Morphological differences in the apposition eyes found in different species of stingless bees (Meliponnini: Apidae: Hymenpotera) with X-ray microtomography

credited to Alban Johansson[1] and Andreas Enström[1] for the SEM image.

Viktor Håkansson[1]

[1]Department of Biology, Lund University

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Supervisors: Gavin Taylor, Emily Baird

As the most diverse group of animals in the world with more than a million describes species, insects varies in many aspects of their ecology. Bees are insects that can be found in many different habitats across the world. Some live in open landscapes while others live in denser vegetation, some are active in the night while others are active during the day. Since bees use their vision to navigate and avoid obstacles, their eyes have to meet different visual requirements to be able to navigate during these different conditions. The aim of this study was to see if any morphological differences can be found in 6 different species of stingless bees living in the tropics where light intensities can be very limited. By creating 3D models of their eyes with X-ray microtomography we calculated the volumes and made measurements of the different parts of the eye. We found that with increased eye length the bees attained more lens and photoreceptors volume as well as thicker lenses. However, the proportion of photoreceptors, lens and cones in the eyes varies between species. Some bees invested more in lens volume and thickness while others invested more in cone volume and thickness and some attained better vision in the center of the eye whereas others had better vision ventrally. Together with previous findings, we suggest different possibilities on how the bees have different adaptations based on their ecology, behavior and eye morphology.

Introduction

Bees are eusocial insects that play one of the most important roles in the ecosystem as being pollinators to a large proportion of the worlds flowers and agricultural products. It has been shown that bees rely on their vision to a great extent to navigate and dodge obstacles in their environment rather than using other sensory organs[1]. All insects have a pair of compound eyes. There are two types of compound eyes: apposition and superposition eyes. Apposition eyes are by far the most common form of eyes in diurnal living insects, including bees, while superposition eyes are more common in nocturnal insects[2,3]. These types of eyes, however, differ a lot from the camera-type eyes as humans possess. Light is refracted into the human eye through the transparent cornea, and continues through the pupil aperture (which can contract to adjust how much light enters the eye), and then diffracts through the crystalline lens. The light focused on the retina in the back of the eye where sensitive photoreceptors convert the light rays into electrical signals that travels through the optical nerves to the brain[4]. Apposition eyes on the other hand are made of several units called ommatidia which can range in number from a couple of hundred up to around 30, 000 in dragonflies. The ommatidia consist of a lens where the light enters and focuses on the crystalline cone and guided into a structure called rhabdom where the photoreceptors are located. The photoreceptors then convert the light into an electrical signal where it, as in humans, travels through optic nerves to the brain and provides a single "pixel" of information given its field of view[2,3]. Although each ommatidia provides a single "pixel" representing their respective field of view, they do not really see all of these individual "pixels" simultaneously, but rather a mosaic of all the "pixels" as one merged image[2]. In this way, apposition eyes give an extremely large field of view, far greater than that of human eyes. But since the apposition eyes are adapted to diurnal lifestyle, when the light intensity drops, the ability to detect light is poor[3]. This is because the thin rhabdoms, the tiny lens size and the small acceptance angle of the ommatidia[3]. To increase sensitivity and resolution, increasing numbers of ommatidia and/or increased lens size and photoreceptors volume are required which at the same time would result in a bigger eye[3].

Previous studies with bumblebees have shown that depending on body size, light conditions and behavior of the bee, the bees have attained different eyes sizes where the bigger bees have attained bigger eyes with more sensitivity[4]. A recent study on wood ants have also shown that the morphology of the eye changes when the body sizes does[5].

However, there have been almost no studies regarding morphological differences in the eye of stingless bees (Meliponnini: Apidae: Hymenpotera). Stingless bees are a group of bees that differ in size from small to medium sized and can be found in the tropical and neotropical regions such as in South America, Australia and Africa where light intensity can be very limited. The stingless bees are eusocial creatures and are also considered as very important pollinators to the flora in the tropics. Many of them also produces honey but as the name implies they don't have a stinger[6].

In this study, we try to find different morphological traits in the stingless bees and try to explain these differences with respect to their ecology and behaviors. Since these bees live in a tropical environment, the light intensity and consistency can be limited on cloudy days in the dense vegetation. Could these bees have adapted to different local habitats? Would bees that live and forage in more open environments have different morphology of the eyes than those in more dense vegetation? Perhaps different species are under different predator pressures and need better vision to avoid these than others? To deal with competition when finding food such as pollen and nectar some might have selected for improved vision to locate these.

We hypothesize that there will be an increased investment in lens size and photoreceptors in bigger eyes to improved sensitivity since the light sensitivity seems to be that of most importance in a very dim light environment. Bees that have invested more in amount of photoreceptors and lens should live in more dim-light regions than those with less photoreceptors. We therefore have studied different species of stingless bees collected in Brazil and made measurements of the volume of the photoreceptors, crystalline cones and lens as well as thickness and length of these structures in different regions over the eye.

Materials and Methods

Preparations and Synchotron X-ray microtomography

Six different species of stingless bees were collected around and in Sao Paulo in Brazil; *Melipona quadrifasciata, Melipona bicolor, Tetragona clavipes, Plebia remota, Trigona spinipes and Tetragonisca angustula.* All the collected bees were female workers. The bees were then chilled to 4°C and the lower part of the heads were then removed. The samples were fixated in a phosphate buffered solution (pH 7.3, 02M) with glutaraldehyde (2%), paraformaldehyde (3%) and glucose (2%) for 2 hours to reduce the possibility of the soft tissue of shrinkage. To increase the contrast for the X-ray tomography, the samples were cleaned in a buffer right before the secondary fixation with OsO_4 for 1 hour. Each samples were then dried with a series of alcohol with concentration ranging from 70 up to 100% where they were kept for 10 minutes in each series and then the samples were transferred to acetone and got coated in epoxy resin (Agar 100) thereafter the outlaying resin were removed. Synchrotron X-ray microtomograhpy was then conducted on the bees using the I13-2 beamline of the Diamond Light Source. By using DAWN v1.7 software the obtained radiographic projections were then reconstructed into 3D volumes using a filtered back projection algorithm that turns the projections into a volume.

Segmentation

The segmentation of the different structures in the apposition eye; cornea lens, crystalline cones and photoreceptors, were performed in the 3D software AMIRA (AMIRA, Mercury Computer System, Berlin, Germany) (Fig. 1). The settings were: Downsampled to voxel size of 5 using a Mitchell filter. In total, 12 left eyes of the bees were segmented in Amira (2 of each species)

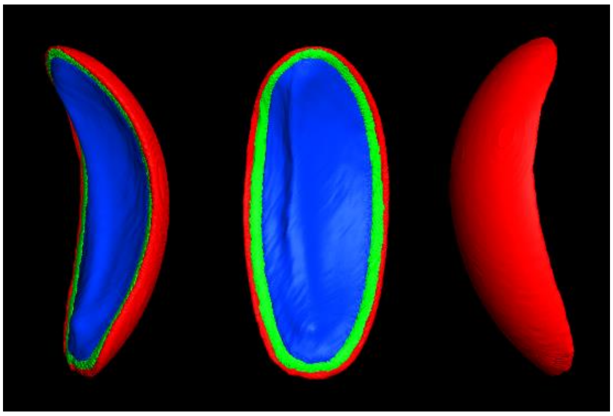

Figure 1. Completely segmented eyes in Amira shown in three different perspectives. Colors represent different parts of the eye. Red: cornea lens, green: crystalline cones and blue: photoreceptors. The species in the picture is *T.angustula*

Measurements

When all the eyes where segmented in AMIRA the thickness of the lens and cones and the length of the photoreceptors were measured in AMIRA's measurement tools. By measuring the total vertical length of each eyes 5 different points in the vertical lane of the eye were located. Since the eye sized varied among the species it was crucial that the measurements were taken in the same relative positions of the eyes. This was done by taking the measurements at 5%, 25%, 50%, 75% and 95% on the total length of the eye (from dorsal to ventral) to find the position on the lens surface. When the 5 points of each eye surface

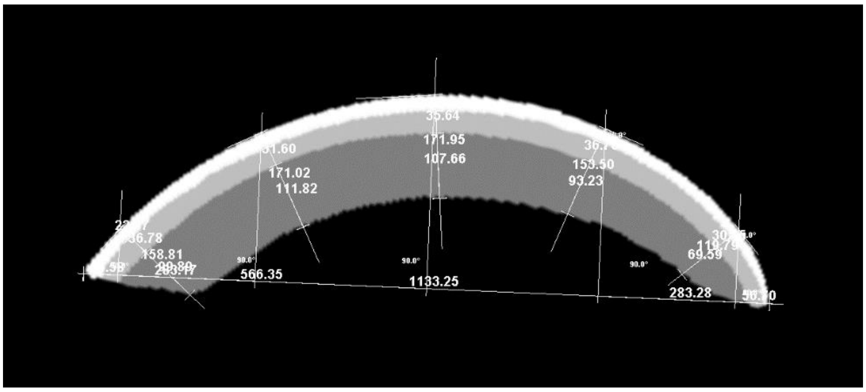

Figure 2. Picture from Amira depicting how the measurements were performed. The species in the picture is *T. angustula*. White colored area; cornea lens, grey; crystalline cones and dark grey; photoreceptors. The left side in the picture represents the dorsal side of the eye and the right side is the ventral.

where located, the tangent to the curved lens was estimated and 90° from the tangent into the eyes, the thickness and length of the cornea lens, crystalline cone and photoreceptors were measured (Fig 2)

Results

Volume

The relative volumes of cones, lens and photoreceptors vary a lot both between species and in some cases within species (Fig 3). The most notable intraspecific differences were found in *T. spinipes* and *T. angustula*. The relative volume photoreceptors varied approximately 5% between the two *T. spinipes* individuals were the one with the higher relative volume photoreceptors had less volume lens and cones compared to the other. In *T. angustula* the relative lens volume was higher than the relative cone volume in one individual but the opposite in the other individual.

The lowest relative volume of photoreceptors was found in *T. angustula* with 44 % of the total eye volume and the highest in *M. bicolor* with nearly 57 %. Interestingly, *M. quadrifasciata* had a very low relative volume of photoreceptors (~ 46%) despite it possessing nearly the same eye length as *M. bicolor*. The highest relative volume lens was similar for *T. Spinipes, M. quadrifasciata* and *M. bicolor* (~ 30 %). Whereas the lowest relative volume of the lens was found in *P. remota* (~ 22 %)

The relative volume cone where similar for *T. clavipes*, *P. remota* and *M. quadrifasciata* (~ 25 %). One individual of *T. spinipes* had notably lower relative volume cone (~ 19 %) and one individual of *T. angustula* had the highest of all species (~ 30%). Both individuals of *M. bicolor* possessed by far the lowest relative volume of cones (one as low as 12 %).

Another thing that is apparent is that the relative volume lens and cones are close to each other in *T. clavipes*, *P. remota* and *T. angustula*, where the relative volume cone is slightly higher than the relative volume lens. In *T. spinipes*, *M. quadrifasciata* and most apparent in *M. bicolor* the relative volume cone is much lower than that of the lens.

Table 1. Eye length, and calculated volumes of the total eye, photoreceptors, cones and lens in all the 12 different bees.

Species	Eye length (µm)	Total eye volume (µm³)	Volume photoreceptors (µm³	Volume cones (µm³	Volume lense (µm³
T. clavipes	1612,97	139595128	69839504	36376500	33379124
T. clavipes	1600,8	131888224	68662736	32183744	31041744
P. remota	1162,73	48939492	24890746	12781498	11267248
P. remota	1247,6	63367127	34462752	15175750	13728625
T. spinipes	1625,38	143290230	74443864	27675246	41171120
T. spinipes	1608,72	139123108	64208744	31355496	43558868
T. angustula	1133,25	56233992	24667622	16808872	14757498
T. angustula	1176,7	68930869	33476872	16472749	18981248
M. quadrifasciata	2340,89	334998744	155779744	79967752	99251248
M. quadrifasciata	2883,84	319961120	145104624	74992496	99864000
M. bicolor	2323,8	351405625	191981750	57181625	102242250
M. bicolor	2347,73	378995861	214143861	46036747	118815252

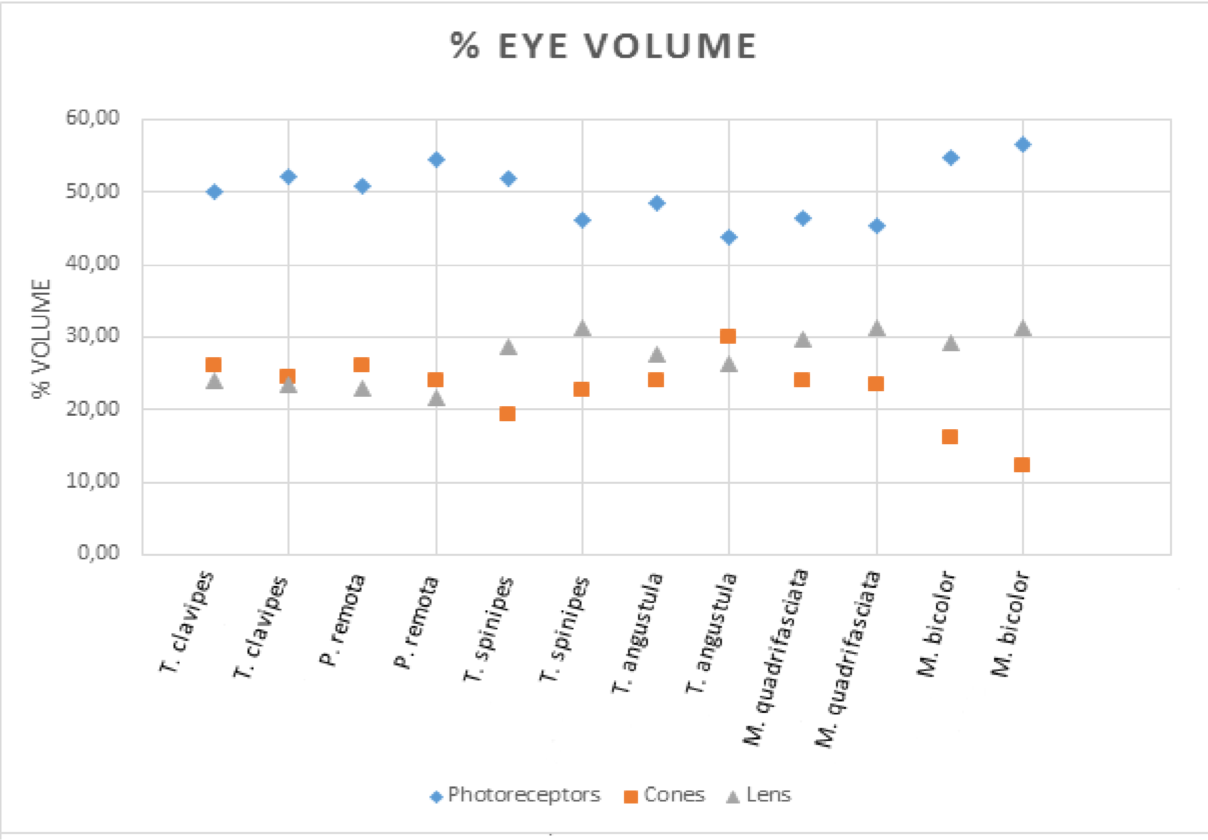

Figure 3. Relative volume of photoreceptors, lens and cones compared to the total eye volume in the 6 different species of stingless bees.

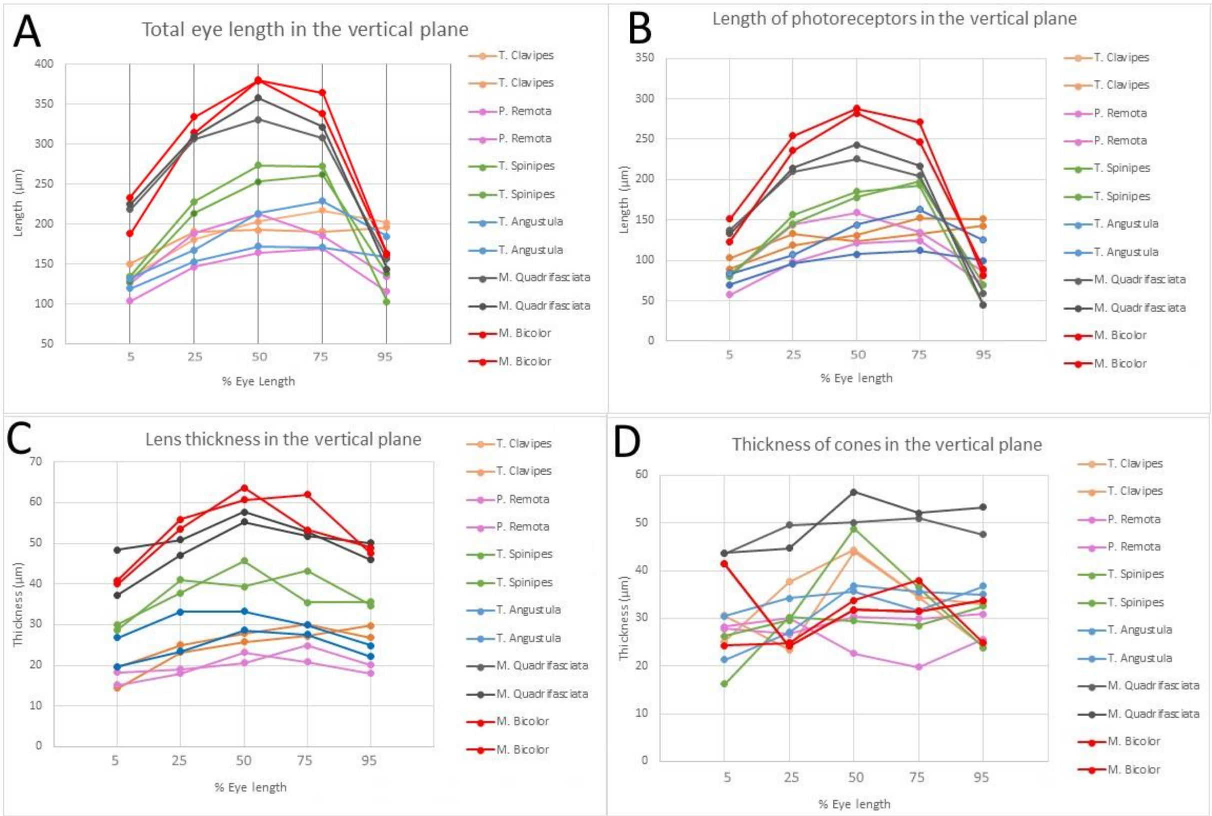

Figure 4. The 5 different measurements points across the eye vertically where going from 5% which is the most dorsal point up to 95% which is the most ventral point of **A** Total eye thickness. **B** photoreceptor length. **C** lens thickness. **D** cone thickness

Length and thickness

The total thickness of the 5 different vertical positions seems to follow similar trends. (Fig. 4A). In all species except for *M. bicolor* and *M. quadrifasciata* (which were the biggest bees) the measurements follows a trend where the thickness increases as we move down vertically on the eye until we reach the 75% measurement point, where the highest measurements where found. This means that the eye is thicker slightly ventral to the center of the eyes in these species. In M. Bicolor and *M. quadrifasciata* the peak was located in the 50% measurement point, meaning that the eyes of these species are instead thicker in the center of the eye.

For the photoreceptor length seems to follow the same trend as for the total thickness of the eye. (Fig. 4A, B). *M. bicolor* and *M. quadrifasciata* both have longer photoreceptors in the center of the eye, whereas the other species seems to have the longest photoreceptors slightly ventral to the center (75% measurements position).

In both total thickness of the eye and the length of the photoreceptors we see a clear trend that with increased eye length, the thicker eye and the longer photoreceptors we observe (Fig. 4A, B and Table 1). However, *T. clavipes* that have almost identical eye length as *T. spinipes* has similar eye thickness and photoreceptor lengths as the smallest bees *P. remota* and *T. angustula.*

The lens thickness across the eye is more dispersed within the species, but all species seems to have the thickest lens at the center of the eye, or slightly ventral to the center (50% and 75 %). One exception is one individual of *T. angustula* where the thickest part of the eye was slightly dorsal of the center.

The cone thickness across the eye seems to vary much across the eye both within species and between species (Fig. 4D). Cone thickness of *M. bicolor* were really low despite having the highest eye lengths together with *M. quadrifasciata*, yet *M. quadrifasciata* has the highest cones thickness over the whole eye of all the bees (Fig. 4D)

Note however, that the photoreceptors in particularly the dorsal and ventral position of the eye were not straight but bent in some cases, especially in the 95% measurement (bottom of the eye). And depending on the curvature of eyes this would make the length measurement on the photoreceptor much lower than it might really be, as seen as the really low values at the 95 % measurement point in *M. bicolor*, *M. quadrifasciata* and *T. spinipes* (Fig. 4A, B and Fig. 5B)

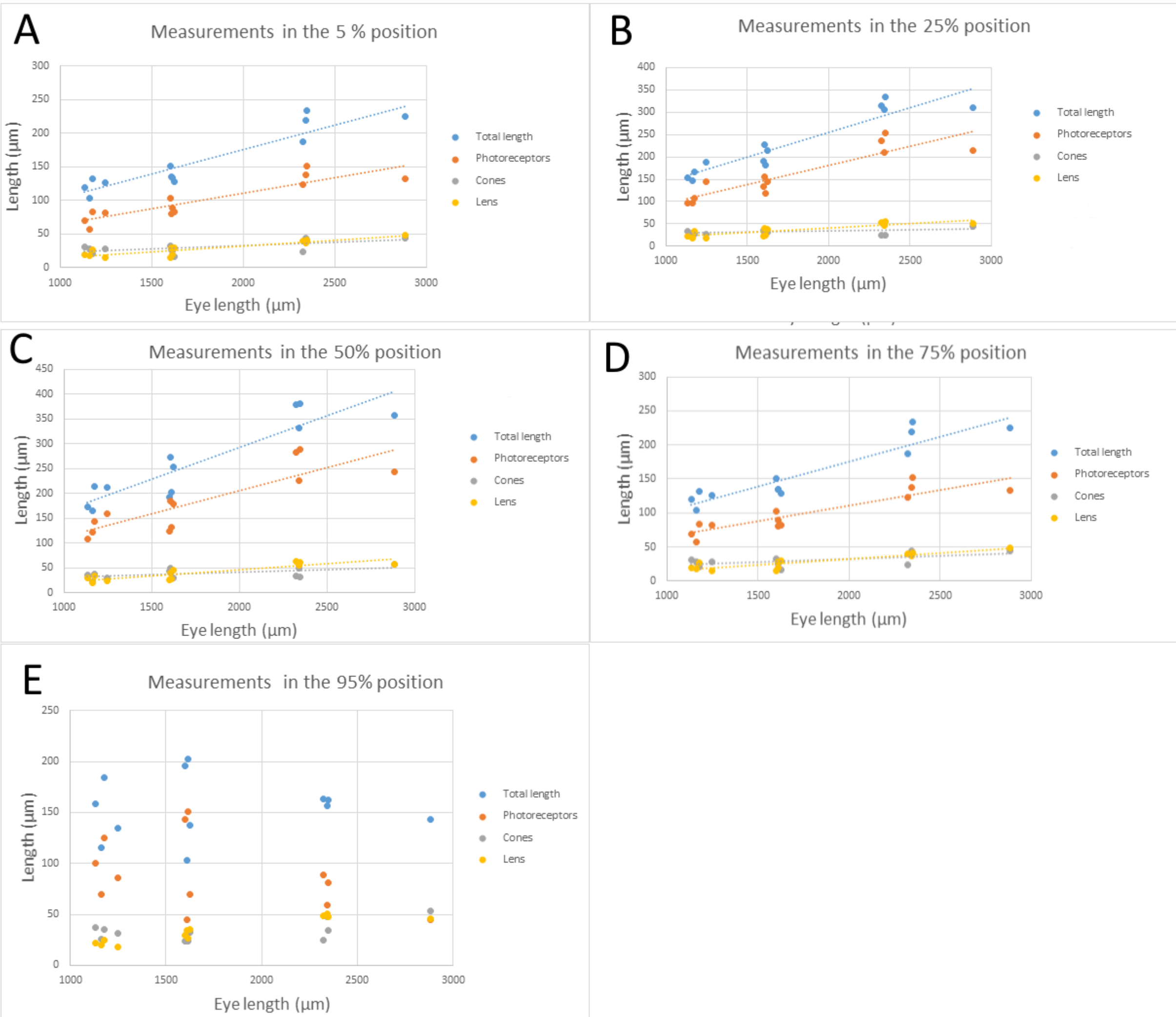

Figure 5. The thickness of the eye, lens, cones and length of photoreceptors against the eye length of each individual bee for the measurement points across the eye with their respective trend lines. **A,** 5 % **B,** 25% **C,** 50% **D**, 75% **E,** 95%)

Scaling

A linear relationship of eye length and eye thickness and photoreceptors length is found across the eye (Fig 5A, B, C, D). As the eye length get bigger so does the eye thickness and the length of the photoreceptors from dorsal to ventral of the eye. The lens thickness also shows a positive linear relationship of eye length and thickness although not as sharp as the photoreceptors, (indicating that when the lens thickness increases the photoreceptor length increases even more). At shorter eye length, the cone thickness seems to be higher than the lens thickness but as the eye length increases the cone thickness tends increase a little slower than that of the lens, resulting in seemingly thinner cones compered to lenses with increased eye length. Especially true in *M. bicolor* where the cones are much thinner than the lens (Fig 4C, D)

Note however, that the photoreceptors in particularly the dorsal and ventral position of the eye were not straight but bent in some cases, especially in the 95% measurement (bottom of the eye). Depending on the curvature of eyes, this would make the length measurement on the photoreceptor much lower than it actually is, as seen as the crashes at the 95 % measurement point in *M. bicolor, M. quadrifasciata* and *T. spinipes* (Fig 4A, B and Fig 5E)

Discussion

Flower detection and navigation

If we assume that more photoreceptor and lens volume represent increased resolution, given that larger eyes usually have more ommatidia, then we see a trend where bigger eyes have more resolution (Table 1). Previous studies have shown that insects with bigger bodies also possess bigger eyes, suggesting that the best way to improve vision is to get bigger eyes[3]. Since all the stingless bees are pollinators it would be a fair assumption to say that better resolution makes it easier to detect flowers and plants. Araúju et al (2004), shows that stingless bee's maximum flight distance increases with body size[7]. With increased flight distance the bigger bees would then presumably navigate through the vegetation more than the smaller bees with shorter flight distance. Bigger eyes with higher resolution might then be useful to assist with longer distance navigation. Since the smaller bees would be closer to the hive when foraging they would then presumably not need as good resolution as the bigger bees since they wouldn't navigate to the same extent.

Adaptation in denser vegetation.

We also found a general trend that lens thickness increased as the eyes got bigger (Table 1, Fig 4 and Fig 5) According to the Lensmaker's equation as the thickness of the lens increases, the focal length becomes shorter which is directly associated with improved sensitivity of the eye[2].

Lensmaker's equation:

$$\frac{1}{f} = (n-1)\left[\frac{1}{R_1} - \frac{1}{R_2} + \frac{(n-1)d}{nR_1R_2}\right],$$

Where:

f is the focal length of the lens
n is the refractive index of the lens material
R_1 is the radius of curvature of the lens surface closest to the light source
R_2 is the radius of curvature of the lens surface farthest from the light source
d is the thickness of the lens

Since stingless bees are generalist pollinators, different species' choice of host plants are surely to overlap, implying that competition might occur between species when foraging[6]. It has been found that within the genus Trigona there is a lot of intraspecific competition when foraging and that it is rather a limitation of food rather than potential nesting places that limits the population size[8]. Our Trigona specie: *T. spinipes* have much thicker lenses compered to *T. clapvies* who has similar eye length (Fig 3, Table 1). We therefore suggest that *T. spinipes* has selected for better sensitivity in the eyes to be able to detect flowers easier during lower light levels compered to *T. clavipes* whom might not be under the same competitive pressure for food or that they instead display a different foraging behavior.

Since the tropics can be very light constricted depending on weather and canopy cover, our hypothesis was that bees with more sensitivity are living in more dense forested areas, while those with less sensitivity are living in more open areas. It has previously been shown that the genus Melipona are foraging plants such as Polygonacae which are located in more dense forests, whereas the genus Trigona forage on plants such as Compositae and Labiatae which are associated with more open vegetation[9]. Since both *M. bicolor* and *M. quadrifasciata* have thicker lenses and bigger eye lengths than *T. spinipes,* this would support our theory that with increased sensitivity, bees live in more dense regions. Interestingly, *M. bicolor* has thicker lens and thinner cones compered to *M. quadrifasciata.* although they both have similar eye length. This observation suggests that of the two, *M. bicolor* has greater sensitivity and presumably have adapted to a particularly more dense vegetation than *M. quadrifasciata.* It could also be that they have different temporal niche, where *M. bicolor* have adapted to forage when its darker (earlier in the morning or later in the evening) to avoid competition. A possible explanation for *M. bicolor* to have evolved such thick lens, thin cones and large photoreceptors could be that it has a unique characteristic compared to the other bees. Almost all bees have a single queen, but *M. bicolor* however have been found to have multiple queens in one colony, resulting in more genetic diversity which then might enhance the species resilience to changes and possibly have improved their ability to adapt to a denser vegetation and forage when it's darker[10].

Shape of the eye

It has previously been shown that insect's eyes are generally associated with higher regions of acuity in either the upward pointing regions or the forward facing regions[11]. However, if we assume that eye thickness of the eye works in the same way then we found that our bees had thickest lens and longest photoreceptors in either the central region or slightly ventral from the center. Following the idea that *M. bicolor* and *M. quadrifasciata* forage in denser forest than the other bees, this might be that better vision in the center of the eye is an adaption to improve navigation in that vegetation. The bees with thicker ventral regions might focus more on finding plants and flowers rather than avoiding obstacle since they might live in a more open vegetation. Since we have only investigated the eye on the vertical plane in five different positions, we don't have enough data to describe the shape of the whole eye. Perl et al 2016 found that in wood ants (*Formica rufa)* the lens diameter scaling across the eye changes with body size, where bigger ants had bigger lenses at the anterior and dorsal regions of the eye[12]. Therefore, the shape of the eyes in the bees we have studied might as well vary in different regions over the eye more than in the region we have been looking on. For example, *M. quadrifasciata* has almost the same cone and lens thickness across the vertical plane yet is has a much higher percentage volume lens than cones, suggesting that there would be shorter cones and thicker lenses somewhere else on the eye (Fig 3 and Fig 4C, D).

Errors and improvements

Biological optics is a complex research field with many laws and rules. In reality, many more parameters rather than lens volumes affect resolution, including lens diameter, curvature of the lens, and angles between the ommatidia. In this project, we have completely modelled the eye and therefore some assumptions we have made might be incorrect. When segmenting the eyes, it was sometimes difficult to distinguish the respective borders of the lens, cones and photoreceptors, leaving the possibilities that the volumes and measurements of the eye could have small errors in them. Because we haven't taken the eyes orientation on the head into account there is a possibility that our measurements and assumptions about their vision also have errors in them. Some insects also have an additional visual organ called ocelli, which have been shown to be of great importance for flight navigation and stability in orchid bees living in the rain forest, as well as in other insects[13]. Therefore, different ocelli structure and adaptations might also correlate to the morphology of the apposition eye. To better understand the actual optical properties in the stingless bees, a more thorough investigation is needed were measurements of the different eye structures should be done over all the whole regions of the eye and parameters such as lens diameters, acceptance angles and rhabdom diameters should be measured

Conclusion

We have shown that photoreceptor and lens volume increases with eye length indicating that bigger eyes have better resolution. Our hypothesis that bees with more sensitivity are more adapted to denser vegetation is supported by our finding that lens thickness increases with eye size with and previous knowledge that bees with more sensitivity are associated with more dense flora. *T. spinipes* have selected for lens volume resulting in better resolution compared to *T. clavipes* with similar eye length possibly to detect flowers easier due being more under more competition pressure for food. We have also shown that bigger eyes seems to have adapted better sensitivity in the center of the eye whereas smaller eyes have better sensitivity slightly ventral from the center.

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