Species and sex differences in eye morphology and visual sensitivity of two nocturnal sweat bee species (*Megalopta* spp., Hymenoptera: Halictidae)

Running title: *Megalopta* eye morphology and sensitivity

Beryl M. Jones[1,2‡], Brett M. Seymoure*[2,3,4‡], Troy J. Comi[5], Ellis R. Loew[6]

1. Program in Ecology, Evolution, and Conservation Biology, University of Illinois, Urbana, Illinois 61801
2. Smithsonian Tropical Research Institute, Panama City, Panama 20521
3. Living Earth Collaborative, Washington University in St. Louis, St. Louis, MO, 63130
4. Sound and Light Ecology Team, Colorado State University, Fort Collins, CO, 80523
5. Department of Chemistry, University of Illinois, Urbana, IL 61801
6. Department of Biomedical Sciences, Cornell University, Ithaca, NY, 14853

‡Authors contributed equally

* corresponding author's information:

Postal Address: Brett Seymoure
Biology Department, Washington University in St. Louis
Campus Box 1137
One Brookings Drive
St. Louis, MO 63130-4899

phone: 269 501 8761

email: brett.seymoure@gmail.com

Abstract

Visually dependent dim-light foraging has evolved repeatedly across taxa, broadening species ecological niches. As most dim-light foraging species evolved from diurnal ancestors, visual sensitivity must increase immensely to compensate for light levels a billion times dimmer than daylight. Some taxa, e.g. bees, are anatomically constrained by their apposition compound eyes, which function well in daylight but not starlight. However, the sweat bee genus *Megalopta* has incredibly sensitive eyes, foraging in light levels up to 9 orders of magnitude dimmer than diurnal relatives. Despite years of behavioral study, variation in visual sensitivity and eye morphology has not been investigated within and across different *Megalopta* species. We describe eye morphology for two sympatric species of *Megalopta*, *M. genalis* and *M. amoena,* which both forage during twilight under little light. We use electroretinograms to find that males, which are smaller than females, have increased retinal sensitivity compared to females. Although males have relatively larger eyes compared with females, morphological features of the eye were not correlated with retinal sensitivity, suggesting males have additional adaptations to improve retinal sensitivity. These findings are foundational for future work into neural and physiological mechanisms that interface with morphology to increase visual sensitivity.

Key words: allometry - compound eye – electroretinograms - eye morphology – facets - nocturnal foraging - visual sensitivity

List of Symbols and Abbreviations: electroretinogram (ERG), light emitting diode (LED)

Introduction

Competition between and within species is a main selective force leading to improved detection of food, mates, and predators (Land & Nilsson, 2012). A common strategy to reduce competition is to change spatial or temporal resource use, exemplified by the flexible foraging strategies of ants (Traniello, 1989), and shifts from diurnal to nocturnal activity in bats and bees (Rydell & Speakman, 1995; Wcislo *et al.*, 2004). As Mayr (Mayr, 1960) argued, species that exploit a new niche invariably establish an array of new selection pressures that result in behavioral changes and concomitant adaptations in morphology and physiology. Thus, the shift in activity pattern from diurnality to nocturnality in organisms that rely upon visual cues will result in adaptations to perceive the nocturnal environment, which has a billion times less light than daytime (Warrant, 1999; Cronin *et al.*, 2014). The ultimate biological problem of shifting from diurnal to nocturnal activity arises from limitations on eyes that cannot collect the requisite number of photons per unit time to perceive contrast in the environment. Here, we investigated the visual abilities of two nocturnal insect species and related eye morphologies that may enable vision in very dim-light conditions.

Seeing in dim light requires extraction of reliable visual information from an inherently unreliable visual environment. Visual contrast deteriorates with declining light levels due to an inadequate number of photons absorbed by photoreceptors, resulting in increased visual noise (Cronin *et al.*, 2014). Visual noise arises from shot noise, dark noise, and photoreceptor noise, which are due to the stochastic nature of photons, the inability for two photoreceptors to discriminate contrast, and incorrect biochemical responses in darkness, respectively (Cronin *et al.*, 2014; O'Carroll & Warrant, 2017). However, many organisms can extract useful visual information under very dim light conditions (Kelber, Balkenius, & Warrant, 2002; Roth & Kelber, 2004; Chuang, Yang, & Tso, 2008). This ability arises from morphological, biochemical and neurological adaptations including large eyes, short focal lengths, longer and wider

photoreceptors, spatial and temporal summation of photoreceptors, longer integration times, and specialized eye types (Greiner, Ribi, & Warrant, 2004a; Frederiksen & Warrant, 2008). Insects have compound eyes that can be divided into two types – superposition and apposition. Due to the structure of superposition eyes, in which photoreceptors collect light from numerous facets, superposition eyes are more sensitive than apposition eyes and thus the most common eye type in nocturnal insects (Warrant & Dacke, 2011; O'Carroll & Warrant, 2017). However, not all nocturnal insects have superposition eyes, as numerous nocturnal insects have evolved from diurnal ancestors, which had apposition compound eyes adapted for bright conditions. These nocturnal insects are further constrained to extract adequate photons from a dark environment (Warrant, 2004). However, the fact that numerous nocturnal insects have apposition eyes is a testament to both the dim-light functional abilities of apposition eyes (Greiner, 2006; Kelber *et al.*, 2011), as well as the selective pressures on organisms to exploit the nocturnal niche.

Apposition eyes have photoreceptors receiving light from a single facet lens, which increases acuity at the cost of sensitivity; this anatomy works well when light is readily available, but is very limiting in dim conditions (Kirschfeld, 1974; Land & Nilsson, 2012). Although apposition eyes are inherently less sensitive than superposition eyes, there are many optical adaptations that improve visual sensitivity, including larger eyes and ommatidial facets, which allow more light to enter the photoreceptors (Greiner *et al.*, 2004a; Frederiksen & Warrant, 2008). Furthermore, longer and wider rhabdoms (the cluster of photoreceptors in an ommatidium) also increase sensitivity by improving the likelihood that a photon will be absorbed by photopigments in the rhabdom; the bigger the rhabdom, the more photopigment to absorb photons (Greiner *et al.*, 2004a; Warrant & Dacke, 2011). Many of these adaptations are found in the nocturnal Indian Carpenter Bee, *Xylocopa tranquebarica*, with large eyes and very wide rhabdoms leading to eyes 27 times more sensitive than *Xylocopa leucothorax*, the related diurnal Indian Carpenter Bee (Somanathan *et al.*, 2008, 2009). Furthermore, eye size tends to

increase with body size, and larger individuals should therefore have better sensitivity (Seymoure, McMillan, & Rutowski; Greiner *et al.*, 2004a). However, larger eyes, larger facets, and other morphological adaptations can only improve sensitivity up to a factor of 1000, which fails to counteract the 9 orders of magnitude reduction of available light at night (Warrant, 1999; Warrant & Nilsson, 2006). Visual systems can further increase sensitivity through neural mechanisms such as enhanced response gain, and spatial and temporal summation of photoreceptors (Laughlin, 1990; Warrant, 1999). Through these mechanisms visual sensitivity can be increased by another factor of 1000 without additional photon capture (Laughlin, 1990), but with a decrease in spatial acuity (Warrant, 1999).

Although the major differences in visual sensitivity between taxa result from visual morphology and physiology, there is also large variation within species, especially between the sexes (Ziemba & Rutowski, 2000; Turlure *et al.*, 2016). In insects, females are often larger than males, yet males tend to have larger eyes (Seymoure *et al.*; Ziemba & Rutowski, 2000; Rutowski, 2000). However, these findings of sexual differences come from non-social insects, and our understanding of sexual differences in eye morphology of social hymenopteran insects is lacking. This is a large knowledge gap in our understanding of nocturnal vision in insects, as most nocturnal apposition eye knowledge is a result of studying female bees and ants and not males (Somanathan & Borges, 2001; Greiner *et al.*, 2004a, 2007; Warrant *et al.*, 2004; Somanathan *et al.*, 2008; Warrant & Dacke, 2011). It is probable that intersexual differences in visual morphology and physiology exist in Hymenoptera, as sexes differ greatly in their natural history and thus likely have different selection pressures directly relevant to vision.

The sweat bee genus *Megalopta* (Hymenoptera: Halictidae) offers a unique system to study the evolutionary effects of selection for dim light foraging. The genus contains multiple nocturnal species, including species that differ in size but live sympatrically and have similar natural histories. *Megalopta* bees have apposition eyes with many of the adaptations mentioned

above to increase sensitivity (Greiner *et al.*, 2004a, 2007; Warrant *et al.*, 2004). *M. genalis* and *M. amoena* both forage through the understory of the forest in very dim light during twilight (Fig. 1; (Roulston, 1997; Kelber *et al.*, 2006; Smith *et al.*, 2012). Kelber et al. (2005) measured the light levels at which these bees foraged to find that individuals can forage in light levels as low as approximately .0005 lux, similar to new moon ambient night lighting with clear skies (Fig. 1). Further, female bees of *M. genalis* and *M. amoena* are facultatively eusocial; some nests are social, with an egg-laying queen that typically does not forage and a non-reproductive worker that acts as the primary forager for the nest (Wcislo *et al.*, 2004; Smith *et al.*, 2012). Queens are on average larger than their solitary counterparts, and it has been suggested that smaller nest-founding females are not queens because they cannot behaviorally dominate offspring to stay in the nest as workers (Arneson & Wcislo, 2003). Furthermore, this body size variation has led to eye size variation, which may result in different visual sensitivities (Wcislo *et al.*, 2004; Tierney *et al.*, 2013). Although the natural history of *Megalopta* females has been studied, little is known about the behavior of males. Males likely rely upon visual information to search for mates, and may be under stronger selection pressure than females to visually cope with low light availability (Ziemba & Rutowski, 2000; Rutowski, 2000).

Many authors have compared the visual adaptations of female *Megalopta genalis* with species occupying non-nocturnal niches (Greiner *et al.*, 2004a; Greiner, Ribi, & Warrant, 2005; Wcislo *et al.*, 2004; Kelber *et al.*, 2006; Frederiksen, Wcislo, & Warrant, 2008; Baird *et al.*, 2011). To date, we lack an understanding of visual system variation within *Megalopta* bees - across individuals of the same sex, between the sexes, and across species. Here, we aim to test the hypothesis that individual differences in absolute visual sensitivity within and between *Megalopta* species are dependent upon size, sex, and eye morphology. In many animals, larger individuals have larger eyes and increased light sensitivity (Rutowski, Gislén, & Warrant, 2009; Cronin *et al.*, 2014). Accordingly, we predict that larger *Megalopta* individuals will also have

increased retinal sensitivity. More specifically, we predict that *M. genalis* will have greater sensitivity than *M. amoena,* and that males will have greater sensitivity than females due to larger eyes and the pressure to visually detect females under dim-light conditions. To test these predictions, we collected males and females of both species at light traps, measured eye size, facet size, number of facets, and estimated retinal sensitivities using electroretinograms. This investigation compares inter- and intraspecific visual sensitivity in *Megalopta* bees to better understand the physiological and behavioral ecology of dim-light vision.

Materials and Methods

Animal Collection

Megalopta genalis and *M. amoena* both occupy tropical rainforests in Central and South America and are commonly captured at the same light traps during their dim-light foraging bouts (Roulston, 1997; Smith *et al.*, 2012). We utilized light trapping methods to collect males and females of both species from May 18-31, 2014 along Pipeline Road in Parque Nacional Soberanía, Panama (collection permit No. SE/A-28-14 from the National Authority for the Environment of the Government of Panama). We mounted mercury vapor bulbs over white sheets to attract *Megalopta* individuals during the peak times of *Megalopta* activity, from 4:45 am – 6:15 am and 6:30 pm – 7:30 pm (Fig. 1; (Wcislo *et al.*, 2004; Kelber *et al.*, 2006). Bees were placed individually into plastic cups with feeders of 50:50 by volume honey:water and transported to a dark room where they were kept at ambient outside temperatures (ca. 21 C to 28 C) for at least 24 hours prior to preparation for electroretinograms.

Electroretinograms (ERG)

To test for overall visual differences between individuals we utilized electroretinogram (ERG) recordings. ERG is a commonly used technique to measure visual sensitivity of insect eyes (Swihart, 1964; Loew, 1975; Eguchi *et al.*, 1982; French *et al.*, 2015) and as we were comparing physiological sensitivities between individuals, much of the uncertainty inherent in ERG measurements is negated as long as recording site and stimulation protocol are identical for all animals (French *et al.*, 2015; Honkanen *et al.*, 2018). We recorded retinal sensitivity using ERG electrodes in the central anterior location of the right eye for each individual. Previous research shows that *Megalopta* females have large facets in this eye region, presumably for increased visual sensitivity (Greiner *et al.*, 2004a).

In order to mitigate circadian effects on visual sensitivity, all bees were assayed during natural morning foraging times, from 4:30 am to 7 am. For at least 24 hours prior to recording, bees were dark-adapted, and all experimental procedures were conducted under red light conditions. We secured individual bees into a cut 200 uL pipette tip with electrical tape such that their head was free, but their thorax and abdomen were fixed inside the tip. This tip was then mounted into a holder within a custom enclosure, diagrammed in Fig. S1. A steel pin (BioQuip Insect Pin, Stainless, #00) was sharpened and used to make a small puncture in the central anterior portion of the right eye lens. The stainless-steel recording electrode (BioQuip Insect Pin, Stainless, #00) was then guided approximately 1mm into the retina through the small puncture. A stainless-steel reference electrode (BioQuip Insect Pin, Stainless, #00) was inserted into the left side of the thorax. These electrodes were attached to a variable-gain, differential amplifier with output digitized to 12-bit precision. A custom program written in LabVIEW (National Instruments) acquired, filtered, and stored the data as well as controlled the stimulus parameters (intensity and duration). The stimulus was provided by a 5mm white-light emitting diode (LED, Fig. S1a) that illuminated the central anterior region of the eye, although other regions of the eye were also indirectly exposed to the light source. The light intensity was

controlled by the voltage output of a digital to analog converter controlling the current to the LED. To confirm correct placement of the electrodes, a low intensity (2.8 volts) white light stimulus was pulsed for 10 milliseconds at 2 flashes per second. When the electrode was placed correctly, a typical ERG response (Fig. S2a) was noted for each flash and the light was turned off. A lack of signal or excessive 60 Hz noise indicated improper placement and the electrode was removed and placed again. If the second placement was also unsuccessful in eliciting a typical response, the bee was not included in the experiment.

Following the above procedure, bees were kept in complete darkness for 5 minutes prior to starting a trial. Bees were then exposed to white-light stimulation, starting at 2.3 volts (10^{-4} lux), and incremented in steps of 0.05 volts until a criterion response was observed (Fig. 1, Fig. S1b-c; Fig. S2; Table S1). Throughout the manuscript we refer to both voltage and lux in order to compare with previous studies; we controlled the voltage of the stimulus, but we also present light values in lux as currently this photometric unit of illuminance is what has been measured in the field for *Megalopta (Kelber et al., 2006)*. We recognize that photometric units are dependent upon the photopic visual sensitivity of humans and are not appropriate for understanding the visual ecology of other organisms (Johnsen, 2012), thus we also include irradiance in photon flux where appropriate (see Fig. 1, Fig. S1b, Fig. S1c, and Table S1). We chose to start at 2.3 volts as this light intensity of 10^{-4} lux is representative of the light conditions at night and thus darker than the twilight conditions when the bees are active (Fig. 1; (Kelber *et al.*, 2006; Veilleux & Cummings, 2012). After the initial response was observed, the voltage was increased incrementally by 0.01 volts to further define the minimum response threshold for each bee. The bee was then stimulated with intensities at 0.05 volt increments until 4.0 volts (6 lux) was reached (see Fig. 1 for range of ERG light intensities in lux and photon flux relative to conditions during foraging periods; see Table S1 for light intensities at voltage steps used during ERGs).

To allow for comparison between the light levels that the individual bees were subjected to during ERGs and the light levels that *Megalopta* experience during their activity bouts, irradiance was measured in the ERG chamber for the range of voltages used. As each bee was tested with a light intensity of 2.3 volts to 4.0 volts, we measured the spectroradiometry in tenths of volts starting with 2.3 volts and ending at 4.0 volts. We measured irradiance, defined as the amount of light striking a surface, with a CR2 UV-VIS_NIR cosine receptor (StellarNet, Tampa, FL) attached via a 1000μm fiber optic cable (F1000-UVVis-SR-1, StellarNet) to a low-light sensitive spectroradiometer (SILVER-Nova-TEC-X2, StellarNet) (Seymoure, Linares, & White, 2019). All measurements were taken in a dark room to confirm that only light from the LED stimulus was illuminating the ERG chamber, and each voltage was replicated with two irradiance measurements. The irradiance measurements were logged as microwatts/cm^2/s/nm and then converted to both lux and photon flux. Lux is a photometric unit that applies the human photopic sensitivity to the spectra and then integrates the spectra (Johnsen, 2012). Photon flux is the integral of the quanta over a specified nanometer range. We integrated across 300nm to 700nm for the values presented in Figure 1 and Supplemental Table 1. We believe that it is important to provide both lux and photon flux as research in this field has used both methods and both have their advantages. Although photon flux is the more biologically relevant measurement for non-primate studies, most research has relied on measuring lux because the equipment for measuring lux is both cheaper and more intuitive. Therefore, to make the data reported here more applicable to previous research, we include both units. Lastly, as we integrated the spectra for each irradiance measurement, the spectral shape is lost. However, we include the spectra in Figure S1c.

ERG data were processed in MATLAB and smoothed with a finite impulse response window lowpass filter generated from the FIR1 function (MATLAB(R) 8.3 and the Signal Processing Toolbox 6.21). A 100-order Hamming window was used with a cutoff frequency of

50 Hz to remove high-frequency noise. The peak response voltage was determined as the difference between the minimum voltage between 15 and 50 ms and the baseline, taken as the average voltage between 100 and 150 ms (see Fig. S2). To estimate sensitivity to light, a linear regression of peak intensity as a function of LED irradiance was performed for trials with LED voltages between 3 volts and 4 volts. Input voltages were converted to irradiance values by linear interpolation between calibration points acquired from 2.3 to 4 volts at 100 millivolt intervals. The slope of the regression was used as the statistic for sensitivity for each individual.

Following processing of the raw ERG data, the noise for each ERG trace was visibly reduced but the shape of each trace was largely unaffected (Fig. S2a vs. S2b). The same processing was used for all ERGs prior to analysis. An example of the processed ERGs for one male and one female *M. genalis* are shown in Fig. S2.

Preparation of corneas

Since the right eye of each bee was punctured for the ERG measurement, the left eye was prepared for facet and corneal measurements. The head of each bee was placed into 20% NaOH overnight, then the cornea was peeled from the cuticle (Seymoure *et al.*; Turlure *et al.*, 2016). Small cuts were made on the perimeter of the cornea until it could be laid flat onto a microscope slide, and the cornea was sealed with CytoSeal 60 (Richard-Allan Scientific, Chesire, UK) and a coverslip as in (Seymoure *et al. 2015*).

Corneas were photographed at 20x magnification to obtain corneal area for each bee by tracing the outline of each cornea. Five regions of the eye were photographed at higher magnification (400x) so that the diameter of facets in each region could be measured by two independent observers (Seymoure *et al.*). For a subset of bees (3 individuals per sex per species; 12 total), individual facets were counted for the entire cornea. Due to imperfections in the corneal preparation and difficulty of facet counting, at least two observers blind to individual

identity counted facets for each measured bee and counts were averaged across observers (R^2=0.93).

Data Analysis

Analysis of electroretinogram data was performed in MATLAB (MATLAB(R) 8.3) as described above. All other statistical analyses were conducted using R (version 3.1.2). Scripts for ERG smoothing and data analysis are available from the authors upon request.

Results

M. genalis was significantly larger based on head width, a commonly used proxy of body size in bees (Kamm, 1974; Alcock, 1984; Plateaux-Quenu & Plateaux, 1993) (Fig. 2a, p=7e-07, ANOVA with Tukey-HSD post-hoc) and had larger corneas than *M. amoena* (Fig. 2b, p=1e-07, ANOVA with Tukey-HSD post-hoc). For both *Megalopta genalis* and *M. amoena,* females were significantly larger than males (Fig. 2a, *M. amoena* p= 0.0108640, *M. genalis* p=0.0000617, ANOVA with Tukey-HSD post-hoc). Within species, no difference in corneal area was found between sexes (Fig. 2b; *M. amoena* p=0.31, *M. genalis* p=0.99, ANOVA with Tukey-HSD post-hoc). The largest absolute corneas belonged to two *M. genalis* males, whereas the smallest corneas were from *M. amoena* females. However, when considering body size, *Megalopta* males of both species had larger eyes than females (Fig. 2c, *M. amoena* p=3e-06, *M. genalis* p=1.47e-06; ANOVA, fixed effect of sex with head width). Larger-bodied individuals had larger eyes (Fig. 3a; p<0.0001, R^2=0.8926 for linear regression), with eye:body allometry scaling elevated in males of both species compared with females (Fig. 3b, Fig. 3c; p<0.0001 for differences in intercept between sexes of both species, p=0.094 and p=0.268 for differences in

slope between sexes in *M. amoena* and *M. genalis,* respectively), consistent with their larger relative corneas.

Facet number was strongly associated with corneal area across sexes and species (Fig. 4a; $p<0.001$, $R^2=0.868$, linear regression) and the larger species, *M. genalis*, had approximately 27% more facets ($p<0.001$, mean of 4454.3 in *M. genalis* compared to mean of 3495.6 in *M. amoena*; ANOVA). Average facet diameter was larger in *M. amoena* males compared to *M. amoena* females (Fig. 4b, Table S2; $p=0.02$, ANOVA with Tukey-HSD post-hoc), driven in part by a difference in anterior facet length, but no difference in facet diameter between *M. genalis* sexes was observed in any eye region ($p=0.79$; ANOVA with Tukey-HSD post-hoc). Overall, anterior, lateral and ventral facets were largest, followed by posterior facets, with dorsal facets significantly smaller than any other region (Fig. 4b). Only anterior facets showed variation explained by sex (Fig. 4b, $p<0.001$, ANOVA with Tukey-HSD post-hoc), while all other regions were not statistically different between sexes.

The range of minimum physiological sensitivity across individuals measured by electroretinogram (ERG) was 2.35 volts (ca. 0.001 lux) to 3.2 volts (ca. 0.1 lux), with an average of 2.56 volts (ca. 0.01 lux) (Fig. 1). No difference in minimum response threshold was observed when comparing sexes or between species (Fig. 5A; $p>0.10$). Males of both species showed an increase in retinal sensitivity, measured from the slope of ERG responses across a range of light levels (Fig. 5b; *M. amoena* $p=0.015$, *M. genalis* $p=0.019$, ANOVA with Tukey-HSD post-hoc). Retinal sensitivity was not explained by corneal area ($p=0.0868$) and was negatively associated with head width across individuals (Fig. 5C; $p=1.445e-04$, $R^2=0.2661$; linear regression).

Discussion

We describe intra- and interspecific variation in visual morphology and sensitivity of crepuscular halictid bees, species with exceptional visual sensitivity despite apposition compound eyes. We found substantial body and eye size variation both within and across sexes of *Megalopta genalis* and *M. amoena,* similar to what has been described for other bee species (Roulston & Cane, 2000). The scaling of eye size relative to head width is elevated in males relative to females, especially in the smaller species, *M. amoena.* This suggests that males of these species may rely upon visual systems more than females, or that females may be more constrained allometrically with respect to eye size. Both species nest in woody stems and vines, and females possess mandibles and associated mandibular muscles for chewing nest tunnels (Wcislo *et al.*, 2004; Tierney *et al.*, 2013). In comparison, males have reduced mandibles and thus may have greater flexibility for both external and internal expansions of visual tissue. Small females may be especially constrained in terms of eye size, since they still require the mandibular strength and support to chew through wood despite their small head size.

Megalopta genalis and *M. amoena* are active during twilight hours when ambient lighting ranges from 0.001 lux to 100 lux (Kelber *et al.*, 2006; Johnsen, 2012; Veilleux & Cummings, 2012). Accordingly, we found that the retinal sensitivity of these two species matches this large range in light intensity. Few individuals had minimal retinal sensitivities near the dimmest recorded observation of *Megalopta* behavior, approximately 0.001 lux (Kelber *et al.*, 2006), while most were sensitive to low light levels of 0.01 lux, which matches published foraging bouts of *Megalopta* (Warrant *et al.*, 2004; Kelber *et al.*, 2006; Veilleux & Cummings, 2012). Our results are consistent with the expectation that *Megalopta* individuals begin to leave the nest as soon as they can detect the visual scene (Greiner *et al.*, 2004a; Kelber *et al.*, 2006; Baird *et al.*, 2015). In addition, we found that morphological features of *Megalopta* eyes differ between

353 sexes and across species, with potential important implications for the ecological success of
354 these species.
355 Males of both species had larger corneas relative to body size than females, consistent
356 with sexual differences in many insect species (Seymoure *et al. 2015*; Ziemba & Rutowski,
357 2000; Rutowski, 2000; Rutowski *et al.*, 2009). The sexual difference in eye size suggests that
358 males invest relatively more in visual systems, which supports the hypothesis that sexual
359 differences in behavior exert differential demands on visual systems of males and females,
360 favoring males with larger eyes for better mate detection (Zeil, 1983; Alcock & Thornhill, 2014).
361 Currently, little is known about male behavior in *Megalopta* species, but if males use vision to
362 locate females and/or select mates, larger eyes would be beneficial both for increasing
363 sensitivity and acuity, which would improve mate detection in dimmer conditions (i.e. earlier in
364 the morning) and from further distances (Warrant & Nilsson, 2006).
365 Although there was considerable variation between sexes and species in eye size, facet
366 number, and facet size, differences in retinal sensitivity were not explained by this
367 morphological variation. The larger species, *M. genalis*, had larger eyes and more facets, yet
368 they did not have larger facets nor more sensitive retinas than their smaller congeners, *M.*
369 *amoena*. Interestingly, males of both species, who are smaller-bodied but have relatively larger
370 eyes than females, showed enhanced retinal sensitivity. Although males appear to invest more
371 in eye tissue, retinal sensitivity was not explained by absolute or relative corneal area,
372 suggesting that neurophysiological adaptations may heavily influence visual sensitivity in
373 *Megalopta,* consistent with previous hypotheses (Greiner *et al.*, 2004b,a, 2005). Although our
374 results suggest that variation in morphology is not predictive of visual sensitivity differences in
375 *Megalopta,* it is also possible that overall retinal sensitivity measurements do not reflect
376 regionally-specific sensitivity of the visual field. We find little evidence for consistent facet size
377 differences at either the sex or species level, but more fine scale measurements of visual acuity

and corneal mapping may identify further differences between species and sexes that explain visual variation in *Megalopta.* Still, the ERG thresholds measured in this study match ecologically relevant light levels with respect to *Megalopta* behavior, suggesting that males have behaviorally relevant increases in overall visual sensitivity compared with females.

Although *M. genalis* was larger than *M. amoena*, no significant differences in facet diameter were identified between species. These results support hypotheses that *Megalopta* individuals are operating at their limits of visual sensitivity (Warrant, 1999; Frederiksen *et al.*, 2008), with minimum morphological constraints in facet size that allow for successful crepuscular foraging. Smaller individuals appear to reduce the number of facets while maintaining facet size across regions of the eye, further sacrificing visual acuity relative to larger individuals while preserving sensitivity (Warrant & Nilsson, 2006). Similarly, although males were smaller than females in both species, males had similarly sized facets across most eye regions compared with females, with males of *M. amoena* having even larger anterior facets. This sexual dimorphism matches findings in multiple species of honey bees where drones have much larger facets than queens and workers (Streinzer *et al.*, 2013). However, the sexual dimorphism in eye morphology in carpenter bees is dependent upon species. Males of two diurnal species, *Xylocopa tenuiscapa* and *X. leucothorax*, have larger or similarly sized facets to females, respectively, whereas males of the nocturnal species, *X. tranquebarica*, have larger eyes than females but similarly sized facets (Somanathan *et al.*, 2017). Our study suggests that in *Megalopta* species, there may be convergence on similarly sized facets between the sexes and across species.

The observed increase in retinal sensitivity for males was not explained by any of the morphological features we measured, suggesting additional mechanisms to increase sensitivity in *Megalopta*. However, there are several morphological mechanisms that could increase retinal sensitivity that we did not measure, including differences in rhabdom width and length which are

known to vary within and across bees (Greiner *et al.*, 2004a; Warrant & Nilsson, 2006; Somanathan *et al.*, 2017). Furthermore, numerous physiological and neurological mechanisms may be at play to increase retinal sensitivity, including neural summation (Theobald *et al.*, 2006). Facultatively nocturnal European hornets (*Vespula vulgaris)* have dim-light vision constrained by eye morphology, however this species likely copes through neural pooling to enable dim light activity (Kelber *et al.*, 2011). Intriguingly, *Megalopta* females have previously been found to possess elevated dendritic arborization of visual interneurons relative to diurnal bee species, with a potential role in spatial summation (Greiner *et al.*, 2004b,a, 2005). Our results suggest that males may have even greater abilities for spatial (and/or temporal) summation of photon capture, enabling them to increase their sensitivity even beyond that of females. Future work comparing both photoreceptor morphology (e.g., rhabdom width) and the neural networks of males and females of nocturnal bee species will provide a fruitful avenue of understanding the relationship between eye morphology, neuronal organization, and visual sensitivity.

Conclusions

Here we report that crepuscular sweat bees have retinal sensitivities that match the dim light environments in which they actively fly. We also discovered visual differences between both sexes and species of *Megalopta* despite similar minimal light thresholds that elicit a retinal response across individuals. Interestingly, we found that males of both species have heightened retinal sensitivity at some light levels, even with their smaller size and smaller corneas. We hypothesize that males experience stronger selection for enhanced retinal sensitivity for finding females and/or navigating their environment. We found little evidence for morphological traits which underlie this increased sensitivity, suggesting that retinal sensitivity in males is enhanced through physiological and neurological mechanisms.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

B.M.J., E.R.L. and B.M.S. designed the study. B.M.J. and B.M.S. performed experiments and wrote the manuscript. T.J.C. analyzed data and helped draft manuscript. All authors edited the manuscript and gave final approval for publication.

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Supplemental Table 1. Intensities of light stimulus in lux and photon flux for the range of voltages used in electroretinograms. As the ERG light stimulus was controlled by voltage, we ran each individual through intensities of light starting with 2.3 V and ending with 4.0 V. However, as voltage is not indicative of light level, we measured the irradiance of light in the ERG chamber and have calculated both photometric (lux) and radiometric (photon flux) units for the light stimulus at a range of voltages.

Supplemental Table 2. Facet diameter measurements given as mean ± standard deviation (unit: μm) for 5 different eye regions, as well as average facet diameter across regions. *Megalopta* species are shown separately, and males and females are separated for each species. P-values below each column are results from a two-sample *t*-test comparing sexes within each species and eye region. N.S. = Not significant.

Figure Legends

Figure 1. Irradiance levels for natural conditions of *Megalopta* and electroretinogram stimuli. Both the photometric unit of illuminance (lux, on left y-axis) and the more biologically appropriate radiometric unit of irradiance (photon flux, on right y-axis) are presented for solar elevations at which *Megalopta* species are active. Light levels for differing environmental conditions (e.g., clear and open habitat to forest understory) are shown based on Kelber et al. 2005, Johnsen 2011 and Seymoure et al. 2019. *Megalopta* leave the nest with light levels as dark as astronomical twilight (~.001 lux) and may return as late as sunrise (~1000 lux). The light intensities for electroretinogram (ERG) measurements ranged from .0005 lux to 6 lux, representing the lower range of light levels relevant to *Megalopta* foraging (all individuals showed high levels of stimulation at 6 lux).

Figure 2. Morphology of *Megalopta* individuals. Comparison of **(A)** head width, **(B)** corneal area, and **(C)** relative corneal area for females and males of *M. amoena* and *M. genalis.* The median of the data is represented by the horizontal line within each rectangle, upper and lower boundaries are interquartile ranges, and whiskers indicate range without statistical outliers. Individual bees are represented as open circles (females) or filled triangles (males) for *M. amoena* in yellow and *M. genalis* in blue. n = 46 (13 *M. genalis* females, 11 *M. genalis* males, 13 *M. amoena* females and 9 *M. amoena* males). *** $p<0.0001$, * $p<0.05$, N.S.=Not significant

(ANOVA with Tukey-HSD post-hoc).

Figure 3. Eye:body allometry for *M. amoena* and *M. genalis.* Lines represent linear regressions (all $p<0.01$) for each species and sex combination (R^2 for *M. genalis* males: 0.8046, *M. genalis* females: 0.8016, *M. amoena* males: 0.6559, *M. amoena* females: 0.5039). For both species, males have a significantly higher intercept, indicative of larger corneal area relative to body size. The slopes of the regressions do not differ among groups (ANCOVA, $p>0.05$). Individual bees are represented as open circles (females) or filled triangles (males) for *M. amoena* in yellow (**A** and **B**) and *M. genalis* in blue (**A** and **C**). n = 45 (13 *M. genalis* females, 11 *M. genalis* males, 12 *M. amoena* females and 9 *M. amoena* males).

Figure 4. Variation in facet number and size across *Megalopta* individuals. (A) Facet number regressed against corneal area for select individuals from both species and sexes. Individual bees are represented as open circles (females) or filled triangles (males) for *M. amoena* in yellow and *M. genalis* in blue. n=3 individuals per group. $R^2=0.868$, $p<0.0001$ for linear regression. (B) Facet diameters in micrometers for each sex and species grouped by eye region. Different letters indicate significant differences among eye regions ($p<0.05$, ANOVA with Tukey-HSD post-hoc), and different numbers represent significant differences within each region ($p<0.05$, ANOVA with Tukey-HSD post-hoc). Numbers are shown only for regions with significant differences among groups.

Figure 5. Comparison of visual sensitivity of *Megalopta* bees. (A) Minimum physiological sensitivity as measured by electroretinogram (ERG) and **(B)** Relative sensitivity (change in ERG response as a function of LED intensity, normalized to the maximum sensitivity observed) for all individuals. For **(A)** and **(B)**, the median of the data is represented by the horizontal line within each rectangle, upper and lower boundaries are interquartile ranges, and whiskers indicate range without statistical outliers. **(C)** Relative sensitivity regressed against head. Individual bees are represented as open circles (females) or filled triangles (males) for *M. amoena* in yellow and *M. genalis* in blue. n = 46 (13 *M. genalis* females, 11 *M. genalis* males, 13 *M. amoena* females and 9 *M. amoena* males). * $p<0.05$ (ANOVA with Tukey-HSD post-hoc).

Supplemental Figure 1. ERG Setup, Voltage, and Spectra. A) Diagram of electroretinogram (ERG) setup. Bee is restrained in a shortened pipette tip, and a reference electrode is inserted

into the lateral thorax. An insect pin is used to make an incision into the anterior portion of the right eye, and a working electrode is inserted through the incision into the retina. The stimulus LED illuminates the right side of the bee with white light. A cork was placed to the right of the light aperture for control recordings. **B)** Spectra of the light stimulus at different intensities across the visually relevant spectrum (300nm-700nm). The spectra are plotted on a log scale to enable viewing of the dim-light intensities. Each spectrum represents one of two measurements at a voltage ranging from 2.3 V to 4.0 V. We present irradiance (photon flux) on the y-axis and select voltages are highlighted next to the respective line. The parenthetical values represent the lux (lx) value for each voltage. **C)** Light levels in photon flux of ERG voltage. The integrated intensities of the ERG stimulus for both the photometric unit of illuminance (lux) and the radiometric unit of irradiance (photon flux). Photon flux was integrated across 300nm to 700nm. The lowest light level matches natural conditions

Supplemental Figure 2. Example of ERG smoothing and analysis from one male and one female of *M. genalis.* A) Unfiltered ERG trace of male *M. genalis* individual at 4.0V. **B)** Trace in panel A following low pass filtering with peak and baseline regions used for sensitivity measurements outlined in dashed boxes. **C)** Control stimuli ERG curves for male *M. genalis* individual (female not shown). **D)** ERG traces of female *M. genalis* (head width: 3.935 mm, corneal area: 3.757 mm^2) when stimulated with light from 2.5 (0.01 lux) to 4.0V (6.12 lux). **E)** Traces of male *M. genalis* (head width: 3.237 mm, corneal area: 3.393 mm^2) when stimulated with light from 2.5 (0.01 lux) to 4.0V (6.12 lux). Inset shows heatmap corresponding to different light intensities and scale for all figures. Note: with increasing light stimulus, the magnitude of response increased and the latency to respond was reduced, shown by the peak shift. Note that the female shown in S2D had a larger head width and corneal area compared with the male in S2E but demonstrated a reduced response relative to the male.

676 **Supplemental Table 1**

Voltage	Lux	Photon Flux	Natural Equivalent
2.3	.0005	4.73E+12	Starry New Moon Sky
2.4	.0045	3.73E+13	Crescent Moon
2.5	.0130	1.08E+14	Quarter Moon
2.6	.0250	2.06E+14	Quarter Moon
2.7	.0386	3.23E+14	Gibbous Moon, Nautical Twilight
2.8	.0528	4.41E+14	Gibbous Moon, Nautical Twilight
2.9	.0690	5.77E+14	Gibbous Moon, Nautical Twilight
3.0	.0960	8.04E+14	Gibbous Moon, Nautical Twilight
3.1	.1912	1.62E+15	Full Moon, Nautical Twilight
3.2	.4552	3.76E+15	Nautical Twilight
3.3	.8593	7.19E+15	Nautical Twilight
3.4	1.3716	1.15E+16	Nautical Twilight
3.5	1.9361	1.62E+16	Nautical Twilight
3.6	2.5338	2.12E+16	Nautical Twilight
3.7	3.1950	2.67E+16	Nautical Twilight
3.8	3.7696	3.14E+16	Civil Twilight
3.9	4.5012	3.78E+16	Civil Twilight
4.0	6.1198	5.15E+16	Civil Twilight

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680 **Supplemental Table 2**

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M. genalis						
	Anterior	Posterior	Ventral	Dorsal	Lateral	Average
Male	38.74±4.75	32.91±1.34	35.98±4.29	27.21±2.94	36.63±3.32	34.29±2.16
Female	34.50±2.13	34.60±2.13	34.90±2.32	28.83±2.59	35.55±2.31	33.68±1.78
	*p=0.0165	*p=0.02484	N.S.	N.S.	N.S.	N.S.
M. amoena						
	Anterior	Posterior	Ventral	Dorsal	Lateral	Average
Male	38.87±4.95	33.62±2.50	36.40±4.86	26.88±4.07	36.93±2.99	34.54±1.91
Female	33.64±2.54	33.74±1.59	34.26±2.09	27.14±2.47	34.36±2.15	32.63±1.18
	*p=0.01408	N.S.	N.S.	N.S.	*p=0.04445	*p=0.01991

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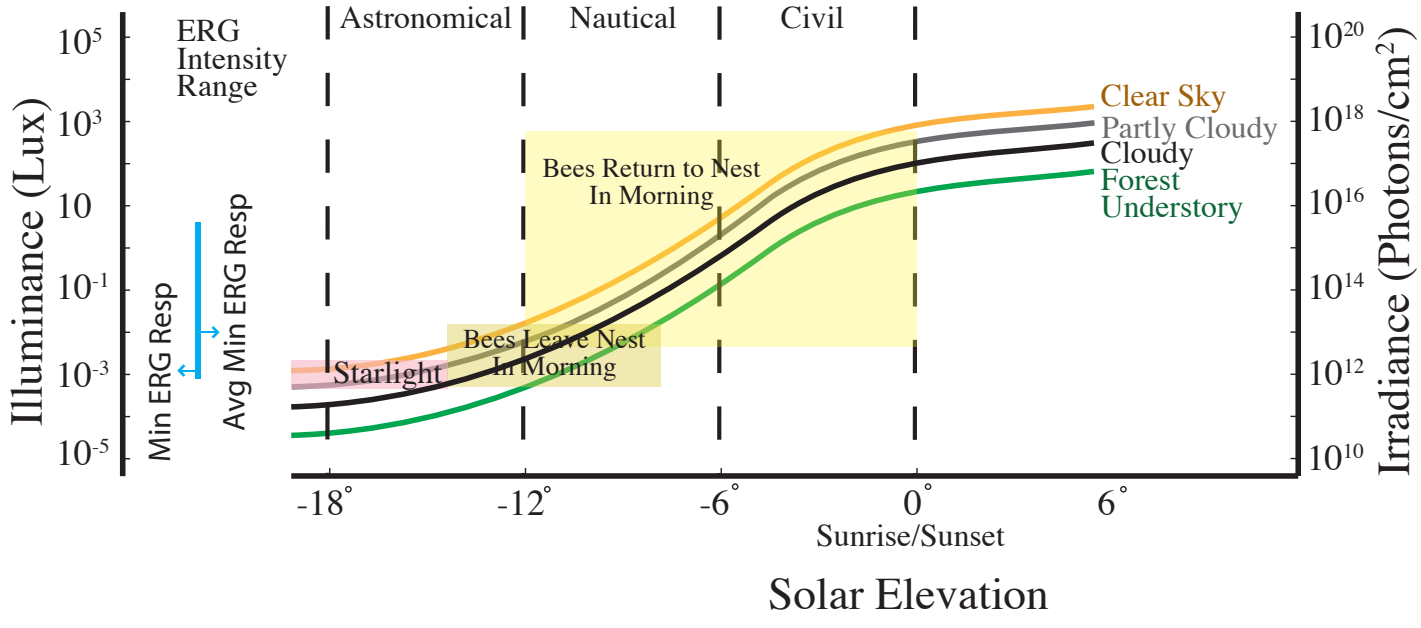

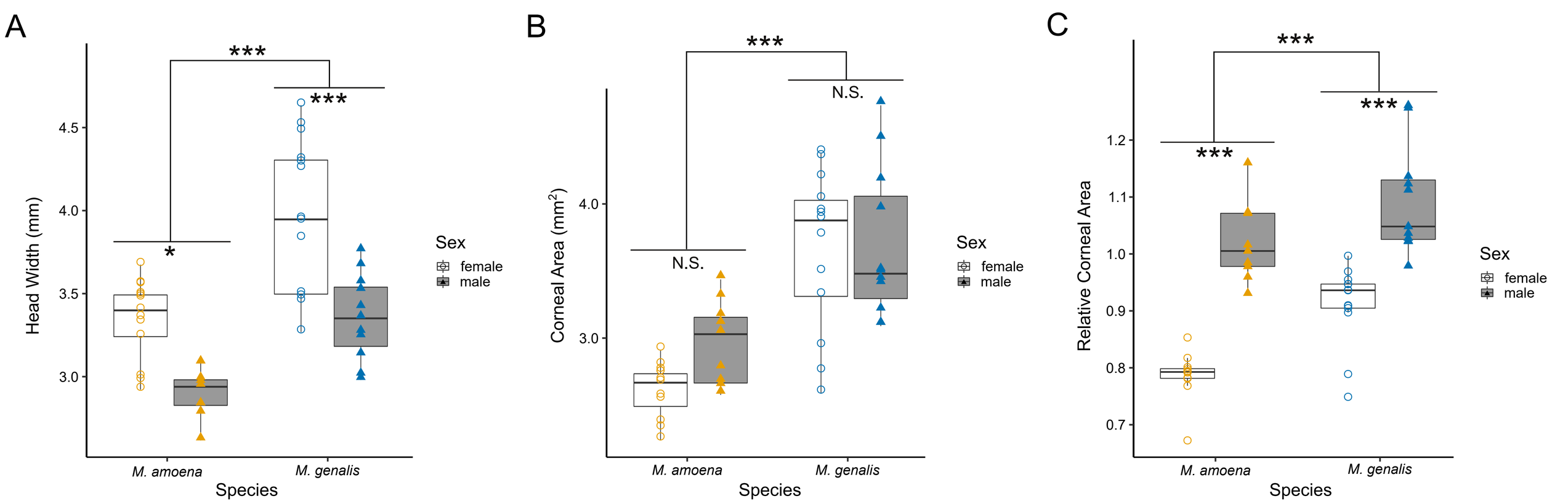

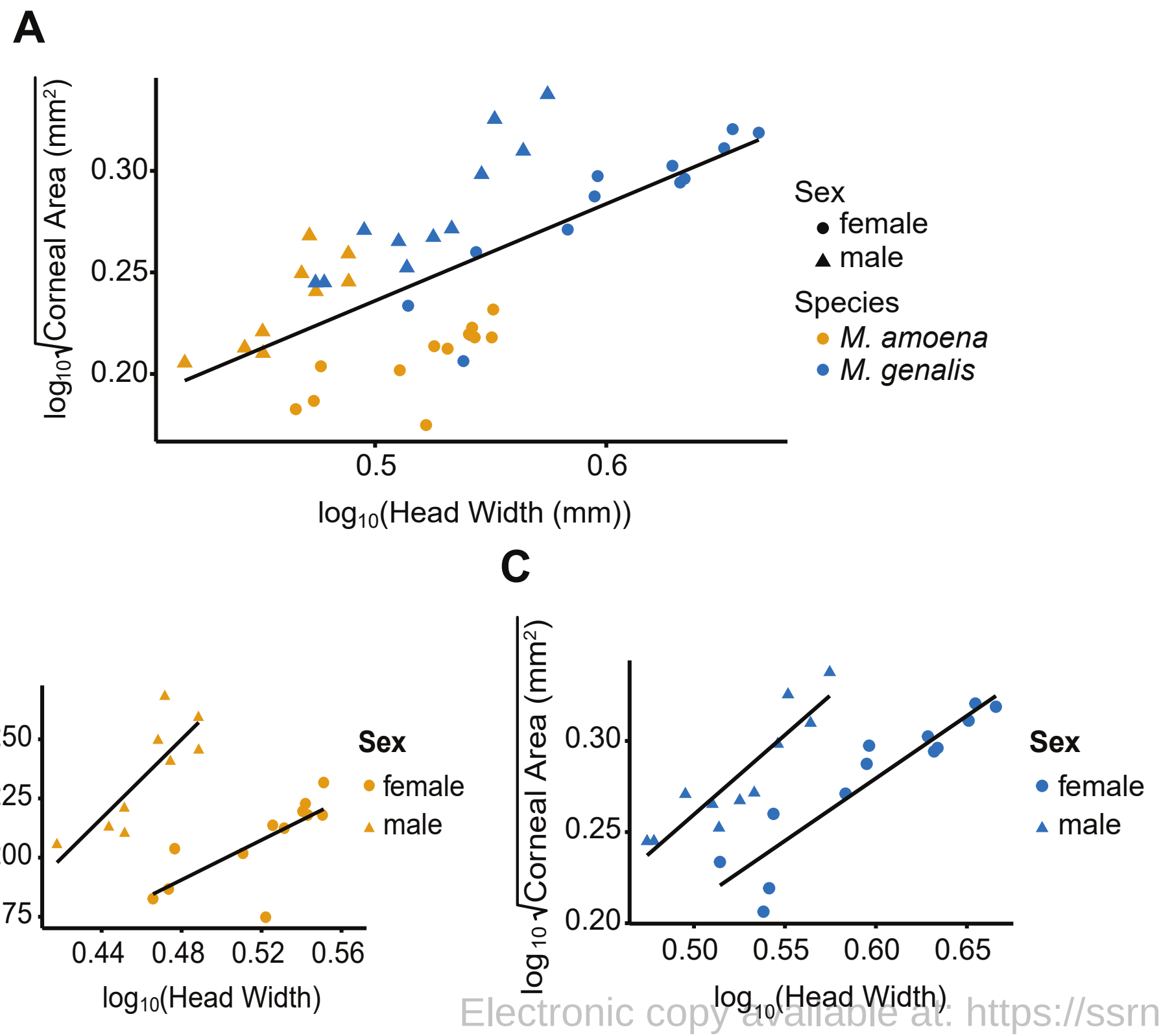

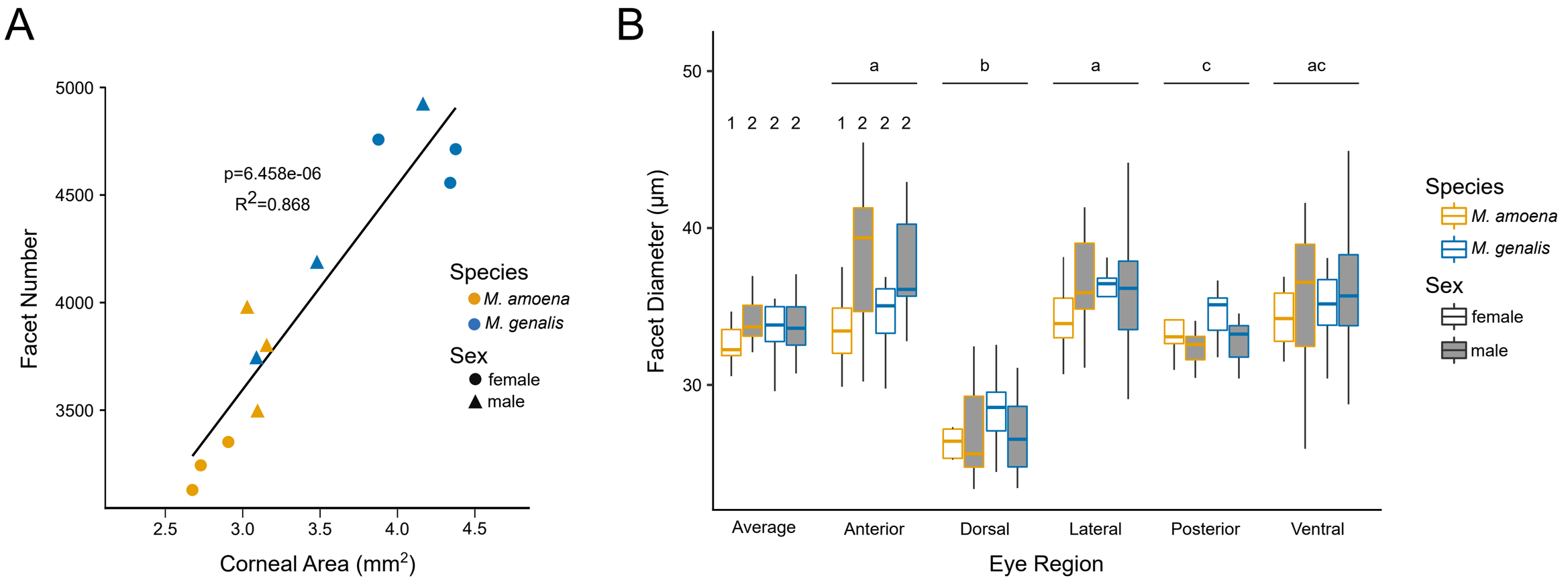

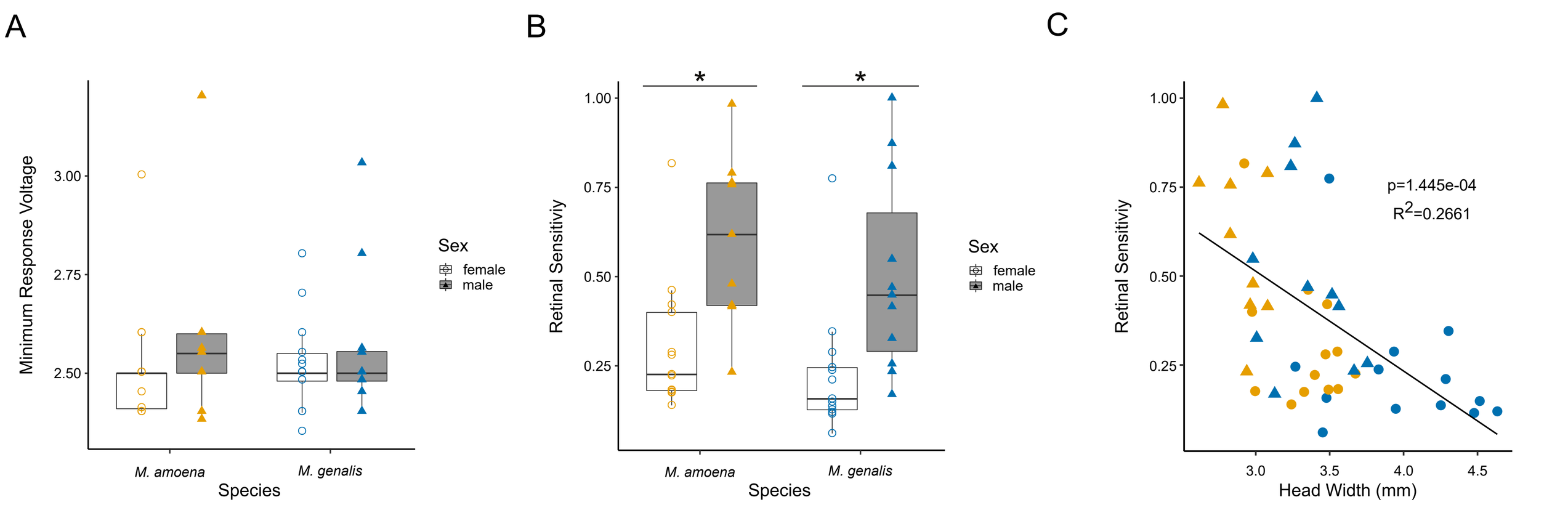

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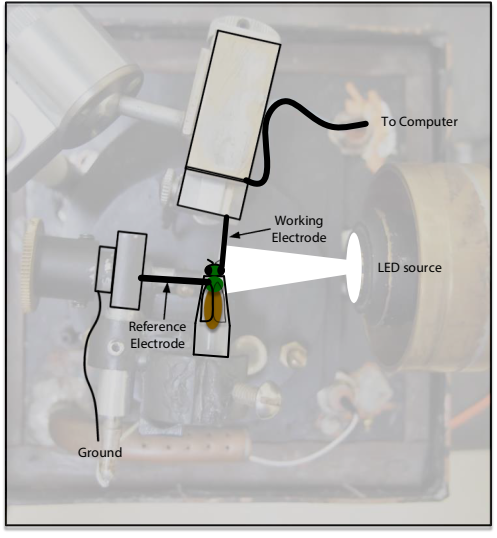

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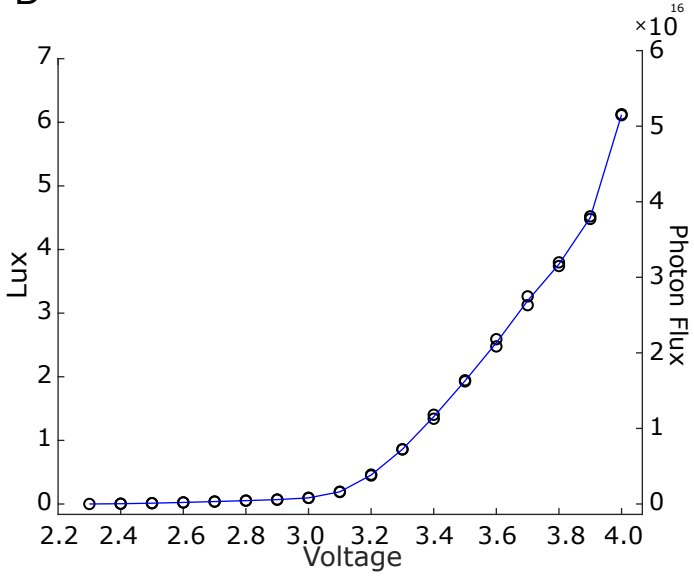

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