, but 57% of specimen exhibited a small degree of fluctuating asymmetry (different numbers of facets in their left and right eyes). The average count for all eyes was 7.963 with a standard deviation of 0.962 and a 95% confidence interval of 8.218 to 7.710. The maximum number of facets counted for one eye was 11 and the minimum was 5. When the formula for binning the number of facets is applied to these maximum and minimum values, the result is 3.398 and 2.609 respectively, both of which round to 3 (the bin value that would be used for analysis). These data, along with the fact that they are nearly identical to that reported elsewhere for *T. major* (Zeve, 1958), suggest that the binning method used is an effective means of conservatively accounting for the lack of variance in the data due to low sample size of the other species in this study.

The majority of Streblidae were found to have facet numbers, which fell into bin 3 (5-12 facets); however those with less than 5 facets (12 taxa in 7 clades) were placed into bins 0, 1 or 2. Additionally, two taxa were placed in bin 4 (13-33 facets) and one in bin 5 (34-90 facets) (Table 1 and Figure 6).

The eye of the Megastrebla species, *M. parvior*, presented a unique case. It appeared superficially to have a single external lens, and therefore one facet; however, if treated as a single facet, the average width was nearly 4 times larger than that found in the next largest streblid eye. This placed it well outside the range of values for all other streblid species. Additionally the total eye area was nearly 10 times larger than the next largest eye of *Joblingia* sp., which possessed 8 facets with an average width of 36.8µm (Table 1 and Figure 6).

The facets of all streblid eyes, except for *M. parvior* (and those which appear to be vestigial) were distinct, sometimes having a small space in between them, and were highly rounded on the surface, nearly hemispherical (Figure 5). The facets of *Drosophila spp.* and *Musca spp.* were also rounded on the surface, but not nearly as much as those of the streblid flies. On the other hand the individual facets of *Glossina palpalis* (Glossinidae; Figure 5A) were almost perfectly flat and the individual facets of *Lipoptena cervi* (Hippoboscidae; Figure 5B) were barely discernible, with no clear boundary between them.

Based on these observations it was concluded that the eyes of *M. parvior* are categorically distinct from the rest of Streblidae. Because of the *M. parvior* data for the number of facets and average facet diameter were excluded from further analysis; however, the total eye area was used because the area of the eye was considered comparable to other bat fly eyes regardless of the structure of the individual facets.

The species with the next largest facets were *Nycterophilia parnelli* (52.72µm; Figure 6E) and *T. caecus* (46.9µm; Figure 6F). Even though the facets of these two species were at the extreme large end of the streblid distribution, they were not unambiguously out-lying; they were therefore included in subsequent analyses as having 1 facet; it is also noted that the facet of *N. parnelli* was irregularly shaped, appearing more like an oblong fold of tissue. This makes the facets of *N. parnelli* and *T. caecus* the only streblid facets that were outside the range between *G. palpalis* and *M. domestica* for facet width.

Statistical Analyses

Correlated Trait Evolution:

Phylogenetic contrasts were conducted to determine if measures of eye size are correlated with either host roost and/or wing morphology. All three eye measurements were found to be significantly positively correlated with wing morphology in both trees (Table 4, Figure 7); as a measure of the strength of this correlation, the r^2 value for these analyses range from 0.29 to 0.41. The number of facets was also found to be significantly positively correlated with the host's roost in the phylogeny resulting from the Bayesian analysis, but, the same analysis using the ML tree produced by RAxML, was shy of traditional levels of significance (p=.079) (Table 4, Figure 7). Neither the average diameter nor ln area were found to be significantly correlated with roost. The r^2 value for these analyses was much smaller, ranging from 0.040 to 0.11 (Table 4, Figure 7).

Ancestral State Reconstructions:

To assess whether or not reduction has been an ongoing process in bat fly evolution , the 95% confidence interval of the ancestral state for each of the three eye traits measured were reconstructed and used to predict the eye morphology observed currently at multiple key nodes in each of the phylogenies: the common node for all Streblidae (S1-S13), the common node for the monophyletic New World Streblidae (except *N. parnelli*, which grouped with Old World Streblidae in this analysis) (S3-S13), and the common node for each of the major in-group nodes previously identified (S1, S2, etc.) (Tables 5 and 6). In cases where clades formed a polytomy at the node, the value was reconstructed for the node forming the polytomy and not each clade individually.

The ancestral state for the number of facets was found to be around bin 4 (13-33 facets) for all of Streblidae and around bin 3 (5-12 facets) for the New World clade (Figure 6). Estimates of the character

value for the numbered clades were generally found to be lower; the 95% confidence intervals were found to be from bin 1 to 3 (1-12 facets). This suggested a general trend towards reduction of the number of facets, but there were several species scattered throughout the phylogeny that fall outside the confidence intervals, and therefore differ significantly from what is expected. This was true for the 5 species (*Brachyotheca lobulata*, Ascodi*pteron sp.*, *Metelasmus pseudopterus*, *Exastinion clovisi*, and *Mastoptera guimaraesi*) from 5 different clades (S1, S2, S5, S9, and S10) in this analysis that lack eyes, but was also true for 7 other species (*N. parnelli*, *T. yunkeri*, *T. caecus*, *T, johnsonae*, *T. galei*, *Paradysheria lineata*, and *P. parvuloides*) from 3 clades (S2, S3, and S11) that possess a reduced number of facets relative to the average. There were also 3 species (*T. petersoni*, *Paratrichobius longicrus*, and *Noctilostrebla tr*aubi) from 2 of the numbered clades (S7 and S11) that exhibited a significant increase in the number of facets relative to what was expected at the common node for their clade, for New World species, or both. The independence of these instances of greater reduction or expansion within the New World streblids was further analyzed using an SH test (see below).

Interestingly, the typical facet diameter for Streblidae (between 10 and 30 um) was no different from that found in *M. domestica* and *D. melanogaster*. However, there were a number of streblid species that fell outside of the 95% confidence interval for at least one of the relevant nodes analyzed (Figure 6). This included significant enlargements in *N. parnelli* and *T. caecus*, as well as 6 other species (*Jobliniga schmidti*, *Anatrichobius scorzai*, *Pseudostrebla ribeiroi*, *Speiseria ambigua*, *S. peytonae*, and *T. frequens*) (representing five clades total: S2, S3, S4, S12, and S13), though the latter 5 of these was only outside the confidence interval for one of the trees analyzed. Additionally, the 5 species without facets (and therefore with measurements of zero microns in diameter), were found to be significantly below the confidence interval.

The 95% confidence intervals of the reconstructed values for ln eye area were notably large: The PP tree estimated the root of all Streblidae to be between 5.52 and 13.24 (251µm^2 and 560,091µm^2); the BS tree estimated these values to be 4.60 and 13.13 (99 µm^2 and 502,033 µm^2). The large confidence intervals result from the huge disparity in size between the outgroup measurements and those of Streblidae. Given this, even though the eyes of *M. parvior* was demonstrably larger (10 times so) than any of the other streblid eyes, their eye's area was not found to be outside the wide confidence interval for any of the relevant reconstructed nodes. The confidence intervals narrowed further up the tree, and had a trend toward reduction; the highest node estimated (for clades S8-S13) had a confidence interval between 4.20 and 8.45 (66-4,691µm^2) in the PP tree and between 1.73 and 8.77 (6-6,433µm^2) in the BS tree. Thus 2 taxa (*Paratrichobius longicrus* and *Pseudostrebla riberoi*)) were found to have significantly larger ln eye area relative to the node for their numbered clade, but only when using one or the other of the trees.

SH Tests:

SH tests were used to assess whether or not the inferred instances of expansion or hastened reduction within the New World streblids were independent. While the SH test revealed no significant difference between the Bayesian tree with the highest likelihood and the ML tree found using RAxML. However, topologies where New World streblids without external facets were constrained to be monophyletic, to test for independent instances of expansion and reduction, were significantly poorer, as were topologies in which the New World streblids with a larger number of facets were similarly constrained (Table 3).

Discussion

Phylogenetic Considerations

The results of the phylogenetic analysis presented here are in agreement with Peterson et. al. (2007) in that bat flies were found to form a monophyletic group, though the Peterson study included a relatively small number of bat fly species. The same result was found in the Bayesian maximum a-posteriori tree in Dittmar et. al. (2006) (Figure 8b), but not in the other two analyses presented in that paper (parsimony consensus and maximum likelihood), each of which found bat flies to be paraphyletic by the inclusion of Hippoboscidae and Glossinidae.

The current analysis also differs slightly from Dittmar et. al. (2006) in that it suggests a slightly different relationship between Old World and New World bat flies. In the 2006 analysis, Old World bat flies and New World bat flies formed separate monophyletic clades. While the current analysis yields strong support for a large New World clade (clades S3-S13), one New World streblid not included in the 2006 analysis, *Nyctrophylia parnelli*, groups with the Old World *Ascodipteron sp*., which in the 2006 analysis groups with the predominately Old World bat fly family Nycteribiidae (*Basilia spp.* being the only New World Nycteribiid). If bat flies originated in the Old World, as suggested by Dittmar et. al. (2006), then this suggests that the arrival of Nycterophilia in the New World represents a separate dispersal event for Streblidae.

Moreover, the current analysis suggests that the Old World *M. parvior* and *B. lobulata*, are sister to a clade consisting of *N. parnelli*, *Ascodipteron sp.*, perhaps Nycteribiidae (due to their association with Ascodipteron in Dittmar et. al. 2006), and the larger clade of New World bat Streblids. If this arrangement is correct, then rather than Old World and New World clades being separate, the New World species branch from within the Old World clade.

With respect to the larger New World clade, these results are in strong agreement with Dittmar et. al. (2006), in that the genus *Strebla* (green text in the tree figures) is found within the genus Trichobius (blue text). However, these trees show *Trichobius species* scattered throughout the New World phylogeny in a polyphyletic manner, mingled with multiple groupings of and with other genera. *Strebla* also forms a paraphyletic clade due to the inclusion of *Paraeuctenodes sp*. and *Metelasmus pseudopterus*.

To some extent, where the current analysis differs from Dittmar et. al. (2006), can be explained by the fact that the basal nodes in Dittmar et al. (2006) lacked statistically significant support (red branches in Figure 8). If the unsupported nodes are collapsed, relevant nodes can be rotated to present less resolved trees that are consistent with each other, and more similar to the current results. When each of the trees from Dittmar et. al. (2006) is thus rearranged (Figure 8d), the resulting trees no longer contradict the potential monophyly of all bat flies. However, with the exception of the maximum a-posteriori tree, the trees still show all Old World bat flies in a sister relationship to all New World species.

While the current analysis suffers relative to the Dittmar et. al. (2006) analysis in that that it does not include the same number of Old World species, it includes some important new species, such as *N. parnelli*. It also adds 5 genes to the 4 that were used in the 2006 analysis. The questions raised by the current analysis, will likely require additional data to be resolved.

Phylogenetic Patterns of Eye Evolution

Fly ancestors to living bat fly species most likely had large eyes with many facets, similar to those of Tsetse flies or most Hippobsocid flies. As with other obligate ectoparasites such as fleas (Benton and

Lee, 1965; Taylor et al., 2005) and lice (Patterson et al., 2007) the shift toward this lifestyle probably meant reduced reliance on visual input, leading to the evolutionary reduction of their eyes. Though drift alone could be sufficient to explain reduction and intra-familial variation, it is possible for selection to affect the process. The analysis of phylogenetic patterns has allowed the identification of potential roles for selection acting in different branches within streblid bat flies.

If the shift towards obligate ectoparasitism eliminated the need to rely on visual input for survival and reproduction, then selection would not act to remove individuals from populations with mutations adversely affecting eye development and function. This removal of purifying selection would lead inexorably to a degradation of eye form and function as these mutations were passed to future generations and moved to fixation in the population. The effect of this process acting within bat fly lineages is evident in species whose eyes appear to be vestigial, and in species that have no visible external eyes. Even though not necessary for this outcome, it is possible that selection could favor individuals with these deleterious mutations, thus hastening their fixation in the population and leading more quickly to the vestigilization or elimination of eyes. The analyses presented here does not allow a determination of which of these possibilities is more likely, but given that eyes are generally considered to be expensive, both for their development and function (Lahti et al., 2009; Buschbeck and Friedrich, 2008), individuals with mutations adversely affecting these processes may have had an energetic advantage leading to their selection. The presence of species in the phylogeny with fewer facets than is predicted by the confidence interval reconstructed for their ancestral nodes could be an indication of hastened elimination through selection (Figure 6).

It is possible that stochastic differences in the rate of reduction could be responsible for the variation in eye morphology found in different bat fly species. If this was the case, one would expect that the

variation in eye parameters such as the number of facets would be random. However, each of the eye parameters measured (number of facets, size of individual facets, and eye size) were significantly correlated with wing morphology (Table 4 and Figure 7), (which has itself been reduced several times in bat fly phylogeny (Figure 6). Furthermore, based on the r^2, about 33% of the variation in eye morphology can be explained by variation in wing morphology. This suggests that visual ability has continued to provide a significant advantage for flight navigation. This further indicates that eye reduction in these lineages has taken place under relaxed but persistent purifying selection. The effect of this process acting within bat fly lineages may be evident in the majority of bat fly lineages, where reduction appears to have halted at an area around 1,000 to 3,000μm^2 with between 5 and 12 facets.

Notwithstanding the correlation between eye and wing morphology, it cannot be concluded that visual abilities are an indispensable requirement for flight navigation. Bat fly species such as *B. lobulata* and *N. parnelli* possess well developed wings, yet either lack eyes entirely or possess only vestigial remnants. Inasmuch as it has been determined that *D. melanogaster* use other senses, such as odor and CO_2 detection, for flight navigation (Duistermars and Frye, 2008; Frye, 2010; Frye et al., 2003; Stewart et al., 2010; Wasserman et al., 2013), within bat flies it is possible that accompanying the reduced reliance on visual stimuli could be an increased reliance on senses such as olfaction and mechanoreception. For bat fly species without functional eyes that have fully formed wings, this reliance on other senses must be total.

It was also hypothesized that eye morphology might be correlated with roosting conditions of their primary host. Because the only eye parameter found to be significantly correlated with roosting conditions was the number of facets, and that only when using the best tree from the Bayesian analysis (Table 4 and Figure 7), the effect of roosting condition on eye evolution cannot be substantiated. Thus

Maa's assertion that bat fly eye morphology is correlated with the roosting conditions of their host cannot be confirmed (Maa, 1971).

Even though exposure to light is a prerequisite for selection favoring eye function, the results of the above analyses indicate that this alone is insufficient to maintain eye development and function unless coupled with some utility such as flight navigation. This suggests a general evolutionary paradigm where bat fly species that have lost their reliance on flight tend to lose their wings, and with the loss of utility for their eyes, have tended also to lose their eyes, even when their hosts roost in locations where they are likely to be exposed to light. At the same time, bat fly species that rely on flight even though their hosts' roosts are unlit (such as dark caves), and are not advantaged by flying to locations where they can be exposed to light, experience purifying selection that maintains their wings, but not their eyes. Therefore, generally speaking, for eyes to be maintained, bat flies would have to depend on flight and either infest hosts that reside in lighted roosts or, if their hosts reside in dark roosts, fly to locations (such as near a cave entrance) where sensitivity to light could provide sufficient advantage to maintain eyes in the population through purifying selection. It is also possible that benefits of visual abilities other than flight navigation could result in sufficient pressure to maintain eyes, but these have not been explored in this study.

Notwithstanding the generally reduced state of bat fly eyes relative to other dipterans, there are at least three independent cases where there has been a secondary expansion in the number of facets. Again considering that eyes are generally thought to be expensive, it is unlikely that expansion would occur randomly within a population. Thus unlike the other scenarios described above, selection must have acted in these lineages in favor of individuals with larger eyes. This finding highlights the advantage of studying reduction in the context of their phylogeny, because if the eyes of these individuals were to be

studied in isolation, the secondary expansion of their eyes after having been initially reduced would almost certainly not be discernible.

One final observation yielding insight into the potential role of selection in bat fly eye evolution is the shape of their external facets. *Glossina sp*. (Figure 5A) and *Lipoptena sp*. (Figure 5B) (representing dipteran families closest to bat flies) both have eyes comprised of many hundreds of facets, the lenses of which are relatively flat. In *Lipoptena sp*. the lenses have the additional feature that the borders between individual lenses are barely discernible. On the other hand, the lenses of most bat fly species in this study are distinctly rounded, nearly hemispherical, and the borders between them are distinct (Figure 5). Similarly hemispherical lenses have been observed in nocturnal mosquito species, where the shape helps to make the eyes more sensitive to light by increasing the angle over which light is gathered for each facet (Kawada et al., 2006; Land et al., 1997; Land et al., 1999; Warrant, 1999). If the shift in lens shape in bat fly species plays a convergent role, then concomitant to the reduction in the number of facets and overall size of the eye, the lenses of individual facets was remodeled in some lineages to make each unit more sensitive to light. The possibility that some bat fly eyes may be remodeled to enhance sensitivity of individual ommatidia is somewhat paradoxical because the process of eye reduction is generally thought to diminish function.

One clear exception among bat fly species to the shape of their external facets is the eye of *M. parvior*. The eyes of this species were excluded from analyses regarding individual facet counts because even though multiple individual facets are not discernible, the eye is too large to be a single ommatidium. One scenario that could explain the appearance of their eyes is that they are comprised of multiple ommatidia, and that the lenses of each are relatively flat and fused at the borders so that the distinction between them cannot be discerned upon external examination. This possibility is supported by the fact

that, after excluding *M. parvior*, a narrow range for facet size among all species sampled was found. Thus Jobling's (1929) earlier report that the eyes of Megastrebla spp. are "unfaceted" (Jobling, 1929) is to be preferred over Maa's (1971) later report that they have 1 large facet per eye, even though they are most likely comprised of multiple ommatidia. Inasmuch as the structure of *M. parvior's* eyes is similar to *Glossina sp.* and *Lipoptena sp.* (only more extreme), it is possible that *M. parvior's* eyes may be plesiomorphic.

Because hemispherical lenses appear to be a general feature of New World streblids, but are not found in *M. parvior*, which is an Old World streblid, selection favoring this feature may have acted on early bat fly lineages that gave rise to New World streblids. However, some Nycteribiids (the second bat fly family) also have lenses that are nearly hemispherical in appearance (personal observation), suggesting that either similar selective pressures were experienced independently in the Nycteribiid linage, or were experienced in even earlier lineages that gave rise to both Nycteribiids and New World streblids. An analysis including an array of both Old and New World streblids that can clearly resolve the early nodes of bat fly evolution is needed to answer this question.

In summary, this analysis has allowed the identification of the nuanced ways in which selection can affect an organ while it is being reduced. It is evident from this analysis that selection can act independently on distinct aspects of the same organ, and that the effects can differ between closely related lineages. Thus even while bat fly eyes were experiencing reduction in size generally (which may have been hastened by selection for the reduced form) selection appears to have been concomitantly acting on some early lineages to modify the structure of the lens, and perhaps the whole ommatidium itself. Furthermore, while selection may have been acting in some lineages to bring about the vestigilization or elimination of eyes, persistent purifying selection acted in other lineages to limit the

extent of reduction to a few facets, while in other lineages it acted secondarily to actually expand the eye after having been initially reduced. Because these finding would have been difficult to ascertain otherwise, these results highlight the advantage of considering extant variability in reduced forms within a phylogenetic context for understanding the history and process of evolutionary reduction.

Figure Captions for Chapter 2

Figure 1: Representative Bat Fly Wing Morphologies

Example bat fly species with fully formed and reduced wings: A-D are fully formed. E-F are reduced. No species are shown that lack wings. A) *Megastrebla parvior*; B) *Strebla christinae*; C) *Trichobius s*p; D) *Nycterophilia parnelli*; E) *Aspidoptera d*elatorei; F) *Megastopoda araeana*.

Figure 2: Example of Eye Measurements

Illustration of how eye measurements were made. The number of facets per eye was enumerated prior to measurements being made so each rater could identify a specific facet by sample and facet number. The width of each facet was measured as its maximum observable length (dashed lines). In this photo, facets 4, 5, and 6 are excluded from analysis of facet width because the bristle in the foreground prohibits a reasonably accurate measurement; otherwise the average width of all facets for a specimen was used for analysis. The area was measured by tracing a polygon around the circumference of the eye (solid line). Because not all facet widths can be measured in this eye, the area for this specimen is also excluded from further analysis.

Figure 3: Streblidae Phylogenetic Tree

Phylogram showing the best tree found during MCMC search by MrBayes – the best unconstrained trees used for SH tests (Table 3). The scale bar is in units of expected change per site. Node labels are as follows. OG1 (Out Group 1): more distantly related dipterans; OG2 (Out Group 2): Representatives of two families of flies (Glossinidae and Hippoboscidae) which are in the same superfamiy as bat flies (Hippoboscoidea); S1: Old world Streblidae; S2: Old world Brachyotheca (which also groups with old world Nycteribiidae (Dittmar et al., 2006)) and new world Nycterophilia; S3-S13: New world Streblidae.

The names of selected taxa have been colored as follows: OG1: red; OG2: orange; Trichobius congeners: blue; Strebla congeners: green. The same node labels and taxa colors are used in all figures. The tree was laderized top-to-bottom, and nodes at the tips have been rotated so that taxa appear in decreasing order of the number of facets and eye size.

Figure 4: Comparison of Posterior Probability and Bootstrap Trees

Comparison of the Posterior Probability (PP) tree generated using MrBayes and the Bootstrap (BS) tree generated using RAxML. All nodes not found to be significant in at least one of the trees have been collapsed. The cutoff values were 95% and 70% for the PP and the BS trees respectively. Nodes have been rotated to match the order of taxa in the phylogram represented in Figure 3. Node labels and text colors are equivalent to those described for Figure 3.

Figure 5: Streblidae Eye SEMs

Scanning Electron Micrographs (SEMs) of the eyes of selected taxa used to obtain eye measurements (number of facets, facet diameter, and eye area). Taxa in the tree are in the same order as in all other tree figures (clockwise = top-to-bottom); node labels and text colors are equivalent to those described for Figure 3. SEMs have been arranged to correspond to the order of taxa in the tree, beginning at the top left and moving clockwise. Letters at the tips of the tree correspond to the letters at the bottom left of each picture before the species names. SEMS were taken at different magnifications; which of the scale bars found under the tree applies to each SEM is indicated by the number in the top left of each SEM. Image D, Q, and AB are not included in the tree. D is a species known to be related to *Megasterbla sp.* (C) (Dittmar et al., 2006); Q is another example of a *Paratrichoius sp.* with well-formed eyes; AB is an *Ascodiptera sp*. with vestigial remnants of external facets.

Figure 6: Data Graphs

Graphical representations of data used for tests of correlated evolution and ancestral state reconstructions. Taxa are ordered left to right as in all other graphical tree representations. The PP tree is shown without support values. Wing and host roost categories are indicated at the top of each taxa name. For wings M = full, B = reduced, and A = absent. For roost T = tree or foliage, G = generalist, L = lighted caves, and D = dark caves. Ancestral states for the three eye parameters found using both the PP and the BS tree are indicated by the tables associated with the nodes for the common ancestor of all Streblidae (S1-S13) and the common ancestor for the major New World Streblidae clade (S3-S13); the ancestral state for additional nodes are found in tables 5 and 6. In each of the three graphs, a diamond was used by default to mark the value found for each taxa. If the tip value was found to be significantly different (outside the 95% confidence interval) from the value determined for any of the relevant nodes a partitioned, color coded circle was used to indicate from which nodes and in what tree the value was significantly different according to the key in the figure.

Figure 7: Plots of Phylogenetic Contrasts

Plots generated by the PDAP module (Garland Jr et al., 2005) implemented within Mesquite (Maddison and Maddison, 2011). Axes of all graphs are indicated as "Y-axis x X-axis" in the title above each graph. The black line is the ordinary least squares regression. The red line indicates the reduced major axis. The green line indicates the major axis line. Plots a-c show the results of contrasting the respective eye parameters (Y-axis) with Roost type using the best tree generated by Bayesian analysis. Plots d-f show the same, but use the maximum likelihood tree found using RAxML. Plots g-i show the results of contrasting the respective eye parameters with bat fly wing morphology using the best tree generated by Bayesian analysis. Plots j-l show the same, but use the maximum likelihood tree found using RAxML.

Figure 8: Agreement Of Dittmar 2006 Trees with The Current Results

Comparison of the trees found in Dittmar et. al (2006). Red nodes in a-c indicate nodes below the levels considered herein to be statistically significant. Tree d is a consensus of a-c with the non-significant nodes collapsed and taxa rotated to match phylogenies presented in this paper; the blue node is not supported in the parsimony consensus tree and the green node is not supported in the maximum a posteriori tree.

Figures for Chapter 2

Figure 1: Representative Bat Fly Wing Morphologies

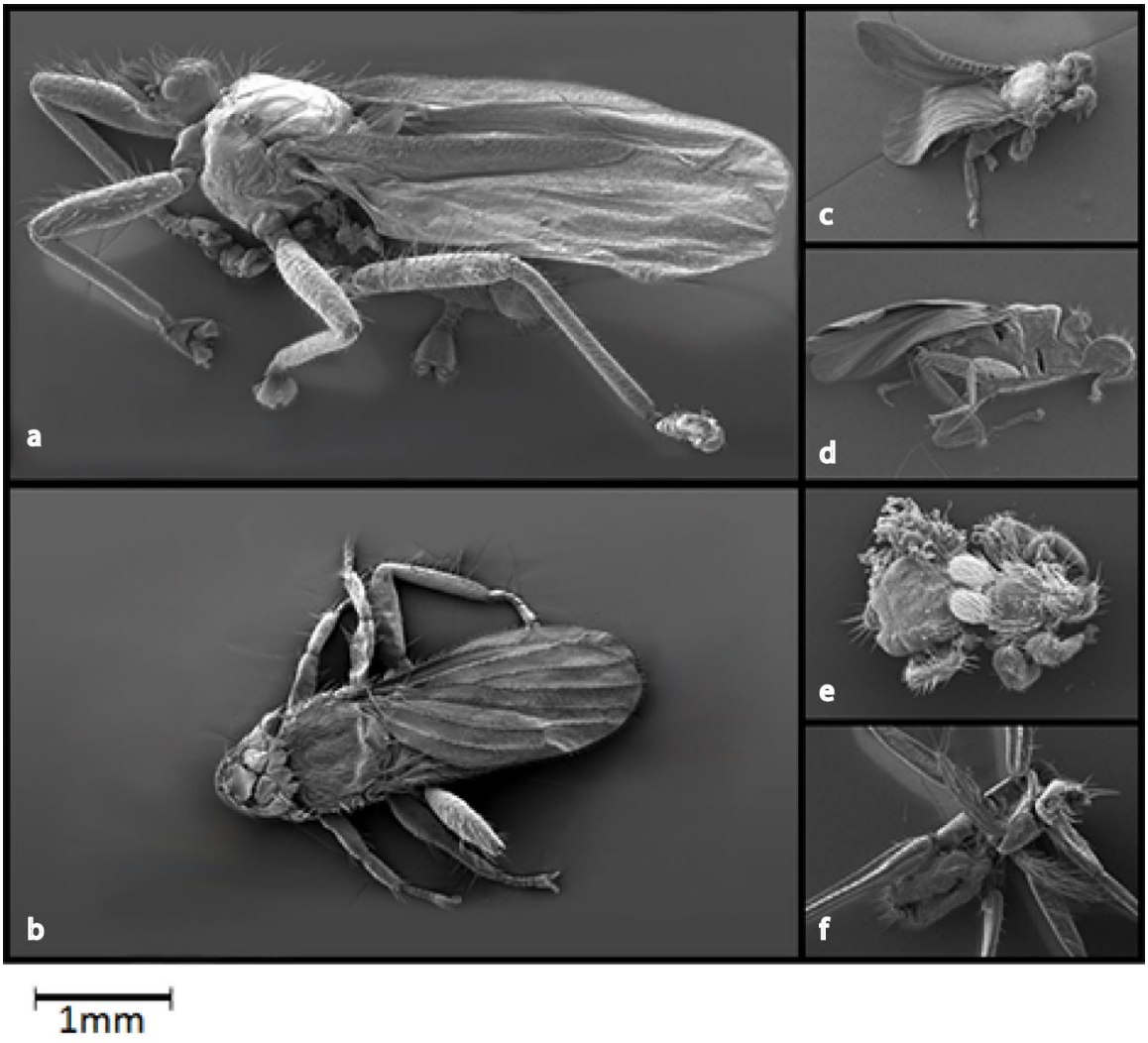


Figure 2: Example of Eye Measurements

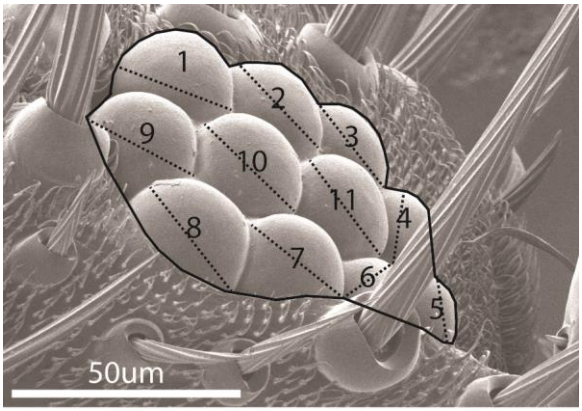


Figure 3: Streblidae Phylogenetic Tree

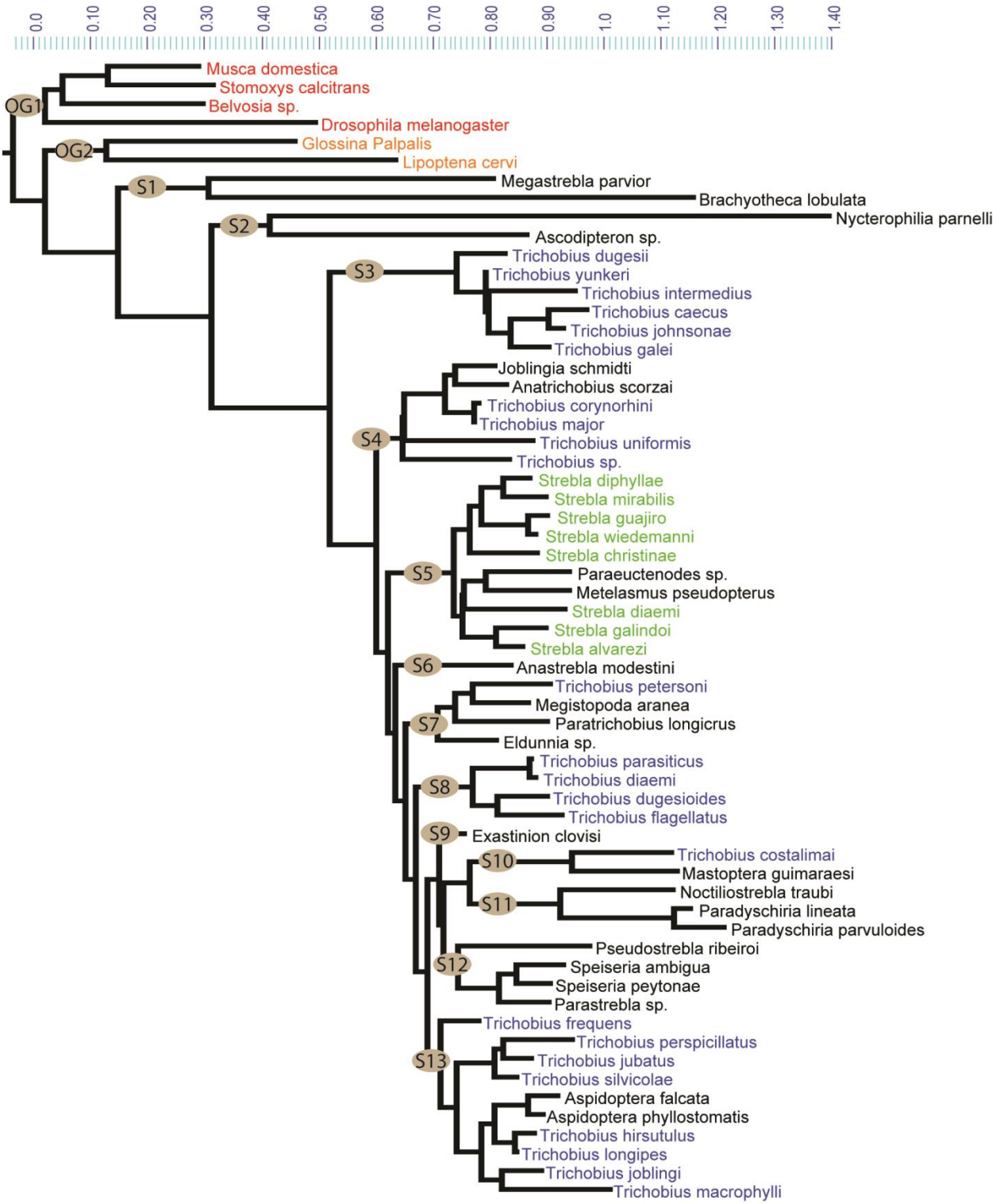


Figure 4: Comparison of Posterior Probability and Bootstrap Trees

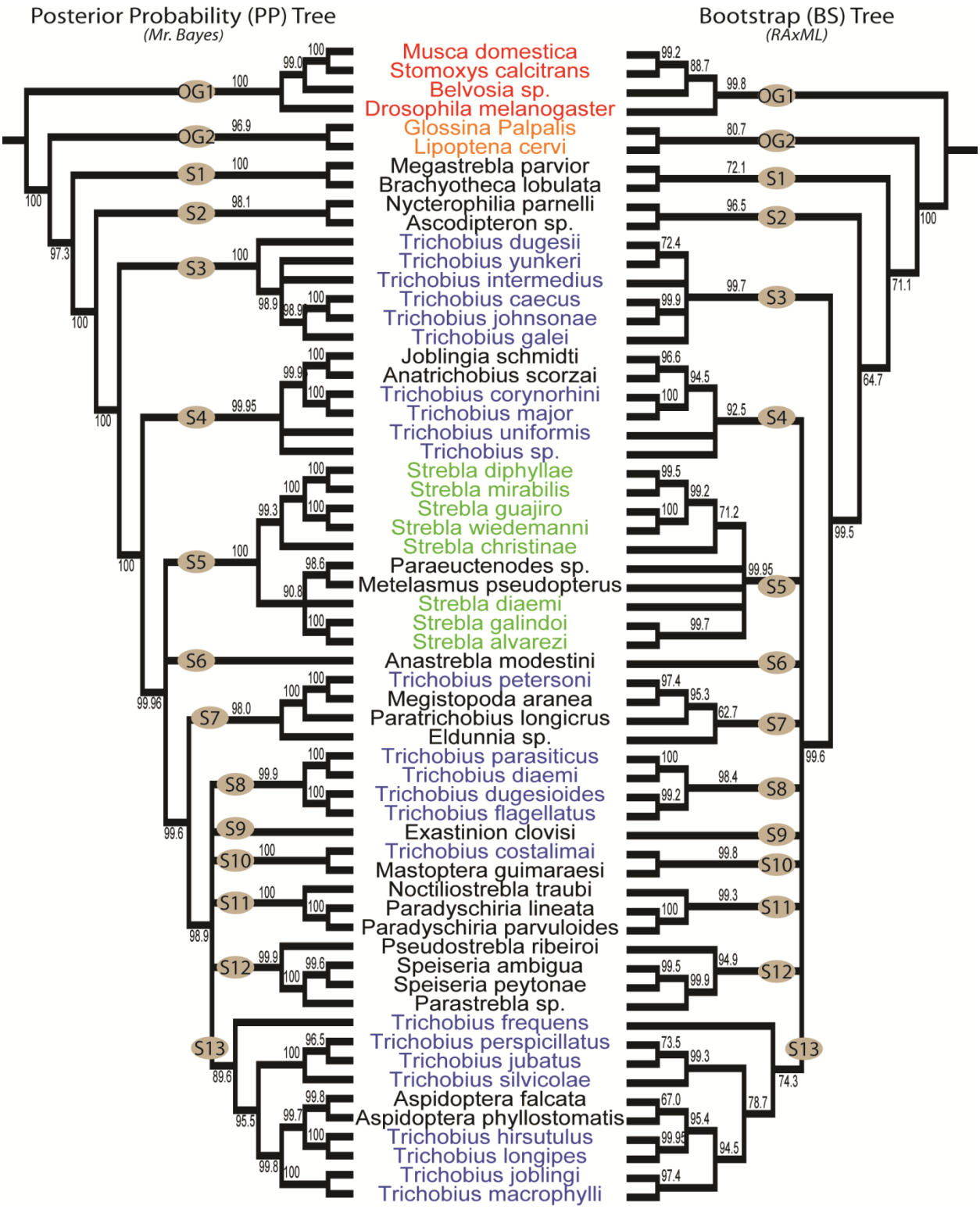


Figure 5: Streblidae Eye SEMs

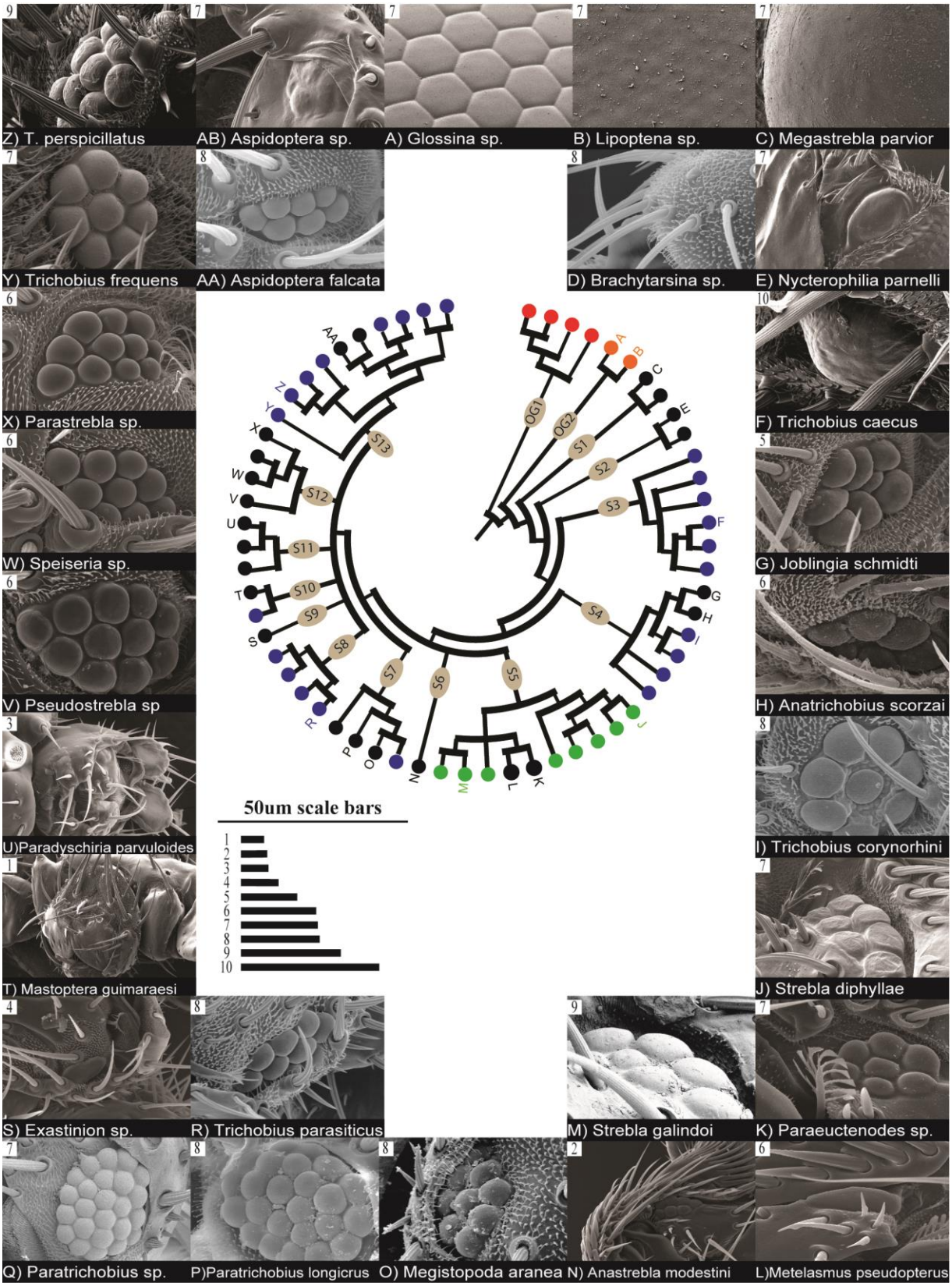


Figure 6: Data Graphs

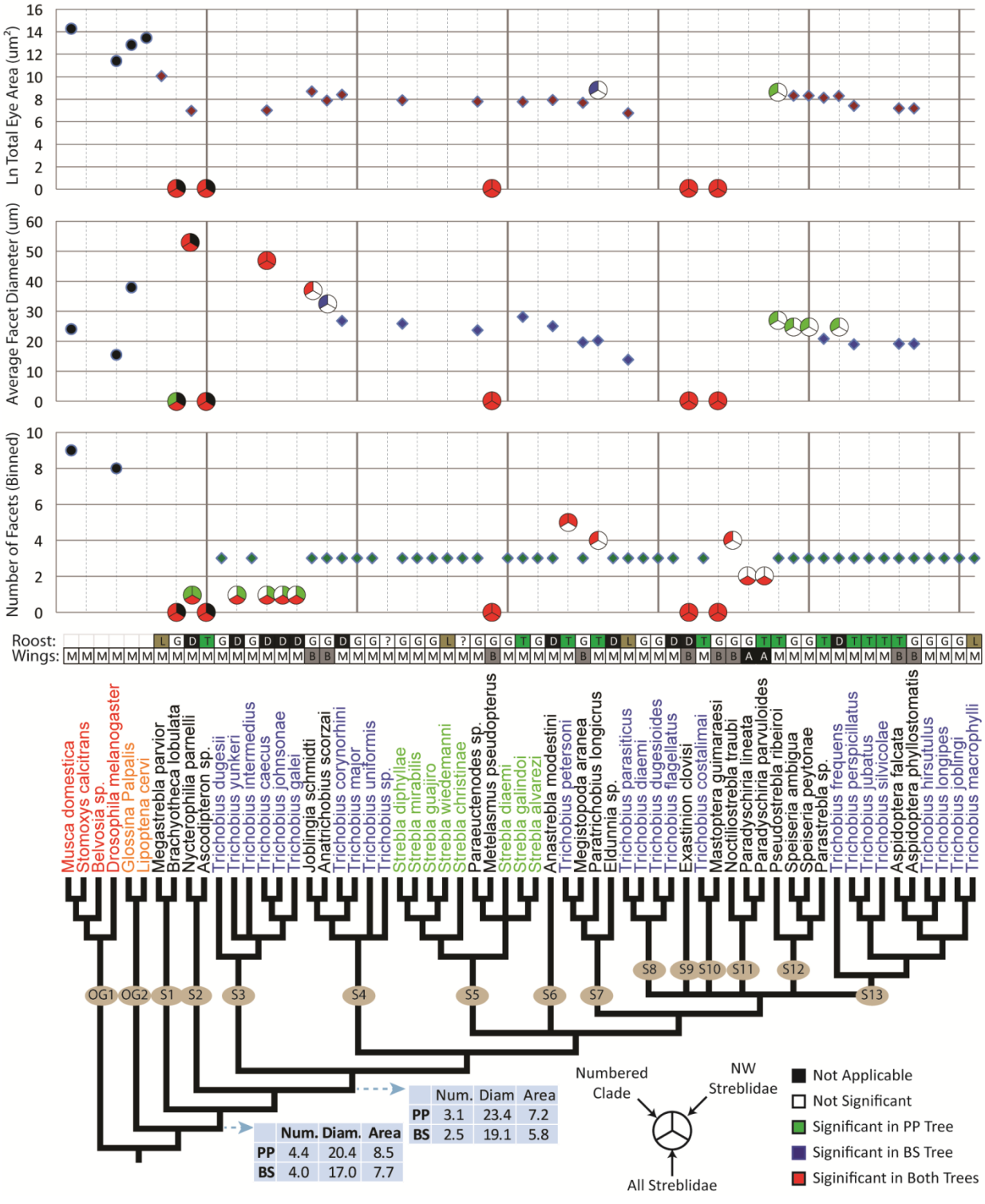


Figure 7: Plots of Phylogenetic Contrasts

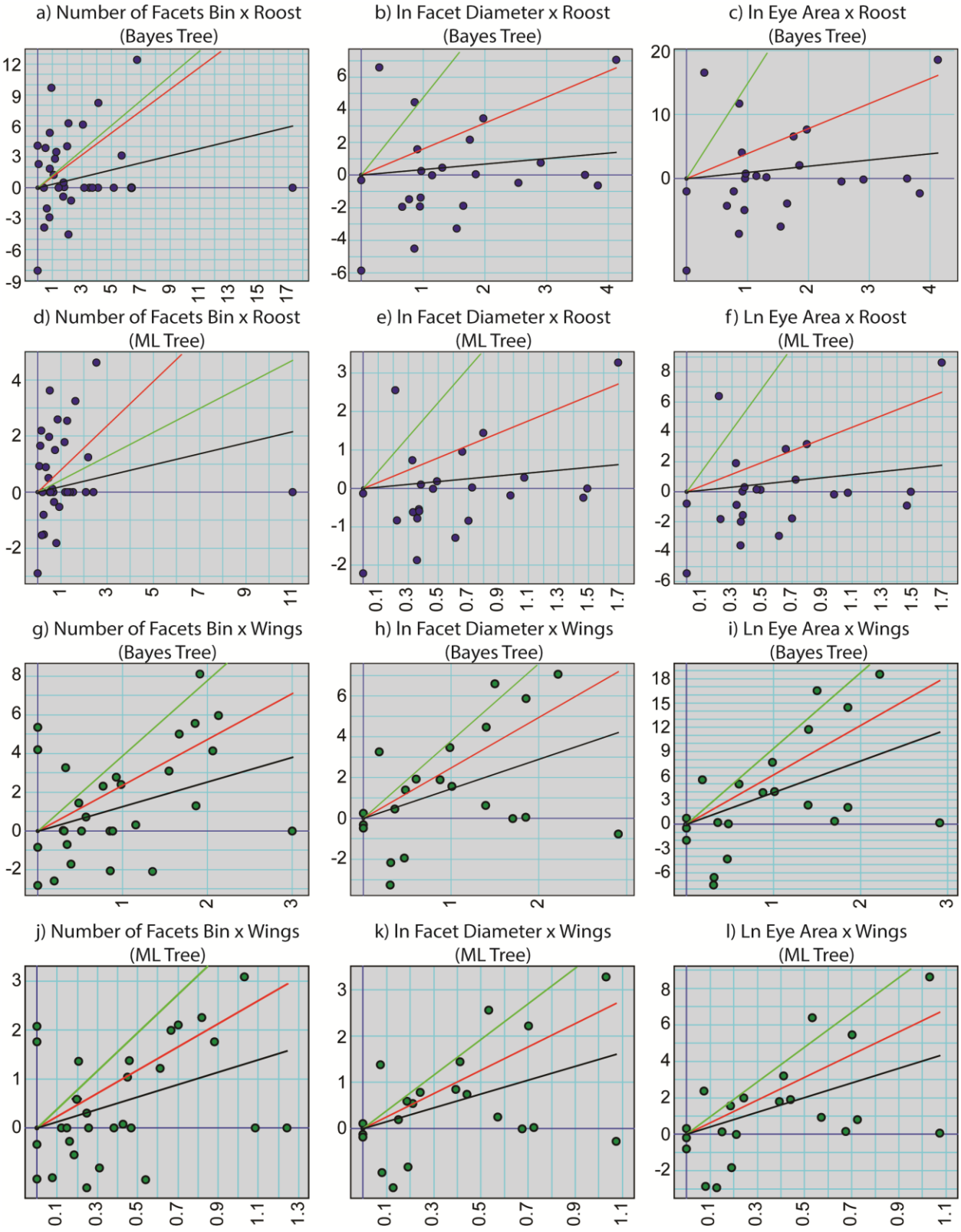


Figure 8: Agreement of Dittmar 2006 Trees with the Current Results

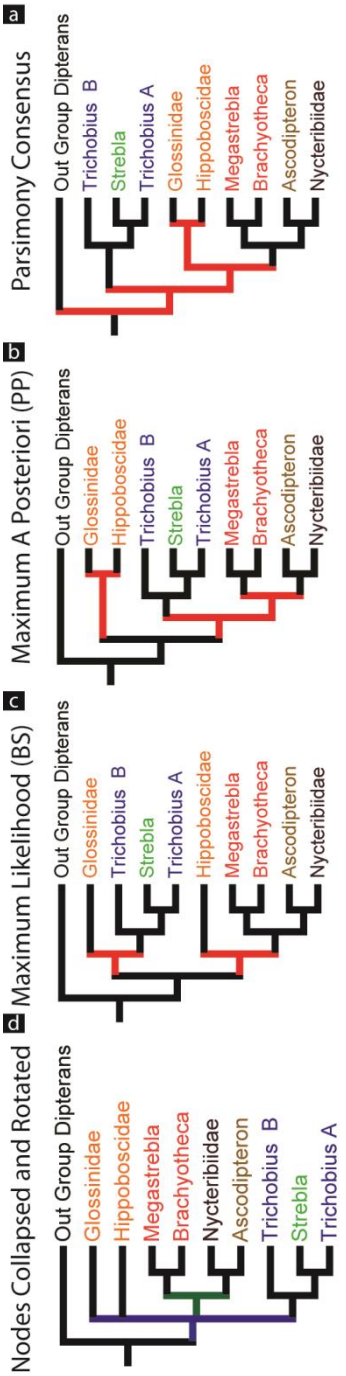


Table Captions for Chapter 2

Table 2: PCR Primers and references for protocols.

List of primers used for PCR amplification of selected loci. References are for primer sources and PCR protocols; n.a. indicates new primers.

Table 1: Taxa, Data, and References

Taxa included in phylogenetic analyses and associated data used for statistical analyses. Data for the number of facets were obtained either through personal observations, or through literature searches. Data on wing morphology and preferred host were obtained from sources cited in the text. Data on the host roost were obtained from the references in the table. A '?' indicates that no data were available. Wing morphology was coded as 0 (A; wingless), 1 (B; reduced) and 2 (M; fully-formed). Roost categories were coded as 3 (tree and foliage roosts), 2 (generalists and lighted caves, and 1 (dark caves).

Table 3: Results of SH Tests

Results of SH tests as implemented in PAUP comparing the best unconstrained trees found using MrBayes and RAxML with the best tree found using the same methods but with the topological constraint indicated. Trees with the "No Eyes Monophyletic" constraint were forced to have New World bat flies that have no externally visible eyes be monophyletic. Trees with the "Expanded Eyes Monophyletic" constraint were forced to have new world bat flies that have experienced an expansion in the number of facets be monophyletic. '-ln L' is short for the negative of the log likelihood of the tree. All constrained trees were found to be significantly poorer than the unconstrained trees.

Table 4: Results of Independent Contrasts for Correlated Evolution

Results of Independent Contrasts testing for correlated evolution of the eye parameter listed with either host roost conditions or bat fly wing morphology. Wings were coded from 0 (no wings) to 2 (fully formed wings) so a positive slope indicates that wing size and eye measurement increased together. Roost site was coded from 1 (dark roosts) to 3 (light roosts) so a positive slope suggests that increasing light conditions of the roost correlates with an increase in the eye parameter indicated.

Table 5: Ancestral States Reconstructions for Relevant Nodes of the BS Tree

Ancestral state reconstructions (point = point estimates) and 95% confidence intervals for selected nodes in the Bootstrap (BS) tree found using RAxML, as implemented by PDAP in Mesquite. Node numbers correspond to the nodes labeled in Figures 1-4. S1-S13 is the node joining all Streblidae. S3-S13 is the node joining all New World Streblidae except *Nycterophilia* (which groups with an Old World species). Other node numbers correspond to the node for each numbered clade, or, if part of a polytomy, the node leading to the polytomy, resulting in shared values for all clades contributing to the polytomy (greyed text).

Table 6: Ancestral States Reconstructions for Relevant Nodes of the PP Tree

Ancestral state reconstructions (point = point estimates) and 95% confidence intervals for selected nodes in the Posterior Probability (PP) tree found using MrBayes, as implemented by PDAP in Mesquite. Node numbers correspond to the nodes labeled in Figures 1-4. S1-S13 is the node joining all Streblidae. S3-S13 is the node joining all new worlds Streblidae except *Nycterophilia* (which groups with an Old World species). Other node numbers correspond to the node for each numbered clade, or, if part of a polytomy, the node leading to the polytomy, resulting in shared values for all clades contributing to the polytomy (greyed text).

Tables for Chapter 2

Table 1: Taxa, Data, and References

Bat Fly Species	Clade Number	Number of Facets	Facet Bin	Average Facet Diameter	Eye Area	ln Eye Area	Wings	Preferred Host	Roost Category	References
Musca domestica	OG1	3000	9	24.08	1,557,740	14.26	M	n.a.	n.a.	(Land, 1997)
Stomoxys calcitrans	OG1	?	?	?	?	?	M	n.a.	n.a.	
Belvosia sp.	OG1	?	?	?	?	?	M	n.a.	n.a.	
Drosophila melanogaster	OG1	700	8	15.51	89,661	11.40	M	n.a.	n.a.	(Choe and Clandinin, 2005; Land, 1997)
Glossina Palpalis	OG2	?	?	37.97	373,539	12.83	M	n.a.	n.a.	
Lipoptena cervi	OG2	?	?	?	691,449	13.45	M	n.a.	n.a.	
Megastrebla parvior	S1	1	?	?	23,208	10.05	M	*Rousettus sp.*	L. Caves	(Maa, 1971),
Brachyotheca lobulata	S1	0	0	0.00	0	0.00	M	*Megaderma lyra lyra*	Gen.	(Myers et al., 2011)
Nycterophilia parnelli	S2	1	1	52.72	1,047	6.95	M	*Pteronotus parnellii*	D. Caves	(Myers et al., 2011)
Ascodipteron sp.	S2	0	0	0.00	0	0.00	M	*Rhinopoma sp.*	Trees	(Barbous, 1910)
Trichobius dugesii	S3	9	3	?	?	?	M	*Glossophaga soricina*	Gen.	(Alvarez, M. R. Willig, J. K. Jones, Jr., and W. D. Webster, 1991)
Trichobius yunkeri	S3	1	1	?	?	?	M	*Pteronotus parnellii*	D. Caves	(Myers et al., 2011)
Trichobius intermedius	S3	7 to 12	3	?	?	?	M	*Artibeus jamaicensis*	Gen.	(Myers et al., 2011)
Trichobius caecus	S3	1	1	46.93	1,105	7.01	M	*Pteronotus parnellii*	D. Caves	(Myers et al., 2011)
Trichobius johnsonae	S3	1	1	?	?	?	M	*Pteronotus gymnonotus*	D. Caves	(Molinari, Aguirre, L., Arroyo

										Cabrales, J., Álvarez Castañeda, S.T., Cuarón, A.D. & de Grammont, P.C., 2008)
Trichobius galei	S3	1	1	?	?	?	M	*Natalus tumidirostris*	D. Caves	(Dávalos, Velazco, P. & Aguirre, L., 2008)
Joblingia schmidti	S4	8	3	36.85	5,897	8.68	B	*Myotis nigricans*	Gen.	(Barquez, Perez, S., Miller, B. & Diaz, M., 2008)
Anatrichobius scorzai	S4	6	3	32.42	2,648	7.88	B	*Myotis keaysi*	Gen.	(Hernandez-Meza et al., 2005)
Trichobius corynorhini	S4	8	3	26.78	4,429	8.40	M	*Corynorhinus mexicanus*	D. Caves	(Myers et al., 2011)
Trichobius major	S4	8	3	?	?	?	M	*Tadarida brasiliensis*	Gen.	(Myers et al., 2011)
Trichobius uniformis	S4	7 to 8	3	?	?	?	M	*Glossophaga soricina*	Gen.	(Alvarez, M. R. Willig, J. K. Jones, Jr., and W. D. Webster, 1991)
Trichobius sp.	S4	?	?	?	?	?	M	*?*	?	
Anastrebla modestini	S5	7	3	24.98	2,747	7.92	M	*Anoura geoffroyi*	D. Caves	(Myers et al., 2011)
Strebla diphyllae	S6	7 to 8	3	25.83	2,697	7.90	M	*Diphylla ecaudata*	Gen.	(Myers et al., 2011)
Strebla mirabilis	S6	7 to 8	3	?	?	?	M	*Trachops cirrhosus*	Gen.	(Myers et al., 2011)
Strebla guajiro	S6	7 to 8	3	?	?	?	M	*Carollia perspicillata*	Gen.	(Myers et al., 2011)
Strebla wiedemanni	S6	8	3	?	?	?	M	*Desmodus rotundus*	L. Caves	(Myers et al., 2011)

Strebla christinae	S6	7	3	?	?	?	M	*Phylloderma stenops*	?	(Sampaio, Lim, B., Peters, S. & Arroyo-Cabrales, J., 2008)
Paraeuctenodes sp.	S6	7	3	23.67	2,394	7.78	M	*Glossophaga soricina*	Gen.	(Alvarez, M. R. Willig, J. K. Jones, Jr., and W. D. Webster, 1991)
Metelasmus pseudopterus	S6	0	0	0.00	0	0.00	B	*Artibeus jamaicensis*	Gen.	(Myers et al., 2011)
Strebla galindoi	S6	6	3	28.11	2,381	7.78	M	*Tonatia saurophila*	Trees	(Sampaio, Lim, B., Peters, S., Miller, B., Cuarón, A.D. & de Grammont, P.C., 2008)
Strebla alvarezi	S6	7 to 8	3	?	?	?	M	*Saccopteryx bilineata*	Gen.	(Myers et al., 2011)
Strebla diaemi	S6	7 to 8	3	?	?	?	M	*Diaemus youngi*	Gen.	(Myers et al., 2011)
Trichobius petersoni	S7	25 to 57	5	?	?	?	M	*Sturnira erythromos*	Trees	(Molinari et. al., 2011)
Megistopoda aranea	S7	9	3	19.62	2,133	7.67	B	*Artibeus jamaicensis*	Gen.	(Myers et al., 2011)
Paratrichobius longicrus	S7	24	4	20.24	6,490	8.78	M	*Artibeus lituratus*	Trees	(Morrison, 1980)
Eldunnia sp.	S7	7	3	?	?	?	M	*Lonchophylla robusta*	D. Caves	(Dávalos, Mantilla, H., Medina, C., Pineda, J. & Rodriguez, B., 2008)
Exastinion clovisi	S8	0	0	0.00	0	0.00	B	*Anoura geoffroyi*	D. Caves	(Myers et al., 2011)

Trichobius costalimai	S9	7 to 12	3	?	?	?	M	*Phyllostomus discolor*	Trees	(Redford, J. F. , 1992)
Mastoptera guimaraesi	S9	0	0	0.00	0	0.00	B	*Phyllostomus hastatus*	Gen.	(Myers et al., 2011)
Noctiliostrebla traubi	S10	14	4	?	?	?	B	*Noctilio leporinus*	Gen.	(Myers et al., 2011)
Paradyschiria lineata	S10	4	2	?	?	?	A	*Noctilio leporinus*	Gen.	(Myers et al., 2011)
Paradyschiria parvuloides	S10	4	2	?	?	?	A	*Noctilio albiventris*	Trees	(Myers et al., 2011)
Trichobius parasiticus	S11	10	3	13.88	852	6.75	M	*Desmodus rotundus*	L. Caves	(Myers et al., 2011)
Trichobius diaemi	S11	7 to 12	3	?	?	?	M	*Diaemus youngi*	Gen.	(Myers et al., 2011)
Trichobius dugesioides	S11	7 to 12	3	?	?	?	M	*Trachops cirrhosus*	Gen.	(Myers et al., 2011)
Trichobius flagellatus	S11	9	3	?	?	?	M	*Lonchorhina aurita*	D. Caves	(Aulagnier, P., 2008; Barquez, M., 2008; Benda, Aulagnier, S. & Palmeirim, J., 2010; Lassieur and E., 1989; Myers et al., 2011)
Pseudostrebla ribeiroi	S12	11	3	26.66	5,808	8.67	M	*Lophostoma silvicolum*	Trees	(Dechmann et al., 2005)
Speiseria ambigua	S12	9	3	25.04	4,073	8.31	M	*Carollia perspicillata*	Gen.	(Myers et al., 2011)
Speiseria peytonae	S12	9	3	25.04	4,073	8.31	M	*Carollia brevicauda*	Gen.	(Bernard and Fenton, 2003; Sampaio, Lim, B. & Peters, S., 2008)

Parastrebla sp.	S12	11	3	20.79	3,368	8.12	M	*Trinycteris nicefori*	Trees	(Tavares and Burneo, 2008)
Trichobius frequens	S13	7 to 12	3	24.66	3,923	8.27	M	*Brachyphylla cavernarum*	D. Caves	(Myers et al., 2011)
Trichobius perspicillatus	S13	9	3	18.97	1,661	7.41	M	*Phyllostomus discolor*	Trees	(Redford, J. F. , 1992)
Trichobius jubatus	S13	7 to 12	3	?	?	?	M	*Eumops patagonicus*	Trees	(Hunt and McWilliams, Troy L. Smith, Kevin G., 2003)
Trichobius silvicolae	S13	7 to 12	3	?	?	?	M	*Lophostoma silvicolum*	Trees	(Dechmann et al., 2005)
Aspidoptera falcata	S13	6 to 7	3	19.14	1,329	7.19	B	*Sturnira lilium*	Trees	(Evelyn and Stiles, 2003; Myers et al., 2011)
Aspidoptera phyllostomatis	S13	8	3	19.14	1,329	7.19	B	*Artibeus jamaicensis*	Gen.	(Myers et al., 2011)
Trichobius hirsutulus	S13	7 to 12	3	?	?	?	M	*Myotis keaysi*	Gen.	(Hernandez-Meza et al., 2005)
Trichobius longipes	S13	7 to 12	3	?	?	?	M	*Phyllostomus hastatus*	Gen.	(Myers et al., 2011)
Trichobius joblingi	S13	7 to 12	3	?	?	?	M	*Carollia perspicillata*	Gen.	(Myers et al., 2011)
Trichobius macrophylli	S13	7 to 12	3	?	?	?	M	*Macrophyllum macrophyllum*	L. Caves	(Myers et al., 2011)

Table 2: PCR Primers and references for protocols

Gene	Forward (5' to 3')		Reverse (5' to 3')		Reference
12s	12sai	AAACTAGGATTAGATACCCTATTAT	12sbi	AAGAGCGACGGGCGATGTGT	n.a.
16s	16sa	CGCCTGTTTATCAAAAACAT	16sb	CTCCGGTTTGAACTCAGATCA	(Bybee et al., 2004; Wheeler et al., 2001)
18s	1F	TACCTGGTTGATCCTGCCAGTAG	3.9	TGCTTTRAGCACTCTAA	(Bybee et al., 2004; Wheeler et al., 2001)
	A0.7	ATTAAAGTTGTTGCGGTT	Bi	GAGTCTCGTTCGTTATCGGA	
	A2.0	ATGGTTGCAAAGCTGAAAC	9r	GATCCTTCCGCAGGTTCACCTAC	
28s	1a	CCCSCGTAAYTTAGGCATAT	4.2b	CCTTGGTCCGTGTTTCAAGACGG	n.a.
	BF1.3a	GGATTTTCTTAGTAGCGGCGAGCG	BF3.9r	ACGGGTCCCGAAGGTATCCTGAATCTT	n.a.
	3a	AGTACGTGAAACCGTTCAGG	B	TCGGAAGGAACCAGCTAC	n.a.
	4.2a	CTAGCATGTGYGCRAGTCATTGG	6.2b	AATAKKAACCRGATTCCCTTTCGC	n.a.
	4.8a	ACCTATTCTCAAACTTTAAATGG	28SRd7b1	GACTTCCCTTACCTACAT	n.a.
Cox2	F-Leu	TCTAATATGGCAGATTAGTGC	R-Lys	GAGACCAGTACTTGCTTTCAGTCATC	(Bybee et al., 2004; Hillis and Dixon, 1991; Wheeler et al., 2001)
CytB	A5	AGGRCAATTATCATTTGAG	MyCyt1.1	AAATATCATTCTGGTTGAATATG	(de la Cruz and Whiting, 2003)
H3	BF	ATGGCTCGTACCAAGCAGAC	BR	ATRTCCTTGGGCATGATTGTTAC	n.a.
CAD	54F	GTNGTNTTYCARACNGGNATGGT	364R	TCNCANGCRAANCCRTGRTTYTG	(Moulton and Wiegmann, 2004)
CO1	Deep6F2.1	GCHCAYCAYATRTTYACHGTWGGW	Deep2R8.1	YCTTTATWDATGRGKTTTAAATCC	n.a.

Table 3: Results of SH Tests

Tree	-ln L	Diff -ln L	P
Bayesian ML - Unconstrained	**59080.97371**	**(best)**	**--**
RAxML ML - Unconstrained	**59081.16941**	**0.1957**	**0.89**
Bayesian ML - No Eyes Monophyletic	**59228.31445**	**147.34073**	**0.000****
RAxML ML - No Eyes Monophyletic	**59172.84285**	**91.86914**	**0.004****
Bayesian ML - Expanded Eyes Monophyletic	**59169.74655**	**88.77283**	**0.005****
RAxML ML - Expanded Eyes Monophyletic	**59220.70262**	**139.72891**	**0.000****

Table 4: Results of Independent Contrasts for Correlated Evolution

Y-Axis Contrast	X-Axis Contrast	Tree	p-value (2-tailed)	Pearson Product-Moment Correlation Coefficient	Least Squares Regression Slope	Least Squares R-squared
Number of Facets (Bin)	**Roost**	**Bayes**	0.0198*	0.33	0.35	0.11
		ML	0.0791	0.25	0.20	0.06
ln Facet Diameter		**Bayes**	0.3252	0.21	0.33	0.04
		ML	0.3407	0.20	0.33	0.04
ln Total Eye Area		**Bayes**	0.2459	0.24	0.96	0.06
		ML	0.2420	0.24	0.99	0.06
Number of Facets (Bin)	**Wings**	**Bayes**	0.0001**	0.54	1.27	0.29
		ML	0.0001**	0.53	1.27	0.29
ln Facet Diameter		**Bayes**	0.0026**	0.59	1.45	0.34
		ML	0.0023**	0.59	1.50	0.35
Ln Total Eye Area		**Bayes**	0.0007**	0.64	3.92	0.41
		ML	0.0007**	0.64	4.02	0.41

Table 5: Ancestral States Reconstructions for Relevant Nodes of the BS Tree

	Number of Facets (Binned)				Average Facet Diameter				Ln Total Eye Area			
Node	Point	SE	95 low	95 high	Point	SE	95 low	95 high	Point	SE	95 low	95 high
S1-S13	4.39	0.92	2.59	6.20	23.40	7.31	9.06	37.73	9.38	1.97	5.52	13.24
S3-S13	3.08	0.81	1.50	4.66	24.67	6.99	10.97	38.38	7.56	1.98	3.68	11.43
S1	3.72	1.13	1.51	5.93	21.05	9.39	2.65	39.46	8.43	2.42	3.69	13.18
S2	2.92	0.98	1.01	4.84	22.37	8.28	6.14	38.60	7.02	2.34	2.44	11.60
S3	2.30	0.74	0.86	3.75	31.57	7.66	16.55	46.58	7.19	2.20	2.88	11.50
S4	2.82	0.50	1.84	3.81	24.77	4.65	15.65	33.88	7.17	1.33	4.55	9.78
S5	2.71	0.46	1.82	3.61	22.59	4.27	14.22	30.96	6.82	1.23	4.42	9.23
S6	2.71	0.46	1.82	3.61	22.59	4.27	14.22	30.96	6.82	1.23	4.42	9.23
S7	2.77	0.45	1.89	3.65	18.15	4.46	9.41	26.88	6.33	1.28	3.82	8.84
S8	2.47	0.34	1.80	3.14	17.01	3.28	10.58	23.44	6.33	1.09	4.20	8.45
S9	2.47	0.34	1.80	3.14	17.01	3.28	10.58	23.44	6.33	1.09	4.20	8.45
S10	2.47	0.34	1.80	3.14	17.01	3.28	10.58	23.44	6.33	1.09	4.20	8.45
S11	2.47	0.34	1.80	3.14	17.01	3.28	10.58	23.44	6.33	1.09	4.20	8.45
S12	2.47	0.34	1.80	3.14	17.01	3.28	10.58	23.44	6.33	1.09	4.20	8.45
S13	2.47	0.34	1.80	3.14	17.01	3.28	10.58	23.44	6.33	1.09	4.20	8.45

Table 6: Ancestral States Reconstructions for Relevant Nodes of the PP Tree

	Number of Facets (Binned)				Average Facet Diameter				Ln Total Eye Area			
Node	Point	SE	95 low	95 high	Point	SE	95 low	95 high	Point	SE	95 low	95 high
S1-S13	3.98	0.98	2.06	5.89	20.75	8.24	4.59	36.91	8.86	2.18	4.60	13.13
S3-S13	2.47	0.92	0.67	4.27	20.36	8.50	3.70	37.01	6.28	2.41	1.56	11.00
S1	3.33	1.10	1.17	5.49	18.46	9.73	-0.62	37.53	7.96	2.45	3.15	12.77
S2	2.36	0.97	0.45	4.26	18.72	8.85	1.38	36.06	5.94	2.49	1.06	10.83
S3	1.78	0.82	0.16	3.39	29.16	8.98	11.57	46.76	6.16	2.58	1.09	11.22
S4	2.13	0.66	0.84	3.41	17.60	6.25	5.35	29.84	5.25	1.80	1.73	8.77
S5	2.13	0.66	0.84	3.41	17.60	6.25	5.35	29.84	5.25	1.80	1.73	8.77
S6	2.13	0.66	0.84	3.41	17.60	6.25	5.35	29.84	5.25	1.80	1.73	8.77
S7	2.13	0.66	0.84	3.41	17.60	6.25	5.35	29.84	5.25	1.80	1.73	8.77
S8	2.13	0.66	0.84	3.41	17.60	6.25	5.35	29.84	5.25	1.80	1.73	8.77
S9	2.13	0.66	0.84	3.41	17.60	6.25	5.35	29.84	5.25	1.80	1.73	8.77
S10	2.13	0.66	0.84	3.41	17.60	6.25	5.35	29.84	5.25	1.80	1.73	8.77
S11	2.13	0.66	0.84	3.41	17.60	6.25	5.35	29.84	5.25	1.80	1.73	8.77
S12	2.13	0.66	0.84	3.41	17.60	6.25	5.35	29.84	5.25	1.80	1.73	8.77
S13	2.13	0.66	0.84	3.41	17.60	6.25	5.35	29.84	5.25	1.80	1.73	8.77

Chapter 3: When Reduction is Remodeling: Histology of a Bat Fly Eye

Abstract

Despite the fact that all bat flies (Streblidae and Nycteribiidae) exhibit marked eye reduction, previous studies have demonstrated that the eyes of at least one species, *Trichobius frequens*, are functional. Because all related Diptera possess the specialized form of apposition compound eye known as neural superposition, this was the hypothesized form for *T. frequens* eyes. However, the histological analysis reveals that even though *T. frequens* have apposition compound eyes, they do not exhibit the neural superposition form. Further, even though their eyes are reduced in terms of the number of ommatidia, they possess several adaptations that enhance the eye's sensitivity at the expense of acuity. Many of these adaptations are convergent with features of distantly related species whose eyes are also adapted to increase sensitivity. This analysis further suggests that reduction of the eye itself may be part of an adaptive remodeling of the entire eye to increase sensitivity.

Abbreviations and Symbols:

Whole Eye Measurements: $\Delta\phi$: interommatidial angle; $\Delta\rho$: acceptance angle; P_{abs}: Photon Absorption Efficiency; *EP*: eye parameter; *R*: whole-eye radius of curvature; FOV: Field of View

Individual Ommatidium Measurements: *D*: lens diameter as viewed from above; *r*: single lens radius of curvature as viewed in transverse sections; H: lens relative hemisphericity; *f*: focal length; *d*: rhabdom diameter; *a*: cross sectional area of the rhabdom microvilli; *L*: rhabdom length

Introduction

Bat flies are a unique group of dipterans in that they are obligate hematophagous ectoparasites of bats. They are also unique in that all bat flies (consisting of two families: Nycteribiidae and Streblidae) exhibit significant eye reduction, and in some cases complete eye loss (chapter 2). The reduced state of their eyes stands in contrast to the vast majority of dipterans that generally have large, well developed eyes.

Notwithstanding the reduced state of bat fly eyes, it has been demonstrated that individuals of one species, *Trichobius frequens*, exhibit a measurable behavioral response to light (Chapter 1). Furthermore, external measures of eye morphology, such as the number of facets, are phylogenetically correlated with wing morphology (Chapter 2). This suggests that selection has acted to maintain eye structure and function in many bat fly species. Thus, in the absence of extra-ocular reception, one would expect that the microstructure of bat fly compound eyes has also been conserved in photosensitive species, such as *T. frequens*.

Phylogenetically, bat flies are among the schizophoral Diptera (united by the presence of a ptilinum), which include house flies (e.g. *Musca domestica*) and fruit flies (e.g. *Drosophila melanogaster*) (Wiegmann et al., 2011), with 3000 and 700 ommatidia respectively (Choe and Clandinin, 2005; Land, 1997). With the possible exception of the parasitic bee louse (Braulidae), which also has significantly reduced eyes (Kaschef, 1959), all schizophoral flies have eyes with a sophisticated neural arrangement known as neural superposition (Figure 1C; Hardie, 1986; Nilsson and Kelber, 2007; Land and Nilsson, 2002; Stavenga, 1975). Thus, if *T. frequens* ommatidia have retained their ancestral form, they would be expected to exhibit neural superposition.

All compound eyes share two features in common. One, for which the eyes are named, is an array of multiple small lenses; the second is a retina composed of light sensitive units called rhabdoms. The rhabdoms in turn are comprised of multiple rhabdomeres, the light-absorbing portion of retinula cells. Individual rhabdomeres are formed by densely packed microvilli whose membranes are rich in the photoreactive protein rhodopsin, and are highly refractile: once light enters a rhabdomere it is maintained within it with little loss by internal reflection (Land, 1997; Land and Fernald, 1992; Land and Nilsson, 2002; Nilsson and Kelber, 2007).

Compound eyes come in two general forms, optical superposition, and apposition; neural superposition (the predicted form for bat flies), is a special case of the apposition form. These forms can be distinguished by the relationship between the lenses and the rhabdoms, which is a result of the arrangement of associated cellular structures such as light-shielding pigment cells, and the crystalline cone (called the pseudocone in dipterans). These differences affect the path light follows from lens to rhabdom. Individual species also differ in the number and arrangement of rhabdomeres comprising the rhabdoms, which is usually constant within a species, and often within species groups (Harzsch et al., 2005; Nilsson, 1989a; Nilsson, 1989b; Nilsson and Kelber, 2007; Paulus, 1979; Paulus, 2000).

The defining feature of optical superposition eyes is that the lenses focus light on a collective retina comprised of an array of rhabdoms (Figure 1a). In an ideal optical superposition eye, all the light entering any of the lenses from a point source would be focused (superimposed) on a single location within the retina. This is accomplished by using the crystalline cone as a secondary refracting structure. This arrangement increases the eyes sensitivity by focusing more of the incident light on the retina; however, inasmuch as the image formed on adjacent rhabdomeres overlaps, it also compromises acuity. Their sensitivity makes optical superposition eyes ideal for arthropods living in low light conditions;

accordingly they are typically found in arthropods that are either crepuscular or nocturnal, such as moths, many beetles, and crustaceans (Land and Nilsson, 2002; Nilsson, 1989a; Nilsson, 1989b).

The defining feature of apposition eyes is that each lens has a one-to-one relationship with a rhabdom upon which it alone focuses incident light; the combination of lens and rhabdom unique to this eye form being called an ommatidium (Figure 1b). This relationship is accomplished primarily by the placement of circumferential pigment cells between individual ommatidia, which prevents light from being leaked between them, and lenses which focus the light entering any ommatidium on the distal tip of the associated rhabdom (Land, 1997; Land and Fernald, 1992; Land and Nilsson, 2002; Nilsson, 1989a; Nilsson, 1989b). This arrangement results in higher acuity, but sacrifices sensitivity because there can be no superposition of light entering neighboring ommatidia from the same source. These types of eyes are thus typically found in diurnal arthropods such as flies, bees, and crabs; they are also found in some crepuscular and nocturnal insects such as mosquitoes, which possess adaptations, such as lens and rhabdom shape, that maximize the potential sensitivity of their eyes (Kawada et al., 2006; Land et al., 1997; Land et al., 1999).

Neural superposition eyes share the same basic structure as other apposition eyes, with the important exception that individual rhabdomeres comprising a rhabdom are not juxtaposed; rather they are separated spatially from each other (Figure 1c) (Land, 1997; Land and Fernald, 1992; Land and Nilsson, 2002; Nilsson and Kelber, 2007; Stavenga, 1975). This arrangement is often referred to as an “open” rhabdom, as that of a typical apposition eye is “closed.” Each of these independent rhabdomeres receives light from a unique field of view, guides it down its length, and initiates a neural response separate from other rhabdomeres in the same ommatidium. Even though there are differences between species, within each species there is a fixed number of rhabdomeres comprising each rhabdom (Nilsson

and Kelber, 2007). In schizophoral dipterans, the rhabdomeres are arranged in a distinct trapezoidal configuration, with 6 outside rhabdomeres (numbered 1-6) responsible for greyscale vision, and two central rhabdomeres (numbered 7 and 8) stacked one on top of the other, that are responsible for wavelength discrimination (Hardie, 1986; Stavenga, 1975).

The reason for the name of these eyes comes from the fact that the field of view observed by any of the central rhabdomeres (7 and 8) is also viewed by 1 of the grey-scale rhabdomeres in each of 6 surrounding ommatidia, in a pattern recursively mirroring the trapezoidal arrangement of rhabdomeres within any one ommatidium (Nilsson, 1989a; Nilsson, 1989b). The axons from the six grey-scale rhabdomeres which share the same field of view all innervate the same column of neurons in the first optical ganglion after the retina, the lamina. Thus, even though the light from a single field of view is not optically superimposed, the information from neighboring rhabdomeres that receive information for a shared field of view is superimposed neurally: thus the name neural superposition. This sophisticated optical and neural arrangement depends on the precise angular relationship between adjacent rhabdoms. The result of this "neural knitting" (Land and Nilsson, 2002) is an increase in sensitivity relative to other apposition eyes, without any loss of resolution. The axons emanating from the 7th and 8th retinula cells do not contribute to the superposition of information as they pass through the first optical ganglion to the second optical ganglion called the medulla. From there information is processed by the lobula and/or the lobular plate (the 3rd and 4th optical ganglia) before being passed to the central brain (Otsuna and Ito, 2006).

It was hypothesized that reduction of *T. frequens* eyes would be seen in the number of ommatidia and in the neural structures responsible for processing visual information, but that individual ommatidia would retain the hallmarks of neural superposition typical of schizophoral dipterans. To test these hypotheses,

head dissections and histological analyses focused on identifying the cell types comprising their eyes, and determining their spatial relationships to each other were used. To the extent that reduction has led to modification of this structure, the null hypothesis of reduction by drift suggests that these differences would diminish function of the eye overall. Contrary to this expectation, histological and optical analyses of *T. frequens* eyes revealed that that their ommatidia do not possess neural superposition eyes, and that they exhibit adaptations that are expected to significantly increase the sensitivity of each ommatidium at the expense of acuity. Interestingly, these changes are convergent with the ommatidia of distantly related organisms that have also adapted to function in low light environments.

Materials and Methods

Sample Collection

Adult bat flies were collected in Cueva de los Culebrones using sticky traps placed near locations in the cave where bat flies are known to deposit pupae, and using sweep nets inside the cave. Flies were also obtained by collecting pupa from the deposition site in the cave, transporting them to the Dittmar Lab at SUNY Buffalo, and allowing them to continue development in an incubator set to 29^{o}C until eclosion. Wild type *D. melanogaster* were used as controls for histological analysis so that the staining/labeling of known structures in *D. melanogaster* could be used to assist the identification of related structures in *T. frequens*. When possible, previously published data for nocturnal mosquitoes were also analyzed as controls.

Specimen Preparation and Preservation

Specimens used for photographing the whole fly were preserved in 100% EtOH. Fly head dissections were completed under a dissecting microscope on teneral flies freshly killed in 100% EtOH, then transferred to PBS+0.1% Tween-20. The brain was exposed by first removing the proboscis to create an opening, then peeling away the external cuticle and removing air sacks and trachea surrounding the brain (Figure 2). Care was taken during the dissections to preserve the eye intact on a piece of the removed cuticle. Optical ganglia and the associated axons were alternately separated from the brain and removed with the eye, or separated from the eye and left attached to the brain during the dissections. Dissected specimens, including detached eyes, were fixed in freshly made 4% PFA + 0.1% Triton X-100 for 4 hours to overnight with gentle rocking. Specimens to be used for paraffin sectioning were similarly fixed, either intact, or partially dissected by removing the proboscis to facilitate exposure to fixative (Figure 2a-c). After fixation, all specimens were dehydrated to 100% EtOH then stored at -20^{o}C until further processing.

Whole and Dissected Fly Imaging

Pictures of intact *T. frequens* and *D. melanogaster* were taken with specimen submerged in 100% EtOH using a Zeiss SteREO Discovery.V12 at the Center of Excellence, SUNY Buffalo. Dissected specimens were photographed during the initial dissection, before fixation.

Paraffin Embedding and Sectioning

Specimens to be sectioned were placed in 100% Methyl Benzoate for 18 hours to soften the external cuticle and eye lenses. They were next infiltrated with Periplast Paraffin beginning with 2x1-hour incubations in serial dilutions of Methyl Benzoate/Periplast, and ending with 4x1-hour incubations in 100% Periplast, all at 57^{o}C. Specimens were then positioned in pre-warmed molds with melted Periplast

so that one eye was either horizontal (cross sections) or vertical (transverse sections) with respect to the mold face. Molds with embedded specimen were allowed to sit at room temperature for several days before transferring to 4°C. Sections were cut on a microtome set at 5µm with molds freshly removed from 4°C. To relax the paraffin ribbons and adhere them to the slides, the area on each slide where the ribbon was to be placed was outlined with a hydrophobic barrier pen; the slides were then placed on a 40°C slide warmer and the outlined area was filled with a bubble of degassed water; the ribbons were then floated on this water bubble and left on the warmer overnight, during which time the water evaporated and the ribbons adhered to the slide.

Staining of Paraffin Sectioned Tissues

Sectioned eyes were either labeled for fluorescent microscopy as with whole mount eyes (see below), or stained with Luxol Fast Blue (0.1% in EtOH) and Cresyl Violet Acetate (0.1% in 1% oxalic acid) using the following protocol modified from Klüver and Barrera's (1954) method for staining nervous tissue. Slides were first deparafinized by submersing 3x5 minutes in Xylene, rinsed 2x5 minutes in 100% EtOH, then soaked in Luxol Fast Blue overnight at 42°C. The following morning, slides were rinsed with brief agitation (3-4 seconds) 2x in 95% EtOH, rinsed with brief agitation 2x in distilled water, dipped (1-2 seconds) in 0.005% Lithium carbonate, dipped in 70% EtOH, then rinsed with brief agitation 2x in distilled water. After the final rinse, slides were placed in the Cresyl Violet Acetate solution for 30 minutes, rinsed 2x in distilled water, resolved for 1.5 minutes in 95% EtOH, washed 2x5 minutes in 100% EtOH, equilibrated 2x5 minutes in Xylene, then cover-slipped using Permount mounting media.

Fluorescent-Labeling

Whole-mount eyes and brains, and sectioned flies were labeled with DyLight 594-AffiniPure Goat Anti-Horseradish Peroxidase (αHRP) from Jackson ImmunoResearch Laboratories; this antibody is known to

cross react with the membranes of neural tissue (Kurosaka et al., 1994; Fabini et al., 2001; Koon et al., 2010; Rumpf et al., 2011). Slides with sectioned tissue were first deparafinized by submersing 3x5 minutes in Xylene, then transferring to 100% EtOH. Slides and whole-mount tissues were serially rehydrated to PBS, transferred to PBS+0.1% Tween-20, then blocked in 10% Normal Goat Serum for 45 minutes. After washing 2x10 minutes with PBS and 1x5 minutes with PBS+ 0.1% Tween-20, tissues were covered with αHRP (1µl/100µl PBS + 0.1% Tween-20 + 0.5%BSA) overnight at 4^{o}C, then washed as before. Tissues were then counterstained with DAPI (300nM in PBS+0.1% Tween-20) for 30 minutes, washed 3x5 minutes in PBS, and mounted with Vectashield (Vector Laboratories). DAPI was found to cause rhabdomeres to fluoresce, it was therefore used to visualize these structures in both *D. melanogaster* and *T. frequens*. Labeled tissues were examined the same day on a Zeiss LSM 710 Confocal microscope with InTune Laser at the UB North Campus Imaging Facility. DAPI was visualized using a 360nm excitation laser and an emission filter of 460nm; αHRP was visualized using a 591nm excitation laser and an emission filter of 616nm. In all cases αHRP is artificially displayed as red and DAPI as cyan.

Eye Measurements

Images of the eyes were evaluated in terms of their morphology, relative sensitivity, relative acuity, and the field of view for the entire eye (see specific measurements below). All measurements were made for both *T. frequens* and *D. melanogaster* on transverse sections stained with Luxol Fast Blue and Cresyl Violet Acetate using either AxioVision 4.8.2 (Carl Zeiss) or ImageJ (Rasband). For some measures it was desirable to compare *T. frequens* to species whose eyes are evolutionarily adapted to functioning in the dark. To this end, data for six species of nocturnal mosquitoes (*Anopheles albimanus*, *A. stephensi*, *A. dirus*, *A. minimus*, *Culex quinquefasciatus*, and *C. pipiens pallens*) (Kawada et al., 2006) are also

presented; when data on relevant parameters have not been reported, measurements were taken on images from Kawada et. al. (2006).

For each parameter measured, specimen were selected that had their eyes optimally oriented for making the given measurement. For each specimen selected, the image of the most ideal section was analyzed, and when multiple measurements could be made from a single image, the average of measurements from that specimen was used for statistical analysis. Not all specimen were well suited for every measurement so the numbers of measurements differ for each parameter. Descriptive statistics for each parameter were calculated using Microsoft Excel. To provide a frame of reference against which the optical features of *T. frequens* eyes could be compared, statistical comparisons were made between them and either *D. melanogaster* or nocturnal mosquitoes; statistical tests were carried out using the R environment for statistical computing (R Development Core Team 2008) using a Welch Two sample t-test.

General Eye Features

To determine the general type of compound eye, descriptions were made of the shape and arrangement of the lens, pseudocone, rhabdom, pigment cells, and neural components connecting each ommatidium to the central brain. Because the rhabdoms of superposition eyes are comprised of cells (retinula cells) whose photosensitive portions (rhabdomeres) are precisely arranged in a distinctive trapezoidal pattern with an open configuration (Figure 1) (Borst, 2009; McIntyre and Caveney, 1998; Nilsson, 1989a; Nilsson, 1989b; Hardie, 1986; Stavenga, 1975), the shape and arrangement of retinula cells and their rhabdoms were specifically examined.

Acuity

The interommatidial angle (Δϕ) determines the maximum potential acuity for a compound eye because it reflects the angular separation two objects must have for their light to land on separate ommatidia, thus allowing them to be distinguished (Land, 1997; Land and Nilsson, 2002; Nilsson and Kelber, 2007; Stavenga, 1975). For *T. frequens*, this value was determined geometrically (Kawada et al., 2006) for 11 specimens as the angle formed by the intersection of lines connecting the midpoints of the rhabdom and lens for adjacent ommatidia (see Figure 12). Pseudopupil measurements are commonly used to determine Δϕ (Land, 1997; Wehner, 1981; Stavenga, 1979; Horridge, 1978; Franceschini, 1975; Franceschini, 1972), but could not be used in this case because the eyes of *T. frequens* have too few facets, and the pseudopupil could not be seen. Sections for *D. melanogaster* were not suitable for this measurement so the previously reported value of 5° (Land, 1997) was used in a one-sample t-test against the measurements made for *T. frequens*.

Sensitivity

An eye's sensitivity is an indication of how responsive it is to light in the environment. Because each ommatidium in apposition eyes acts as a discrete unit, responding independently to light, the sensitivity of the eye as a whole depends on the ability for individual ommatidia to capture light. Sensitivity is a function of three parameters (Franceschini, 1972; Land, 1997; Land and Nilsson, 2002; Nilsson and Kelber, 2007; Sakura et al., 2003): 1) The lens diameter as seen from above (D); 2) the acceptance angle (Δρ) – the angle over which the rhabdom receives light; and 3) the proportion of photons absorbed by the rhabdom (P_{abs}). The sensitivity of an ommatidium can be calculated using the formula:

Formula 1:

$$Sensitivity = 0.62 \times \mathrm{D}^2 \times \Delta\rho^2 \times P_{abs}$$

Where 0.62 = $(\pi/4)^2$ and is due to the circular cross section of both the aperture and the receptor (Land and Nilsson, 2002)

Sensitivity (Lens Diameter): Sensitivity increases with D because as it gets larger more light is brought into the ommatidium; if there is no iris limiting the effective D, then the only way to increase sensitivity through D is to make the ommatidium larger, and thus the whole eye (Land and Nilsson, 2002). Accordingly, many species of nocturnal insects exhibit large D relative to their diurnal counterparts (Greiner et al., 2007; Warrant, 1999; Warrant, 2008; Warrant and Dacke, 2011). D was measured as the width along the base of each lens as seen in transverse sections through the eye (N=11 for *T. frequens*; N=4 for *D. melanogaster*; N=6 for Nocturnal mosquitoes).

Sensitivity (Acceptance Angle and Related Measures): The acceptance angle (Δρ) is a function of two parameters: 1) the refractive power of the lens, which is measured as the inverse of its focal length (*f*); and 2) the diameter of the rhabdom (*d*). It can be calculated (Land and Nilsson, 2002) as:

Formula 2:

$$\Delta\rho = \frac{d}{f}$$

Because *f* can be difficult to determine for small lenses, it was not measured and therefore Δρ could not be calculated directly. Instead the interommatidial angle, Δϕ, was used as an estimate of Δρ, because each is expected to be a close approximation of the other. This is so because if Δρ is smaller than Δϕ, sensitivity is lost without any gain in resolution, and if Δρ is larger, then neighboring ommatidia sample

overlapping points in space, compromising acuity. When the two are optimized, increasing sensitivity by increasing Δρ also increases Δϕ, resulting in a decrease in resolution. Thus acuity and sensitivity have an inverse relationship. Because of this Δϕ, as described above, was used as an estimate of Δρ.

Sensitivity (Absorption Efficiency): Photon absorption Efficiency, P_{abs}, is a function of how well the photosensitive pigment absorbs photons and the density of the pigment in the rhabdom microvilli. These factors being equal, P_{abs} can be increased by increasing the volume of the rhabdom, either by increasing the cross sectional area of pigment-containing microvilli, or by making the rhabdom longer (Kawada et al., 2006; Sakura et al., 2003). P_{abs} can be calculated as

Formula 3:

$$P_{abs} = \mathrm{a}(1 - e^{-\kappa L})$$

where *a* is the cross sectional area of the rhabdom, *L* is the length, and κ is a the coefficient of absorption reflecting the absorption properties of the pigment (Sakura et al., 2003). Because *a* can be problematic to measure due to irregularities in the shape of the rhabdom, *d* was measured as a related approximation of its value (N=8 for *T. frequens*; N=4 for *D. melanogaster*). *L* was also measured as the length of the rhabdom in transverse sections (N=8 for *T. frequens*; N=6 for *D. melanogaster*). κ is unknown for *T. frequens*, but is generally assumed to be relatively constant across species (Land et al., 1999), therefore *d* and *L* were used to assess this aspect of sensitivity.

Alternate Indicators of Sensitivity

Eye Parameter: The eye parameter (EP) was developed as a way of assessing the acuity of an apposition compound eye, or for regions of an eye, with measurements that can be made externally (Fordyce and Cronin, 1993). It is calculated as the product of lens diameter and the interommatidial angle:

Formula 4:

$$EP = \mathrm{D} \times \Delta\phi$$

Because increasing both D and Δϕ lead to an increase in light gathering ability of an eye, EP has also been used as an indication of the sensitivity of ommatidia (Buschbeck et al., 2003; Kawada et al., 2006). EP was calculated for each suitable specimen as the product of its average D and average Δϕ in radians (N=11 for *T. frequens*; N=4 for *D. melanogaster*).

Relative Hemisphericity: For a lens of a given refractive index, the refractive power is a function of the inverse of its radius of curvature (r) (Fowles, 1975). When comparing two lenses with the same D, the lens with a smaller *r* will have greater refractive power, and thus a smaller *f*. Similarly (if *d* is also held constant) lenses with a smaller *r* will contribute to a larger Δρ (formula 2), and hence enhanced sensitivity. If while holding D constant, r is decreased, lenses become more hemispherical (Land et al., 1997; Land et al., 1999), as is seen in nocturnal mosquitoes when compared to diurnal counterparts (Kawada et al., 2006). Based on the geometry of the transformation that occurs as *r* is decreased, the minimum *r* for a given ommatidium is ½ D (personal observation), at which point the lens is a hemisphere, and the refractive power of a thin lens of a given refractive index cannot be increased any further (Figure 3). Therefore the parameter *relative hemisphericity* (H) was derived as a measure of the degree to which a lens of any size is hemispherical, and therefore adapted for sensitivity; it is calculated as:

Formula 5:

$$H = \frac{D}{2r}$$

Because the dimensionless value of H approaches 1 as *r* gets smaller relative to any D, H values can be compared across species of varying D. To determine H, *r* was measured by fitting osculating circles to the outer curvature of individual lenses (Figure 3), then measuring its radius using ImageJ (Rasband) (N=11 for *T. frequens*; N=4 for *D. melanogaster).* H was calculated independently for each specimen by dividing its average D by its average 2*r* (N=11 for *T. frequens*; N=4 for *D. melanogaster*). Because nocturnal mosquitoes have been noted for their hemispherical lenses, and are generally accepted as being adapted for sensitivity, H was also calculated for this group as a frame of reference (N=6).

Field of View

The field of view (FOV) of an eye is the angle over which the whole eye samples the environment. It is a function of the proportion of the whole-eye's radius of curvature (R) over which facets can be found (Duparre, 2006; Duparre, 2004). Because $\Delta\rho \approx \Delta\phi$ when the field of view for individual ommatidia do not overlap, the FOV was calculated by multiplying the number of facets seen in cross section by $\Delta\phi$ (N=11 for *T. frequens*). R was measured in the same manner as *r* except that an osculating circle was fit to the outside of the entire eye for each specimen measured (N=6 for *T. frequens*; N=5 for *D. melanogaster*).

Results

General Anatomy

To test the hypothesis that the optical processing centers of *T. frequens* brain would be reduced along with their eyes, the optical processing centers of *T. frequens* brain were compared to *D. melanogaster*. Side by side comparison of *D. melanogaster* with *T. frequens* (Figure 4) showed that even though that the bodies of the two species were of similar size (validating the comparision of optical structures), not only were the eyes of *T. frequens* significantly smaller, but so was their entire head. Dissections

exposing the brain and optical components of each species (Figures 6a-b) revealed that the difference in head size was primarily due to a reduction in the visual components of *T. frequens* brain. Rough measurements using these images (Figure 6b-c) showed the central brain of *D. melanogaster* was about 253 µm wide by 245µm long, and occupied an area of approximately 46,800 μm^2 (Figure 6c); these measurements were close to those found for *T. frequens* with a central brain that was 295µm wide by 239 µm long, and occupied an area of approximately 55,000 μm^2 (Figure 6b). On the other hand, the optical components of the two species were markedly different in size. Excluding the retina in both species (because they were mostly dissected away), the left and right optical components of *D. melanogaster's* brain were each approximately 224 µm long by 153 µm wide, occupying an area of approximately 50,200 μm^2 (Figure 6c); this means that the optical centers associated with each eye of *D. melanogaster* were approximately the same size as the central brain. However, the greatly reduced optical components *of T. frequens* brain were a small fraction of the central brain size. These optical components can be seen in as axonal attachments extending from the retina to a single reduced optical ganglion (probably corresponding to the medulla of *D. melanogaster)* (Figure 6a, 6d, 6e); this optical ganglion in turn sends a thin axonal projection into the central brain. Measurements using these latter images showed that the optical ganglion of *T. frequens* was approximately 51µm long by 19 µm wide, and occupied an area of approximately 1,000 μm^2 in the picture. The optical ganglion of *T. frequens* could also be seen in histological sections (Figure 7), with a distinct cellular region at the distal edge (closest to the rhabdoms) evident by the dark stained nuclei, and a white matter region, which narrows to form the projection that goes to the brain.

It was also noted that even though the eyes of *T. frequens* do not exhibit obvious red pigmentation in images of undisected specimens (Figure 4), red pigment is seen upon dissection, so the difference observed may reflect visibility of the pigment through the lenses of the eye. Furthermore, the SEMs of *T.*

frequens eyes (Figure 5) showed that they lack the eye bristles possessed by *D. melanogaster*, and that the facets are not hexagonally packed, as is typical of most compound eyes; rather, individual facets of *T. frequens* were rounded and the entire eye was usually comprised of 8 facets, with 7 surrounding a central 1; however, eyes with different numbers of facets have been observed (Chapter 2).

Eye Measurements and Lens Structure

To determine whether deviations from the expected neural superposition structure have a functional significance, as well as to enable a comparison with other compound eyes, particularly dipterans, a number of optically related measurements were analyzed for their effect on the acuity and sensitivity of *T. frequens* eyes. Among the measurements distinguishing *T. frequens* from the other dipterans was their very wide $\Delta\phi$ (and thus presumably $\Delta\rho$) at 33.65° (95% CI: 36.93 - 30.37), and correspondingly large EP of 13.75 rad-µm (95% CI: 15.58 - 11.91) (Table 1). This compares to the reported $\Delta\phi$ for *D. melanogaster* of 5° (Land, 1997), which when applied to measurements of lens diameter for individual specimen, resulted in an average EP of only 1.43 rad-µm (95% CI: 1.56 - 1.30) (Table 1).

In addition to the large difference in the number of facets between these species (8 compared to 700 respectively), the reduction which has taken place along the bat fly line is also manifest by the large difference in R: 51.81µm (95%CI: 58.25 - 45.37) for *T. frequens* and 152.48µm (186.87 - 118.09) for *D. melanogaster* (Table 1). The wide confidence interval for *D. melanogaster* was due to the fact that the value varies widely depending on the axis of the section. Reduction in the number of facets and in the radius of curvature also resulted in a slightly diminished FOV. In any of the transverse sections cutting through *T. frequens's* eye, only 3 complete ommatidia were seen. Since $\Delta\phi$ was measured to be about 34°, this corresponded to a FOV of about 102° (assuming $\Delta\rho \approx \Delta\phi$). This is compared to the nearly 180° FOV of *D. melanogaster* (Borst, 2009).

This reduction notwithstanding, *T. frequens* possessed significantly wider lenses than *D. melanogaster:* 22.87µm (95%CI: 24.52 - 21.22) compared to 16.37µm (95%CI: 17.89 - 14.85). Because the lens radius of curvature was only slightly larger (although still significant, Table 1) for *T. frequens*, 12.41µm (95%CI: 13.85 - 10.97) compared to 10.27µm (95%CI: 10.92 - 9.62), the result was lenses with a significantly larger H: 0.93 (95%CI: 0.97 - 0.90) compared to 0.80 (95%CI: 0.86 - 0.74) These values in *T. frequens* were very similar to those measured for five species of nocturnal mosquitoes with an average D of 24.52µm (range: 22.02-27.01), an average *r* of 13.06µm (range: 11.72-14.4), and a corresponding average H value of 0.939 (range: 0.974-0.905).

The pseudocone of *T. frequens* was also distinctly different from that of *D. melanogaster*. The pseudocone of *D. melanogaster* has such a low refractive index that it appears as seemingly empty space below the lenses (Figure 7e). The pseudocone of *T. frequens*, on the other hand, was highly compressed and pushed up against the proximal surface of the corneal lens; this was observed consistently in all sections as manifest by a narrow gap between the two. In addition, while the lenses of *D. melanogaster* absorbed some of the stains used (indicated by their bluish appearance), neither the lenses nor the pseudocones of *T. frequens* appeared to have absorbed any of the stain.

Structure of the Ommatidia and Rhabdoms

To determine whether or not the ommatidia of *T. frequens* exhibit neural superposition, the structure of the rhabdom was specifically examined. It was found that the rhabdoms of *T. frequens* also had a distinct structure relative to those of all dipterans previously described. While the open rhabdom configuration could be seen in cross sections of *D. melanogaster* eyes (circles in Figure 8b,d), corresponding sections of *T. frequens* revealed closed rhabdoms which were flower-like in appearance

('r' in Figure 8a,c), having petalloid structures radiating out from a common center. Similarly in transverse sections ('r' in Figure 7e), the long thread-like rhabdomeres comprising the rhabdoms of *D. melanogaster* could be seen clearly running perpendicular to the surface of the eye, but the rhabdom of *T. frequens* typically appeared as solid blocks; although with proper focus the edges of the petals could sometimes be seen running the length of the rhabdom (white arrow in Figure 7c).

Inspection of 32 fluorescently labeled rhabdoms, from four different specimens where all petals could be clearly counted, showed that the numbers of petals was variable, ranging from 11 to 18, and having an average of 13.6 (st. dev. 2.23; 95% CI 12.79 – 14.33) (Figure 9). It was also observed that the shape of the rhabdom was not consistent. Some rhabdoms were circular with petals radiating out from the center in a more or less symmetrical pattern, while others had an overall elliptical shape (Figure 9). There was no evidence that retinula cells or rhabdoms were stacked as the central rhabdoms are in other dipterans.

Optical sections taken on a confocal microscope of the same eye at different focal planes revealed three distinct sets of cells are discernible by their nuclear labeling (Figure 10). First is a set of nuclei surrounding each ommatidium just below the lens (Figure 10d) where there is a primary ring of nuclei around each rhabdom and a secondary set of nuclei associated with cells in-between each of the ommatidia (Figure 10c, white arrows). These cells did not label with αHRP, suggesting that they are not neuronal; rather these are the primary and secondary pigment cells, also apparent in distal locations in the stained sections (black arrow in Figures 7a, 8a).

Nearer the base (proximal edge) of the rhabdom (Figure 10b), a distinct set of nuclei for neuronal cells identified as the retinula cells (membranes labeled with αHRP) were apparent, surrounding each

rhabdom. The membranes of these cells revealed them to be tear-drop shaped (Figures 11a-c and 11d-f), with the inner "V" portion of the cells (where the plasma membrane pinches together) combine to form the rhabdom. Thus each of the petals of the rhabdoms are formed by the juxtaposed membranes of two adjacent retinula cells. Because of this, the number of petals for each rhabdom equaled the number of retinula cells comprising the rhabdom, and the number of retinula cells comprising each ommatidium was variable, as the numbers of petals was variable. The juxtaposed membranes of adjacent cells were often visible as a line down the middle of each petal which had more intense labeling (Figure 11a). Generally speaking the number of retinula nuclei matched the numbers of cells inferred by counting petals; however, it was difficult to get precise counts because not all nuclei are in the same plane, and shifting the focus made associating nuclei with rhabdoms problematic.

It was found that most of the rhabdomeres were not labeled with αHRP (Figures 11b and 11e). While the possibility that this may have a functional significance cannot be ruled out, observations suggest that the number and orientation of rhabdomeres which were stained were random (compare Figures 11b, 11e, 11 and 11h).

It was also observed that many retinula cell nuclei were visible without distinct αHRP labeling (Figure 11b). This lack of staining occured for two reasons. First, the non-rhabdomeric portion of the retinula cells only stained near their basal (proximal) edge so that no tear-drop stained cells were visible in distal sections (Figure 11h). Second, the retinula cell nuclei were staggered somewhat proximo-distally so they were often not all visible in the same plane. Nonetheless, in some instances (Figure 11i and 11b) rhabdoms were observed where most of the associated retinula cells were labeled in the same plane.

These data together suggest that the variable staining of the rhabdomeres was simply due to the fact that they were rather dense, and may therefore be difficult for antibodies to penetrate. This hypothesis was supported by the fact that when eyes were physically sectioned and labeled with DAPI and αHRP, the entire rhabdom was labeled, presumably because physically sectioning the rhabdom gave αHRP access to bind to targets within the rhabdom (Figure 11j).

Another set of cells comprising each ommatidium was identified at the base of each rhabdom with axonal projections extending to the optic ganglion (Figure 10a). Even though these cells did not appear to contribute to the rhabdom, the shape and contribution of these cells was somewhat unclear. Because these basal cells were between the retinula cells and the optic ganglion, and because axons could clearly be seen emanating from them, a tentative conclusion was that the basal cells constitute a sparse lamina that processes information from the retinula cells before communicating with the optic ganglion; related possibilities are discussed below.

Finally, several axons at the base of the eye appeared to form loops between neighboring ommatidia (Figure 7a-b). While these neurons may be involved in processing visual information at this level, they were not observed in the fluorescent labeled whole-mount eyes; this may have been because these specimens were observed in optical cross sections where the loops may not be readily apparent.

The above differences in rhabdom structure notwithstanding, it was found that the rhabdom diameter (d, Table 1) in *T. frequens*'s eye (12.39µm; 95%CI: 13.55 - 11.24) was not significantly wider than those of *D. melanogaster* (10.1µm; 95%CI: 11.66 - 8.55). However, the rhabdom of *T. frequens* were significantly shorter: 17.48µm (95%CI: 19.82 - 15.13) compared to 82.96µm (95%CI: 89.99 - 75.92) in *D. melanogaster* (Table 1).

Discussion

Observations from dissected specimens and various histological examinations clearly illustrate that while the optical components of the brain and the number of facets have been significantly reduced in *T. frequens*, the remaining ommatidia have been dramatically remodeled. Even though *T. frequens* eyes still exhibit the apposition architecture, they have closed rhabdoms, and therefore do not use neural superposition. Other features of the eye also exhibit adaptations that increase the eyes sensitivity. While this is most clear through consideration of the optical properties of *T. frequens* eyes, it seems likely that the rhabdom has responded to the same selective pressures, and that its present structure may therefore also be adaptive.

Optical Factors Contributing to Increased Sensitivity

The first variable that could be modified to increase sensitivity is the diameter of the lens (D) (Formula 1). Relative to *D. melanogaster*, the lenses of *T. frequens* are significantly wider (16.37µm compared to 22.87µm), but this may be misleading in some respects because *D. melanogaster* has narrower lenses than other diurnal dipterans; *M. domestica*, for instance, has facets that are 24.08µm wide, and the lenses of the tsetse fly, *Glossina palpalis*, which are more closely related to bat flies, are wider still at 37.97µm (Chapter 2). Larger lens dimensions for other bat fly species have also been reported (chapter 2). These data suggest that it is unlikely that D in *T. frequens* has been increased relative to common bat fly ancestor species. Nonetheless, *T. frequens* lenses are generally comparable to those of nocturnal mosquitoes, which though somewhat larger on average, range from 20 µm in *A. minimus* to 29.3 µm in *A. albimanus* (Land et al., 1999).

Even though the lens diameters of *T. frequens* are relatively unremarkable, they are notably more hemispherical than those of *D. melanogaster*, resembling the hemisphericity of nocturnal mosquitoes. This was quantified in terms of relative hemisphericity (H) and indeed both the lenses of *T. frequens* (0.93) and nocturnal mosquitoes (0.94) approach the theoretical maximum of 1. By comparison, H measured for *D. melanogaster* was 0.80. The large relative hemisphericity of *T. frequens* seems to be the general rule for most new world bat flies (Chapter 2). Importantly, the closely related species of Tsetse and Hippoboscid flies have lenses that are almost perfectly flat by comparison (chapter 2), further suggesting bat fly lenses have been modified through their evolution to be more hemispherical.

Increasing the curvature of a lens increases its refractive power, resulting in a shorter focal length (*f*). Because the acceptance angle (Δρ) is inversely related to *f*, lenses of a given diameter with greater H will have an increased sensitivity through Δρ, the second variable in formula 1. Although Δρ was not measured directly, the very wide Δϕ of 33.65^{o} further suggests that sensitivity is increased in *T. frequens* through Δρ, because Δρ and Δϕ are expected to be approximately equal (Land and Nilsson, 2002).

The only previously reported insect group with Δϕ values as large as *T. frequens* are Collembola, with values ranging from 25-57^{o} (Land, 1997). Thus, both Collembola and *T. frequens*, have Δϕ that are significantly larger than the next largest reported values, which are for nocturnal mosquitoes at around 7^{o} (the largest being for *A. albimans* at 8.2^{o} (Kawada et al., 2006). The Δϕ of other dipterans pale in comparison: 5^{o} for *D. melanogaster*, 2.25^{o} for *M. domestica*, and 0.6^{o} for *Syritta pipiens* (one of the smallest ever reported) (Kawada et al., 2006). Other arthropods with large Δϕ include the deep sea isopods (genus *Cirolana*),whose Δρ have been reported to be around 47^{o} (Land anssd Nilsson, 2002).

The EP as a related measure of light capturing ability further confirms the heightened sensitivity of *T. frequens* with a value of 13.75 rad-µm, which is greater than both *D. melanogaster* (1.43 rad-µm) and the nocturnal mosquito *A. albimans* (4.2 rad-µm). While the value for Strepsipterans is significantly larger (31rad-µm), because each of their facets is actually an eyelet (Buschbeck, 2005; Buschbeck et al., 2003) comparison would be unjustified.

Further indication that the refractive power of *T. frequens* lenses have been increased, thus increasing sensitivity through Δρ, comes from the observation that their pseudocone is highly compressed against the back of the corneal lens. This suggests that the pseudocone may be acting as a secondary lens, providing greater focusing power to each ommatidium as a whole. In contrast, the primary function of the pseudocones in *D. melanogaster* is to provide vitreous space for light rays to converge on the distal tip of the rhabdom (Nilsson and Kelber, 2007). Corresponding to their inferred shorter *f*, the rhabdoms of *T. frequens* (discussed below) are found almost immediately below the compound lens. Finally, the observation that the lenses of *T. frequens* did not absorb any of the stain, while those of *D. melanogaster* did, may suggest that the lens of *T. frequens* are more dense, which could contribute to a larger refractive index (Fowles, 1975).

Structure of the Bat Fly Rhabdom

The final parameter by which sensitivity could be increased is by modifying P_{abs} (Formula 1). Given the altered structure of the bat fly rhabdom relative to related dipterans, it seems unlikely that P_{abs} has been unaffected. Just as the many adaptations affecting Δρ result in an eye with greater sensitivity, it is reasonable to hypothesize that the observed modifications to the rhabdom also increase sensitivity.

One of the notable changes in the bat fly rhabdom is that they are closed, not having any space between the individual rhabdomeres that comprise it. While this means they clearly do not exhibit neural superposition, it also means that there is an increased cross-sectional area of microvilli within the rhabdom, even though the diameter of the rhabdom (*d*) is not significantly different than that of *D. melanogaster*. In terms of sensitivity, the resulting increase in *a* is adaptive, particularly given that *a* has the greatest effect on P_{abs} (to the extent that the rhabdoms are wider, *a* increases exponentially because the radius is squared in determining the area of a circle; see formula 3).

While the above observations suggest enhanced sensitivity of *T. frequens* ommatidia relative to *D. melanogaster*, *T. frequens* rhabdoms are significantly shorter than those of *D. melanogaster*. This observation is somewhat paradoxical because by itself, shorter rhabdoms make ommatidia less sensitive. Nonetheless, shorter rhabdoms have also been observed in a number of species whose eyes are adapted for increased sensitivity, including nocturnal mosquitoes. Even though the rhabdoms of *T. frequens* have a roughly rectangular appearance in transverse sections, and those of nocturnal mosquitoes are triangular, being narrower distally (closer to the lens), their sizes are comparable; *A. gambiae's* facets have been reported to be 5µm distally, 15µm wide proximally, and 20µm long (Land et al., 1997). Because shorter rhabdoms tend to decrease sensitivity, it has been unclear why this has been observed in organsims such as *T. frequens* and nocturnal mosquitoes, whose eyes are apparently adapted for sensitivity. However, observations of the bat fly eye suggest one possible explanation. It was noted while modeling their eyes that increasing $\Delta\phi$ poses a spatial constraint on the length of the rhabdoms: When $\Delta\phi$ is larger, it forces the rhabdoms to be shorter to prevent them from running into each other. This effect is seen in cross sections of the bat fly eye, and in Figure 12, where measurements were used to construct a scaled transverse-section of *T. frequens* ommatidia. Conversely, increasing sensitivity by increasing D means that the rhabdoms can be longer. This means

that gains in sensitivity by increasing D can ultimately be greater than gains from equal increases in Δρ, but it requires that they eye become much larger (Land and Nilsson, 2002). On the other hand, when acuity can be decreased without a loss in fitness, increasing sensitivity through Δρ avoids the problem of developing and maintaining larger eyes. Furthermore, because the effect of *L* on P_{abs} is less than the effect of *a*, the necessary shortening of the rhabdoms when Δϕ is smaller does not negate the gains in sensitivity, particularly when *a* is increased. Thus even though having shorter rhabdoms may be an undesirable consequence of increasing sensitivity through Δρ, it is a necessary trade off.

In light of this, when acuity can be sacrificed, increasing sensitivity through Δρ may allow, or even favor, the eye becoming smaller in terms of the number of facets. This is possible because the sensitivity of a typical apposition eye is determined by the sensitivity of individual ommatidia, not the number of ommatidia. These observations suggests that the reduction of *T. frequens* eyes could have resulted from positive selection for increased sensitivity by increasing Δρ.

Additionally, modifications to the shape of the rhabdom could also be an important factor in determining P_{abs}. This has been clearly demonstrated in nocturnal mosquitoes where the conical shape of the rhabdomeres increases light capture when Δρ is large (Land et al., 1997). This raises the question as to whether or not the flower-like shape of *T. frequens* rhabdoms could also contribute to P_{abs}. This question is punctuated by the fact that similarly structured rhabdoms are observed in a number of species that are active in low-light environments. Most notable among these is the lateral eye of *Limulus* crabs (Limulidae) (Ichikawa and Tateda, 1982b), but the list also includes the compound eye of the dung beetle *Scarabaeus zambesianus* (Dacke et al., 2003), and the larval stemmata of several Lepidoptera species (Ichikawa and Tateda, 1982a). Analysis of the similarities and differences between species which

share the flower-like rhabdom may yield insights into the structure and function of *T. frequens* rhabdoms, as well as the principles and constraints governing rhabdom evolution in general.

In *Limulus*, the petals are formed in the same way as *T. frequens*, with each petal being made by part of two juxtaposed retinula cells, but other species form their petals differently (Ichikawa and Tateda, 1982b). Even though the significance of the flower-like shape is unclear, because it is found convergently in different species where enhanced sensitivity would be advantageous, and because it is sometimes achieved through different cellular arrangements, it is possible that the flower shape itself is adaptive. As with the conical rhabdoms of nocturnal mosquitoes, it is possible that experiments with the flower shape may demonstrate that it plays a role in improving photon capture.

An additional insight into the structure of *T. frequens* rhabdoms may come by further comparison with *Limulus*. *Limulus* lateral eyes also have 2nd order neurons, called eccentric cells, at the base of their rhabdoms that contribute to the neuronal output from each ommatidium. These cells may be analogous to the basal cells identified in *T. frequens* rhabdoms. The fact that eccentric cells contribute to the center of each rhabdom in *Limulus* suggests the possibility that the basal cells in *T. frequens* may also contribute to the rhabdom. Further analysis is needed to clarify the role of the basal cells in *T. frequens*.

Limulus eyes also exhibit neural loops between rhabdoms that are known to enhance contrast through lateral inhibition (Hartline et al., 1956). Given the other parallels between *T. frequens* eye structure and *Limulus*, this suggests that the loops observed in stained sections of *T. frequens* may also be responsible for lateral inhibition.

Another similarity between *Limulus* and *T. frequens* is that the number of petals/retinula cells comprising the rhabdoms is variable (ranging from 4-20 in *Limulus*) (Hartline et al., 1956). This is interesting because all other dipteran species have a fixed number of cells comprising each rhabdom (Nilsson and Kelber, 2007). Because *Limulus* is both color and polarization blind, while species with fixed numbers of retinula cells usually have color and/or polarization sensitivity, it has been postulated that having a fixed number of retinula cells facilitates retinula differentiation in a way that makes color vision and polarity detection possible (Nilsson and Kelber, 2007). Therefore, there may be a connection between selection for sensitivity and the relaxation of selective pressure for a fixed number of cells, because the ability to detect color and polarity are less important. Because all other Schizophoral dipterans have color vision, determining whether or not *T. frequens* has color vision may shed light on this hypothesis.

Implications for the Ancestral Arthropod Eye

Based both on their placement phylogenetically within Arthropoda, and based on arguments concerning the structure of their eyes, it has been suggested that the eyes of *Limulus* (Chelicerata) are most similar to the ancestral arthropod compound eye. Specifically, Harzsch et. al (2005) have argued that the large and variable cell numbers found in *Limulus* (and many Myriapod species) suggests that their eyes are closer to the ancestral form: *Limulus* eyes lack a pseudocone (i.e. crystalline cone) but have in its place about 100 vitreous cells which fill the space between the lens and the rhabdom; they also have 200-300 pigment cells shielding each rhabdom. This stands in contrast to the compound eyes of many insects and crustaceans, which generally have a smaller and fixed number of cells (Harzsch et al., 2005; Nilsson, 1989a; Nilsson, 1989b; Nilsson and Kelber, 2007; Paulus, 1979, 2000). However, Paulus (2000) has suggested that because the petal-like rhabdom is seen convergently in a number of species, it is likely to

be derived even in *Limulus*, leading to the conclusion that the typical insect and crustacean eye may be closer to the ancestral form.

Discovering flower-like rhabdoms in *T. frequens*, which are clearly derived from neural superposition eyes, would seem to favor the model of Paulus. However, the view that this study demonstrates the evolutionary lability of compound eye structure is preferred. This may mean that it could be impossible to determine the ancestral form of arthropod eyes from structural arguments related to the phylogenetic relationships of extant species. Rather, the question may need to be answered through studies on the evolution of developmental processes within this phylum.

Conclusion

Each of the above observations clearly stands in contrast to the common assumptions about evolutionary reduction. *T. frequens* ommatidia are neither in a state of destruction, nor are they simply being maintained as a miniaturized form of larger eyes with smaller numbers of ommatidia. Rather, nearly every aspect of the eye from the structure of the lens and pseudocone, to the total size of the eye and the structure of the rhabdom is remodeled in a way that enhances sensitivity. It is unlikely that these adaptive changes are the consequence of drift, or relaxed or negative selection. Instead, it is more likely that positive selection had a role in the observed reduction by actively selecting for sensitivity. This conclusion supports earlier findings regarding behavioral response of *T. frequens* to light (Chapter 1), and the phylogenetic patterns evident in the evolution of bat fly eyes (Chapter 2).

Related arguments about the role of selection in reduction have been made in relation to the blind mole-rat (*Spalax ehrenberghi*). Even though degeneration of visual pathways along with ocular reduction has made *S. ehrenberghi* blind, their eyes retain function in photoperiodic detection. While

some parts of the brain involved both in processing these circadian rhythms and in image processing are reduced, other parts of the brain have experienced hypertrophy as a compensatory mechanism for processing photoperiodic information (Cooper et al., 1993). Although positive selection has clearly played a role in this selective hypertrophy to maintain basal function, the same positive selection has not actually brought about reduction of the eye. Thus for the mole-rat, eye reduction fits the null model of drift and negative selection leading to deconstruction while for *T. frequens*, reduction may have resulted from positive selection for enhancing the sensitivity function of the eye.

These findings suggest that selection may also play an adaptive role in the reduction of compound eyes in other species, as a part of their active remodeling. More generally, old assumptions must be challenged before drawing conclusions about the functional significance and the evolutionary forces affecting the reduction of any organ.

Finally, the analysis presented here also brings to light the special case presented by apposition compound eyes when it comes to analyzing changes in their size. Because the number of facets, the width of the lenses, the optics of each ommatidium, the angular relationship of ommatidia to each other, and the structure of the rhabdom each play unique roles in compound eye function, and variably affect the size of the eye, each must be analyzed fully when seeking to understand specific cases of evolutionary reduction.

Acknowledgments

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Rodriguez (Universidad Interamericana de Bayamon) for logistic support in Puerto Rico. The confocal microscope is supported by National Science Foundation Major Research Instrumentation Grant # DBI0923133. Research related to this publication was funded by SUNY Buffalo, and the National Science Foundation through a grant awarded to KD.

Figure Captions for Chapter 3

Figure 1: Representative Compound Eyes

Diagram comparing the three basic types of apposition eyes: a) Optical Superposition eyes are adapted for sensitivity because light from the same source can be focused on the same rhabdom, regardless of the lens through which it passes. b) Apposition eyes are adapted for resolution because of the one-to-one relationship between lenses and rhabdoms. c) Neural superposition eyes are apposition eyes which increase sensitivity through the open rhabdom configuration and neuronal pooling of rhabdomeres with the same field of view.

Figure 2: Dissection Process

Images showing various stages of dissecting out the bat fly brain and optical components. a) Ventral view of *T. frequens* before dissection; arrow points to the proboscis. b) *T. frequens* with proboscis (arrow) pulled up and partially removed. c) *T. frequens* with proboscis removed, creating a hole (arrow) in the ventral part of the head that provides a location where the cuticle can begin to be peeled from around the brain. d) *T. frequens* with the cuticle partially removed, revealing the brain and optical components. i: eye (ommatidia); ii: optical ganglion; iii: optical nerve; iv: brain.

Figure 3: Relative Hemisphericity

Relative hemisphericity (H) is a measure of the degree to which a lens of a given D is hemispherical, and therefore adapted for enhanced sensitivity (see text). When D is held constant and *r* is decreased, lenses become more hemispherical. When *r* is reduced such that 2r = D, the lens is a hemisphere, and r cannot be reduced further without reducing the size of the lens. Lenses are all drawn to have the same D but different values of *r*; dashed circles are osculating circles drawn to each lens so that *r* can be

calculated. a) A relatively flat lens with a large *r* and therefore small H. b) Lens with intermediate *r*. c) Lens where H has reached its maximum value of 1 because 2*r* = D; the lens is therefore a hemisphere.

Figure 4: Fruit Fly and Bat Fly Comparison

Side by side comparison of D. melanogaster (left in each image) and *T. frequens* (right in each image). The same flies are shown in a and b at different magnification.

Figure 5: SEM of T. frequens Eye

Scanning electron micrographs of *T. frequens*, showing details of the external eye structure.

Figure 6: Brain Dissections Showing Reduced Optic Lobes

Images of dissected *T. frequens* and *D. melanogaster* brains and optical ganglia: a) Dissection of *T. frequens* head with the brain still attached to the body. b-c) Comparision of the dissected brains of *T. frequens* (b) and *D. melanogaster* (c). Notice that the central brains are roughly the same size with the primary difference being the size of the optical ganglia. d) Brain from (a) which has been removed from the body and labeled with αHRP to highlight the optical components still attached on the right side. e) Same as (d) at higher magnification. Abbreviations: r=retina, m=medulla, b=central brain, lb=lobula, lp=lobular plate.

Figure 7: Histology of Lateral Sections

Paraffin sections of *T. frequens* and *D. melanogaster* stained with luxol fast blue; all images have the same scale except c: a-c) Lateral sections of *T. frequens* eyes. a) Black arrow points to an apical pigment cell; white arrow points to a neural loop possible providing negative feedback between rhabdoms. b) white arrow points to a neural loop as in (a); black arrow points to the pseudocone revealed by the

corneal lens which has been serendipitously pulled away c) top rhabdom in (b) shown at higher magnification and at a different focal plane (scale bar = 20µm); black arrow points to the narrow space between the corneal lens and the pseudocone; white arrow points to the elongated edges of the petals which form a flower-like rhabdom in cross section (see Figure 8). d-e) lateral section of *D. melanogaster* brain and eyes. d) optical ganglia leading to the brain. e) lateral view of ommatidia; the black arrow points to the distal tip of a rhabdom; the seemingly empty space above this is the pseudocone; below it is the retina comprised of string-like rhabdomeres. Abbreviations: r=retina, m=medulla, b=central brain, lb=lobula, lp=lobular plate.

Figure 8: Histology of Cross Sections

Cross section of *T. frequens* and *D. melanogaster* ommatidia showing the structure of the rhabdoms: a-b) Paraffin sections stained with luxol fast blue with the same magnification. a) Distal section of *T. frequens* eye showing the petals and flower-like structure of the rhabdom; because rhabdoms are found just below the lenses, sections showing the apical portion of the rhabdoms include much of the lenses; black arrow points to pigment cells between the ommatidia. b) Section through *D. melanogaster* eye showing several ommatidia at different proximo-distal levels; the lenses, pseudocone, and open configuration of the rhabdomeres can clearly be seen; the black circle encloses a single rhabdom mid-level. c) Optical cross section of a whole mount *T. frequens* eye labeled with DAPI (cyan) showing the petals and flower-like appearance of the rhabdom and nuclei of pigment cells (black arrow). d) Paraffin section of *D. melanogaster* eye labeled with DAPI showing ommatidia at different proximo-distal levels, similar to b; the solid circle encloses a single rhabdom at its distal end; the dashed circle encloses another rhabdom at a more proximal level. Abbreviations: r=rhabdom, l=lens, p=pseudocone.

Figure 9: Variation in Bat Fly Rhabdom Morphology

Optical sections of *T. frequens* rhabdoms stained with DAPI showing the variation in the shape and the number of petals comprising the flower-like rhabdoms. Images a-d have 13, 15, 15, and 16 petals respectively. All images are at the same magnification

Figure 10: Bat Fly Ommatidia

A series of optical sections showing a whole mount eye of *T. frequens* at various planes; tissue was labeled with αHRP (red; neuronal cell membranes) and DAPI (cyan; nuclei and rhabdoms). The eye was mounted with the lens down so sections progress from the bottom (proximal) portion of the eye to the top (distal). a) Optical section below (proximal to) the eye looking out; axonal projections (white arrows) can be seen exiting the base of the rhabdoms and entering the medulla; nuclei for basal cells having membranes labeled with αHRP can be seen in groups covering each of the rhabdoms. b) Optical section near the bottom of each rhabdom; a ring of nuclei corresponding to the retinula cells and having membranes labeled with αHRP can be seen surrounding each of the rhabdoms (e.g. dashed circle); rhabdoms are seen faintly stained with DAPI; not all rhabdoms/ retinula cells are in the same plane. c) Optical section near the top of each rhabdom, just below the lens; two distinct sets of non-neural nuclei (i.e. no αHRP labeling) for pigment cells are visible: one set forms a ring around each of the rhabdoms (outer white arrows) and the other is found in between each of the rhabdoms (middle white arrow); portions of the rhabdom are labeled with αHRP (see text for explanation). d) Optical section at the plane of the lenses; rhabdoms can be seen in the center of each lens with the nuclei for pigment cells (same as outer arrows in c surrounding them. Abbreviations: m=medulla, r=rhabdom.

Figure 11: Structure of the Bat Fly Rhabdom

T. frequens eyes stained with αHRP (red) and Dapi (cyan) revealing the structure of the rhabdom. a-c) Dapi (a), αHRP (c), and merge (b) of a *T. frequens* rhabdom showing the shape of retinula cells. αHRP labels two tear-drop shaped retinula cells showing how the inner rhabdomeric portions form ½ of each of two neighboring petals; the position of nuclei of stained cells are indicated with white asterisk in each image; not all retinula cells are in the same plane in the image and the rhabdomeric portion of different retinula cells are stained randomly (see text for explanation). d-f) Same as a-c with one retinula cell membrane labeled. g) Optical section in the proximal plane of a *T. frequens* rhabdom stained only with αHRP; the outlines of cell bodies can be seen surrounding the central rhabdom, and the rhabdomeric portion of several retinula cells are labeled with variable intensities. h) Same as g except that the image is taken at a distal plane. i) Optical section of a *T. frequens* rhabdom stained with αHRP and DAPI; even though many of the circumferential retinula cell bodies can be seen with membranes extending into the rhabdom (white arrows), none of the corresponding rhabdomeric membranes are labeled. j) Proximal paraffin section of a *T. frequens* rhabdom labeled with αHRP and DAPI; physically sectioning the rhabdom has allowed intense staining with both labels (white); the membranes of two retinula cells (white arrows) can be seen extending into and forming part of the rhabdomere.

Figure 12: Schematic representation of a T. frequens *ommatidium*

Illustration of *T. frequens* eye and rhabdom. a) Representation of a transverse section through three *T. frequens* ommatidia drawn to scale using the measurements shown. White circles represent nuclei observed in Figure 10; black cells are pigment cells. b) Representation of a proximal cross section through a *T. frequens* rhabdom showing how each petal is formed by the juxtaposition of two retinula cells.

Figure Images for Chapter 3

Figure 1: Representative Compound Eyes

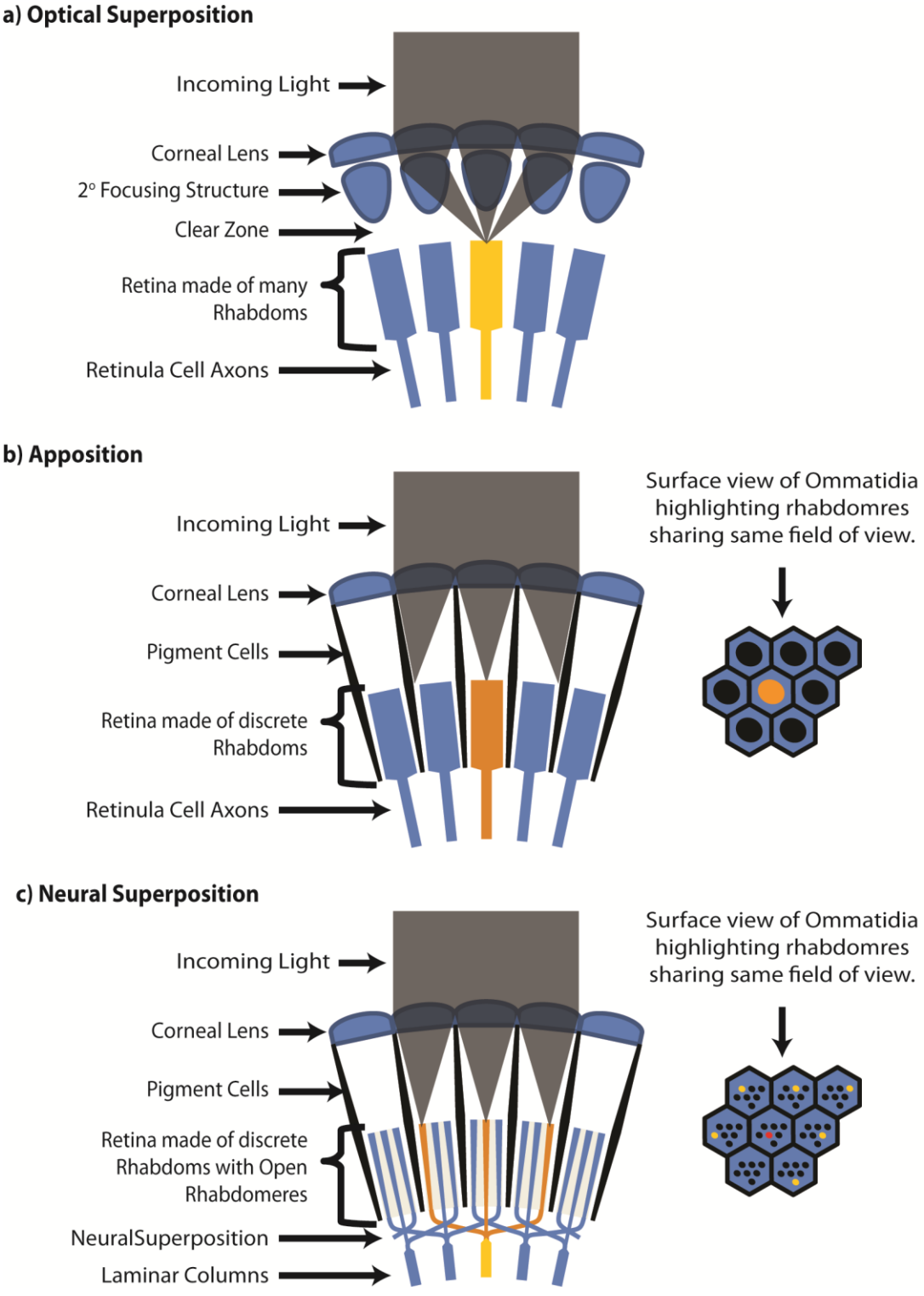

Figure 2: Dissection Process

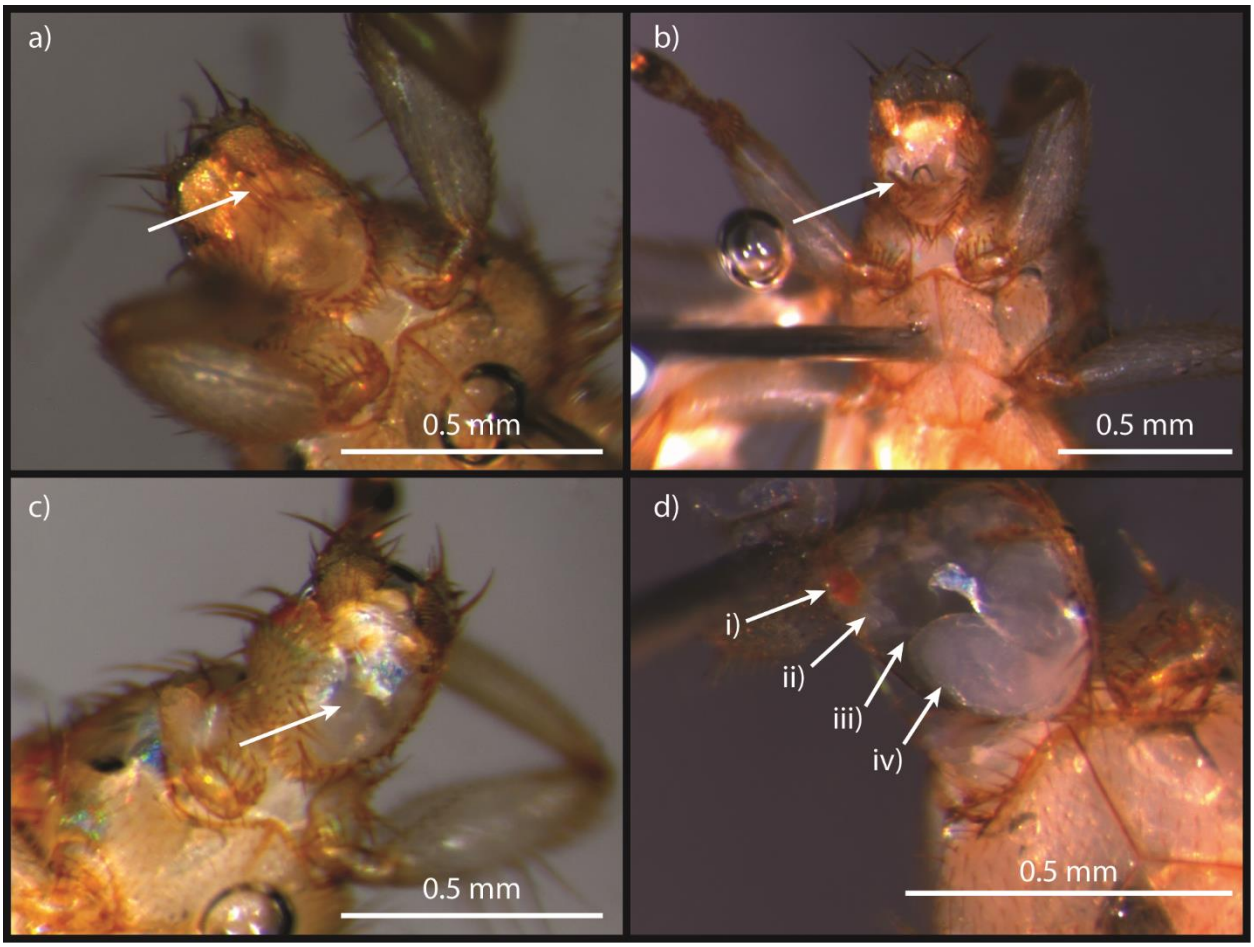

Figure 3: Relative Hemisphericity

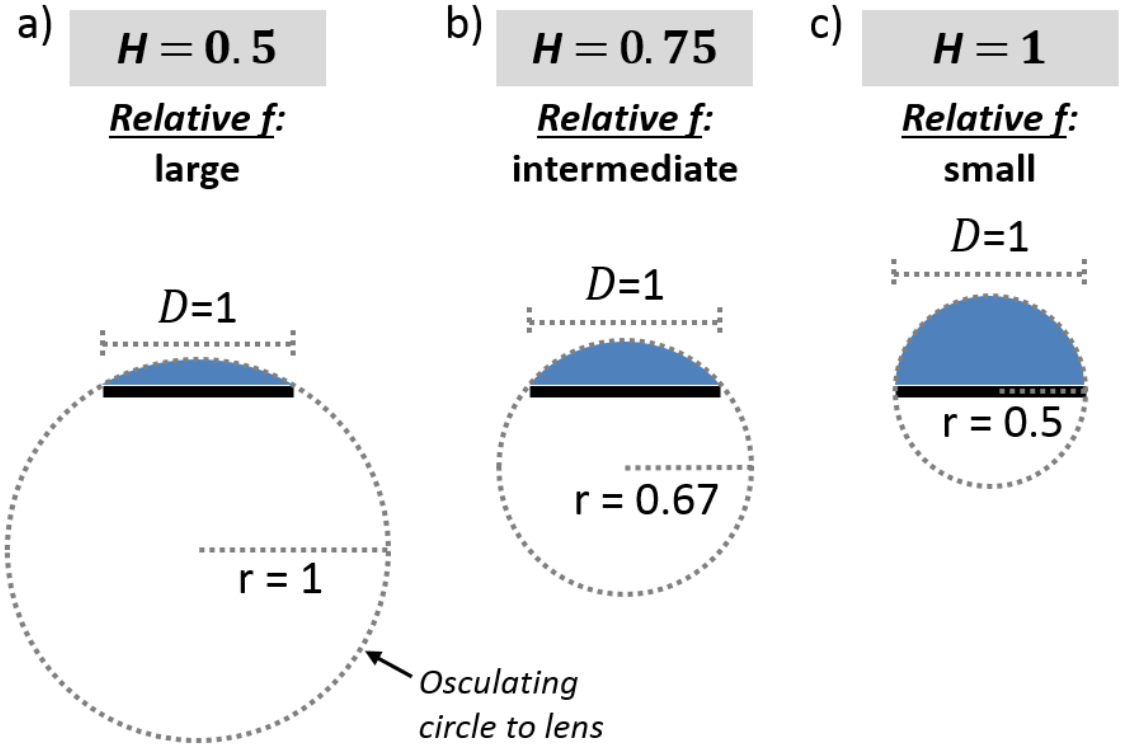

Figure 4: Fruit Fly and Bat Fly Comparison

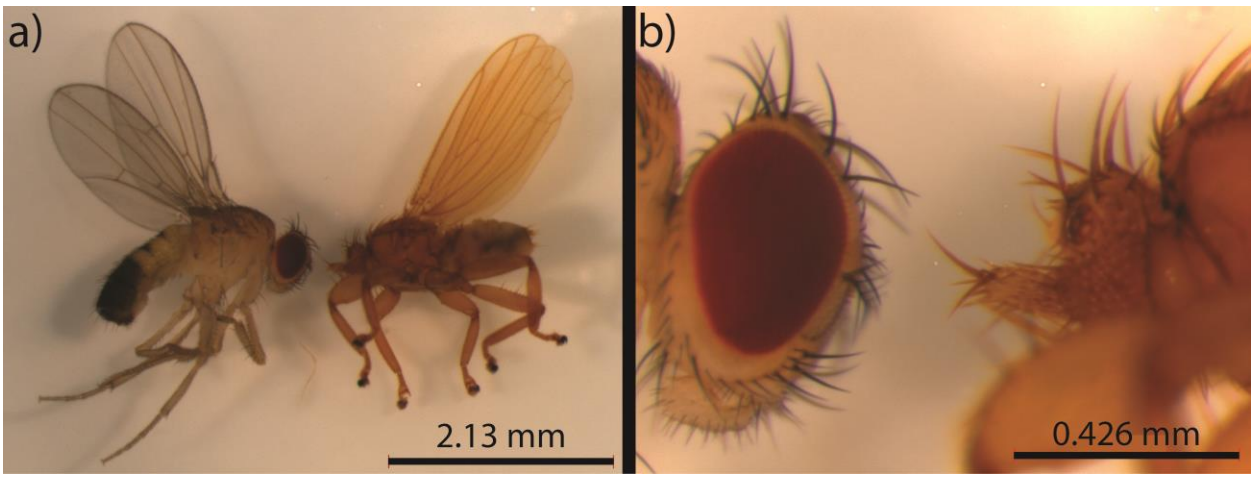

Figure 5: SEM of T. frequens Eye

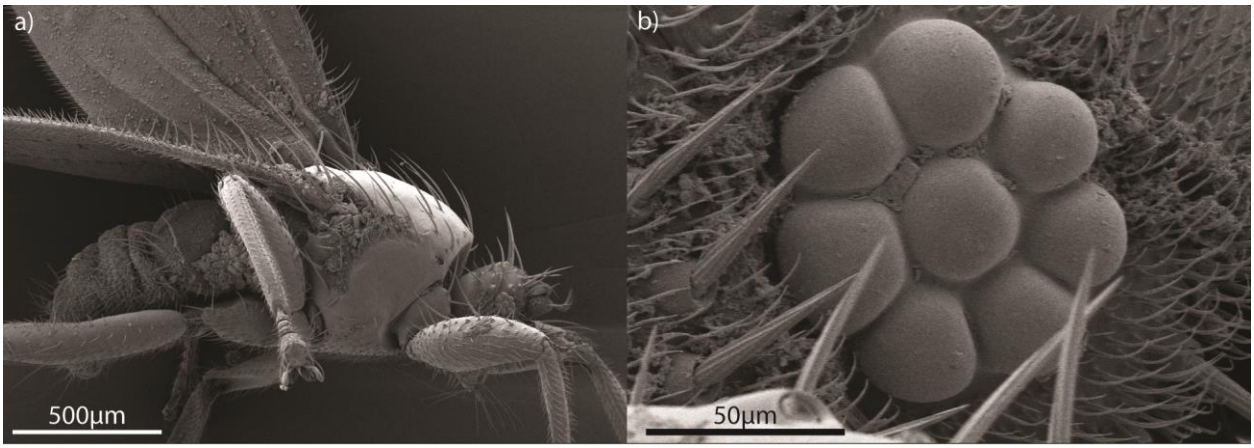

Figure 6: Brain Dissections Showing Reduced Optic Lobes

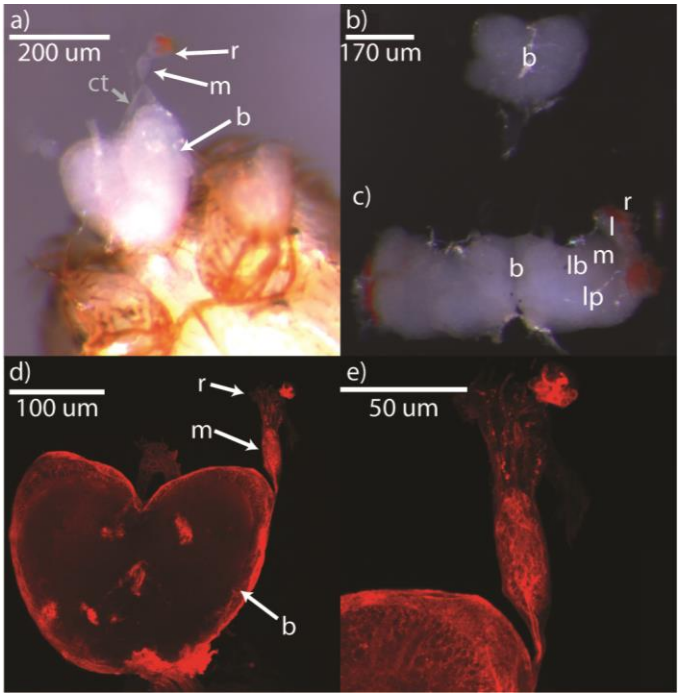

Figure 7: Histology of Lateral Sections

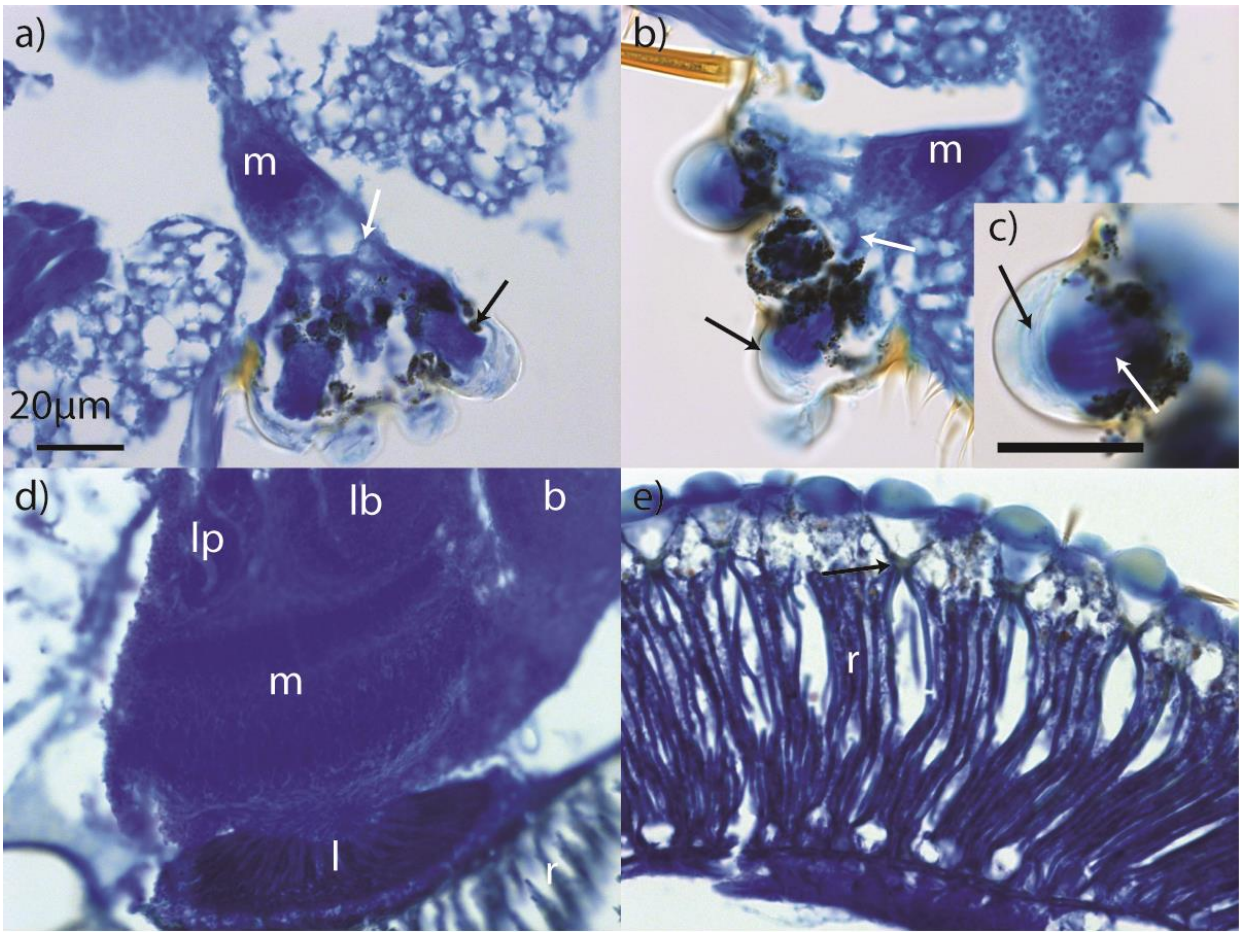

Figure 8: Histology of Cross Sections

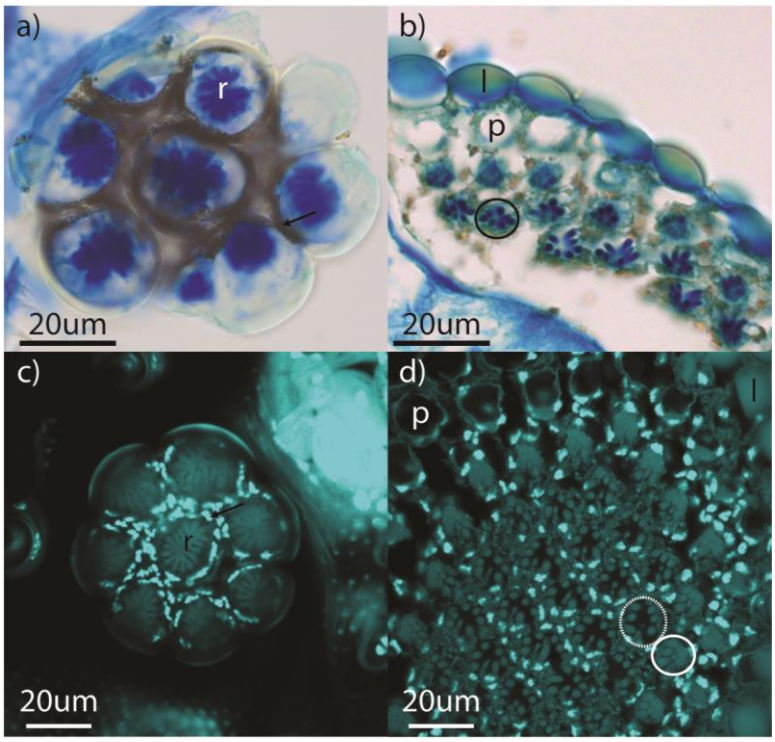

Figure 9: Variation in Bat Fly Rhabdom Morphology

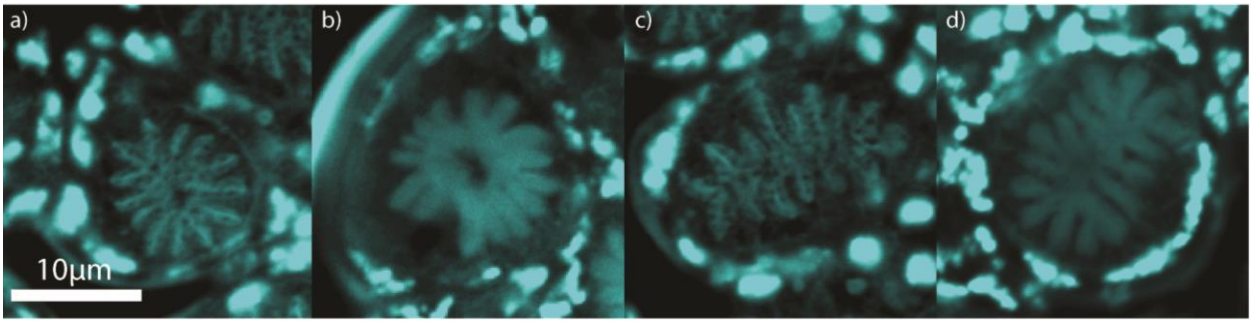

Figure 10: Bat Fly Ommatidia

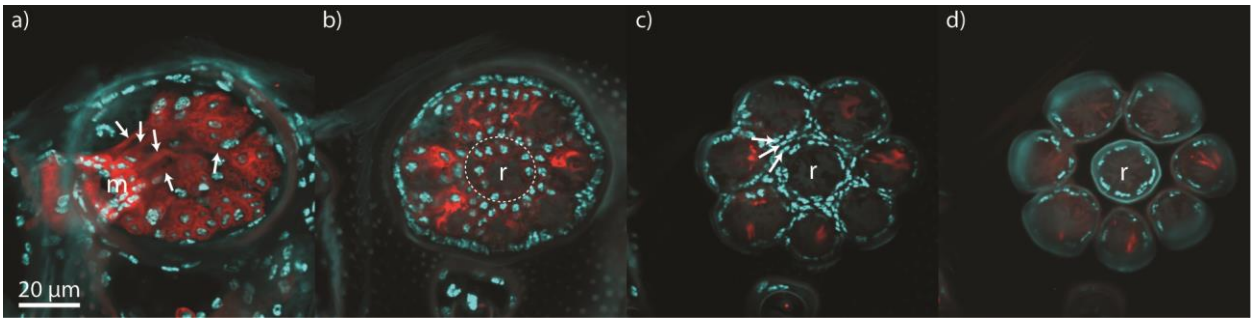

Figure 11: Structure of the Bat Fly Rhabdom

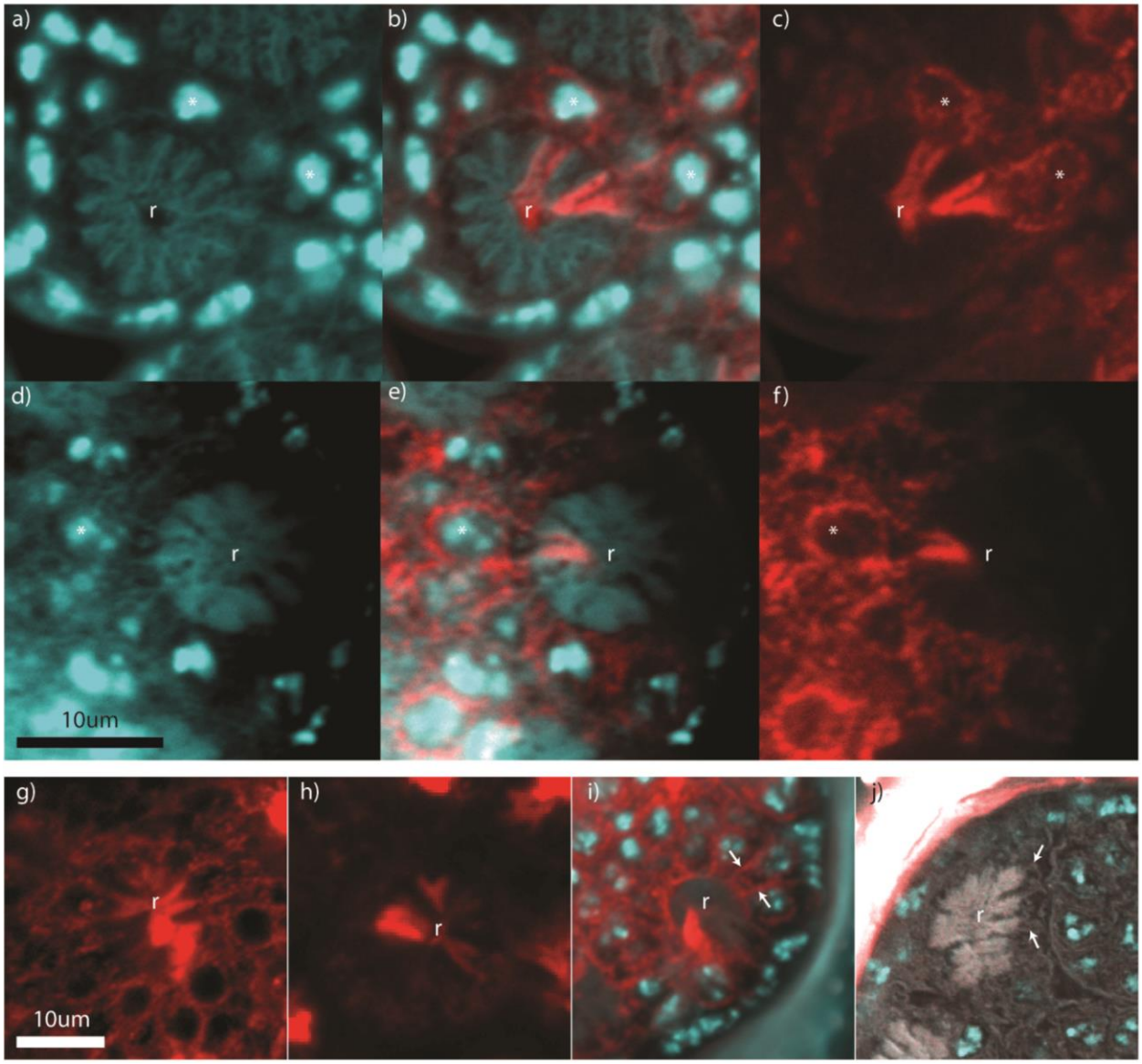

Figure 12: Schematic representation of a T. frequens ommatidium

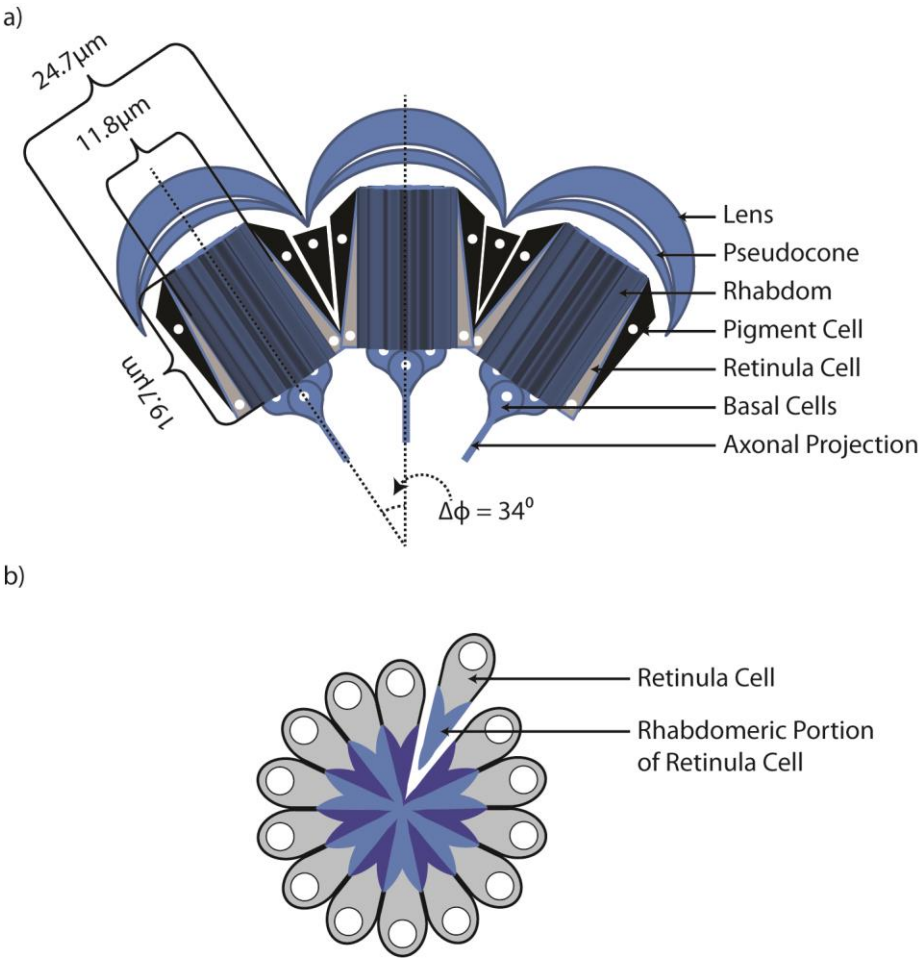

Table Caption for Chapter 3

Table 1: Results of eye Measurements

Eye measurements made from histological sections of *T. frequens* and *D. melanogaster*. **Column Headings**: N= Number of Specimens; Avg. = Aveage; St. Dev. = Standard Deviation; 95% CI = 95% Confidence interval. **Row Headings**: D = lens diameter as seen from above; *r* = lens radius of curvature; *d* = rhabdom diameter; L = rhabdom length; R = whole eye radius of curvature; $\Delta\phi$ = interommatidial angle; EP = eye parameter; H = relative hemisphericity. **Other Symbols**: †= value reported in Land 1997; ** = highly significant; n.s. = not significant. See text for explanations.

Table for Chapter 3

Table 1: Results of eye Measurements

	T. frequens				***D. melanogaster***				P-value
	N	Avg.	St. Dev.	95% CI	N	Avg.	St. Dev.	95% CI	
D (µm)	11	22.87	2.79	24.52 - 21.22	4	16.37	1.55	17.89 - 14.85	**0.00020
r (µm)	11	12.41	2.43	13.85 - 10.97	4	10.27	0.66	10.92 - 9.62	**0.020
d (µm)	8	12.39	1.67	13.55 - 11.24	4	10.1	1.59	11.66 - 8.55	0.057
L (µm)	8	17.48	3.38	19.82 - 15.13	6	82.96	8.79	89.99 - 75.92	**1.98 x 10-6
R (µm)	6	51.81	8.04	58.25 - 45.37	5	152.48	39.23	186.87 - 118.09	**0.0040
Δϕ (Deg.)	11	33.65	5.55	36.93 - 30.37	na	5†	na	na	**9.7 x 10-9
EP (rad-µm)	11	13.75	3.11	15.58 - 11.91	4	1.43	0.14	1.56 - 1.3	**1.1 x 10-7
H	11	0.93	0.06	0.97 - 0.9	4	0.8	0.06	0.86 - 0.74	**0.0094

Conclusion

The body of research presented here uses multiple lines of inquiry to shed light on the evolution of eyes in bat flies. Behavioral studies demonstrate that the reduced eyes of one bat fly species, *T. frequens*, are functional, at least in the sense that they initiate a behavioral response to light. Comparison to the documented behaviors of other dipteran species indicate that their behavior is also unique, both in that they no longer exhibit photo-tropotaxis, and their response results in net movement away from light via either klinotaxis or kinesis.

The variation in reduced states among bat fly's eyes also made possible a phylogenetic analysis of their reduction. These studies revealed that the variability in bat fly eye phenotypes is in part due to a correlation with wing morphology, which suggests that the demands of flight may contribute to selective pressures for eye maintenance. The phylogenetic analysis also show that the so-called reduced eyes of a few bat fly species may have secondarily experienced an evolutionary increase the numbers of facets they possess.

Histological analysis *T. frequens* eyes has also been used to discover that unlike schizophoral dipterans, bat flies do not exhibit neural superposition. Instead, their ommatidia have been remodeled to convergently resemble the apposition compound eyes of other organisms that are adapted for sensitivity. In the case of *T. frequens* (and presumably other bat flies), because visual acuity could be sacrificed, reduction of the eye may have actually been part of an active remodeling that resulted in a samller eye with increased overall sensitivity.

In addition to these specific conclusions, these studies add to the literature on eye evolution by being one of the few that focuses on the evolutionary reduction of compound eyes. It also illustrates the

potential phylogenetic analysis has for illuminating not only the process of evolution, but also the factors that affect the process. This in turn highlights the value of identifying groups of related organisms that exhibit variability in the characteristic to be studied.

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