Bachelor thesis, MOBK01, VT16

Biologiska institutionen

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A comparative study of preparation methods used for bee heads prior to microCT imaging as well comparing eye to head ratio in five different bee species.

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The complexity and importance of the eyes of a rather simple organism, the bee, is apparent when looking at the adaptation to their environment. The so called compound eyes consists of multiple facets, each having their own point of view and combined results in a larger image. For being able to study the magnificent eyes on a bee's head, there have been in recent studies an increase by X-ray tomographic imaging. Tomographic imaging, in this case micro computed tomography (CT), has enabled for scientists to virtually in 3D study both the external as well internal structures of a subject of interest. To get a fine external structure of a bee's head, one need to prepare it in a way that it does not lose any parts or contain artefacts before the microCT. Thus, the main aim with this report was to think of a preparation method that would be suitable for analyzing the bee's head surface. By doing so, another old method was compared for a basis to determine if there was in fact any advantages by using this new method. The old method had some issues retaining the surface where either the mandibles or antenna were lost during the preparations. The new method showed prominent results where the external structure remained intact however the internal structure was somewhat damaged. The secondary aim was to compare the eye to head ratio between five different bee species and to see what advantages the eye size might have in their specific habitat, to know what they are seeing and how their technique is utilized by navigating in dense rainforests. But more studies must be done in this field to draw any real conclusions.

Introduction

Eyes are undoubtedly the most important organ of an insect. The otherwise relatively undeveloped invertebrates would not be able to survive in their different habitats. Their eyes enables them to interact with the surrounding by avoiding obstacles, finding food, looking for predators or a potential partner for mating [1]. As for the bees, they need to be able to navigate through tight areas in the forest or their beehive to not collide. Throughout the evolution this has been made possible, where the bees and other insects are utilizing the eye design called compound eyes [2]. The compound eyes are consisting of certain units called ommatidia. The ommatidias are all identical with having a lens, a crystalline cone for focusing the incoming light into the mutual photoreceptor called the rhabdom. To sensor the photons of light that has been focused into the rhadom, the rhodopsin molecules that are present there in all eyes, are responsible for sensing and subsequently through biochemical events

generate a picture to perceive the world [3]. The ommatidia, each have a certain spatial view which they are seeing, meaning that one ommatidium in e.g. bees, which may have as many as thousands of ommatidia, functions each as one pixel to the whole image. For bees, one can further divide their group of eyes into the so called apposition eyes. The apposition eyes are good tools for species living in bright habitats, having their ommatidia separated by a small layer of light-absorbing pigment. This makes it so that each lens has its own view, its own pixel, without having any disturbance from neighbouring ommatidium [1][4]. Bees does however not only use their apposition eyes for navigating during flight, but are also helped by their ocellus. The ocellus is consisting of a photoreceptor with a single lens positioned on the surface of the body. It is sensing light intensity and is incapable of actually seeing, where the bees possess three photoreceptors (ocelli) on top of their head [1]. Recent studies suggests that the ocelli functions as a compass for the bees, giving information horizontally above but as well having fronto-dorsal view. This provides an excellent and complex navigation system needed for a species living in dense rainforests with only a small portion of light penetrating through [5].

The basis of much of the recently and upcoming concluded information in different biological fields, have mainly been determined through the high resolution analysis by micro computed tomography (CT) X-ray imaging [6]. With the microCT one can analyze the anatomy of, in our case, a bee eye both internal- and -externally. Thus, the microCT is a good tool to investigate what the visual systems are providing with navigation for a certain bee specie by displaying a three-dimensional (3D) representation of the subject. The information that is provided by the high resolution CT-scanning, are multiple virtual digital slices of the specimen of interest [7]. In recent years, the X-ray tomographic imaging has become even more effective by using a synchrotron at the Diamond Light Source, thus enabling more rapid scanning of multiple samples for analysis [8]. However, the samples prepared for the CT-scanning might not always be intact, as pieces might have fallen of or shattered during the preparation steps. This is of course not ideal, thus there is much emphasis on preventing it to happen before the scanning, to get the most out of the sample of interest and not to spoil a segment that otherwise would have been interesting to examine.

Thus in our study, we are comparing different preparation methods used for the microCT imaging as well comparing the eye to head relation in five different species. When doing so, a previous method used by our supervisor when examining the ocelli of *E. Imperialis* is made as a comparison [5]. During this article, our method will be named as the "new" method and the compared one as the "old" method. We hypothesized that the new method would result in better presented structures for the CT-scanning, having less complications than the old. First, we performed a new mounting method of the heads onto steel needles instead of perspex blocks, where over 100 samples had been collected in Brazil and Panama. From these, 94 were later mounted and only five different species (*Euglossa Cordata, Melipona Bicolor, Tetragona Clavipes, Nannotrigona Testaceicornis,* and *Trigona Spinipes*) were scanned through microCT due to time limitation with having no synchrotron at our disposal. *Tetragona Clavipes* was scanned with the old method. The preparation steps before the mounting and the following imaging, was done in a dehydration series and critical point drying. Through computer analysis by Amira, the scanned samples were compared to the old method's 3D images. Secondly, when studying the samples, the eye- to -head relation were measured and compared to see if there is any distinct size difference. This was done to investigate what advantages the eye size might have in the environment where the certain bee species lives.

Material and methods

(a) New method

The samples to be prepared for analysis had previously been collected in Brazil and Panama by our supervisor. A total of 94 heads were mounted from over 100 samples that were collected. From these, five specimens were imaged by X-ray computed tomography and later computationally analyzed.

The specimens of the collected bee heads of various species were dehydrated in 5 different series, all made with the same procedure. First, the bee heads were cleaned with paint brushes to remove any dust and unwanted particles. The samples were then dehydrated in a series; firstly for 10 minutes in 80% ethanol, then at 96% and finally with 100% ethanol. To further remove any particles from the bee heads, they were cleaned for 30 seconds with an ultrasonic cleaner, Branson 1200, and then stored at +4° C over night in 70% ethanol.

To prepare the bee heads for imaging by x-ray CT and remove any remaining artefacts, the samples were dried with critical point drying using the Bal-Tec CPD 030. The critical point drying was used because it neatly preserves the surface of a biological sample. The drying step included 5 quick steps in 9° C liquid carbon dioxide for 2 minutes, followed by a longer treatment of around 30 minutes and finally 3-5 steps of 10 minutes treatment. Liquid carbon dioxide is used because its lower critical point from aqueous phase to gaseous phase of 31° C at 74 bar compared to water being at 374° C at 229 bar. The CO_2 is however not soluble with water, thus the samples were beforehand transferred into ethanol. The procedure was completed when no scent of ethanol was left and only gaseous phase of CO_2 was remaining, thus different amount of steps were exerted [9]. When finished, the samples were kept safely in a tray.

After the dehydration and drying of the samples, the heads were carefully mounted onto paper that had been placed on top of a steel pin with glue. When mounted, the samples were analyzed with microCT (ZEISS Xradia 520 Versa 3D X-ray microscope). Because the tomography was time limiting only a few samples were analyzed. One head each of the species *Euglossa Cordata, Melipona Bicolor, Nannotrigona Testaceicornis, Tetragona Clavipes* and *Trigona Spinipes* were chosen for analysis since some of the same species had been analyzed by the previous method, thus making a good comparison.

From the x-ray tomography, data was collected and computationally analyzed with the 3D software program Amira (version 5.3). To visualize the specimens in Amira, the isosurface was determined, where the isosurface is a tool that displays the 3D structure of the specimen at a certain value. The orthoslice tool, which displays a single slice of the internal structure, was used to examine how intact the specimens were. Multiple cleaning steps in Amira was performed to remove excessive glue or resin that was attached to the bee heads as a consequence of the mounting methods. After the cleanup of the glue, the eye and head size were measured. The eye area was calculated with a certain area tool and the head width were measured with a three dimensional line. Thus, to obtain the eye to head size ratio, a common unit was needed, resulting in that the square root of the eye area was compared to the head width.

(b) Old method

The samples were treated with cold anaesthetic and fixed with a mixture as in Curbio [5], where 3% paraformaldehyde, 2% glutaraldehyde and 2% glucose were treated for approximately 2 hours in phosphate buffer. A washing step was then carried out prior to the secondary fixation were 2% of OsO_4 was applied during 1 hour. After the secondary fixation, a dehydration series was carried out according to the new method (see above). No critical point drying was performed. The specimens were

instead embedded with epoxy resin where the outer resin was removed. When mounting the heads, they were placed onto perspex blocks and attached with resin.

The microCT imaging was done by the synchrotron in Diamond Light Source, thus more samples were enabled for analyzation. Computational optical analysis was visualized by using Amira and then calculated with a script in MATLAB [5].

(c) Indicative test

To indicate that the addition of alcohol followed by the dehydration and critical point drying of the samples did not in fact comprise or enhance the head size, an indicative test was performed. First, five bumblebees of *Bombus Terrestris* were collected and dissected. Pictures were taken of their heads using stereomicroscopy, Nikon SMZ18, before (fresh bees) and after dehydration as well critical point drying (dried bees). The head size was measured with six different measurements using ImageJ, where the dried bees were compared to the fresh bees. The difference was obtained in percentage where the probability of error (the p value) was set to be statistically significant at $p = 0.05$.

Results and Discussion

Indicative test

After the dehydration and critical point drying of the *B. Terrestris* samples from the indicative test, the size difference were measured using ImageJ. When measuring the eye length, it was made computational by hand which results in a slight difference every time one measures and reads the value. Thus, to ensure that they were somewhat done correctly, different measurements of the bee head (**Figure 1**) were done five times each to minimize the human error.

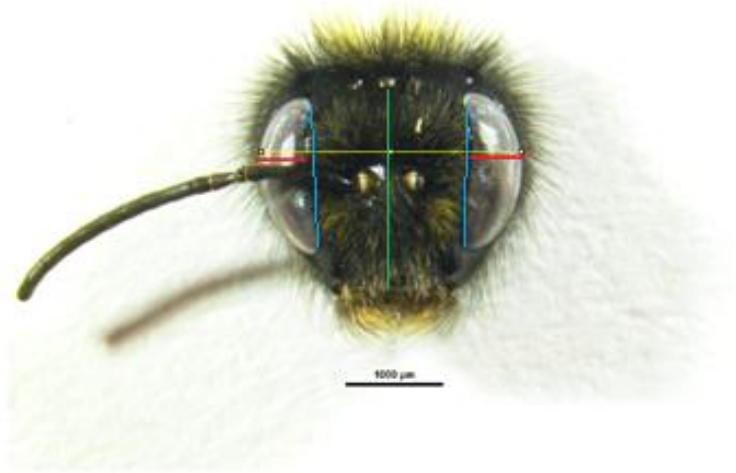

Figure 1. Measurements of the bumblebees taken accordingly. The width across from eye to eye (shown in yellow line), the width and length of each eye (shown in red and blue lines) as well the length across eye were measured. The small black scale seen is indicating the length of 1000 µm.

With the measurements from ImageJ, the size of the dried bees (being those who had been dehydrated as well critical point dried) was calculated relatively in percentage to the fresh bees (bees with no treatment) (**Figure 2)**. The indicative test did show that there was in fact no significant difference between the dried to the fresh bees. The result was true within the significant difference of five percent

(p = 0.05). When measuring the length it was sometimes harder to distinguish where to put the ruler in ImageJ, e.g. when trying to measure the length across the eye with the bees having lot of hair interfering on the frontal side of the head. Thus, if one would use an even more reliable source of measuring, there would undoubtedly not be any difference of the head and eye size between the different treatments.

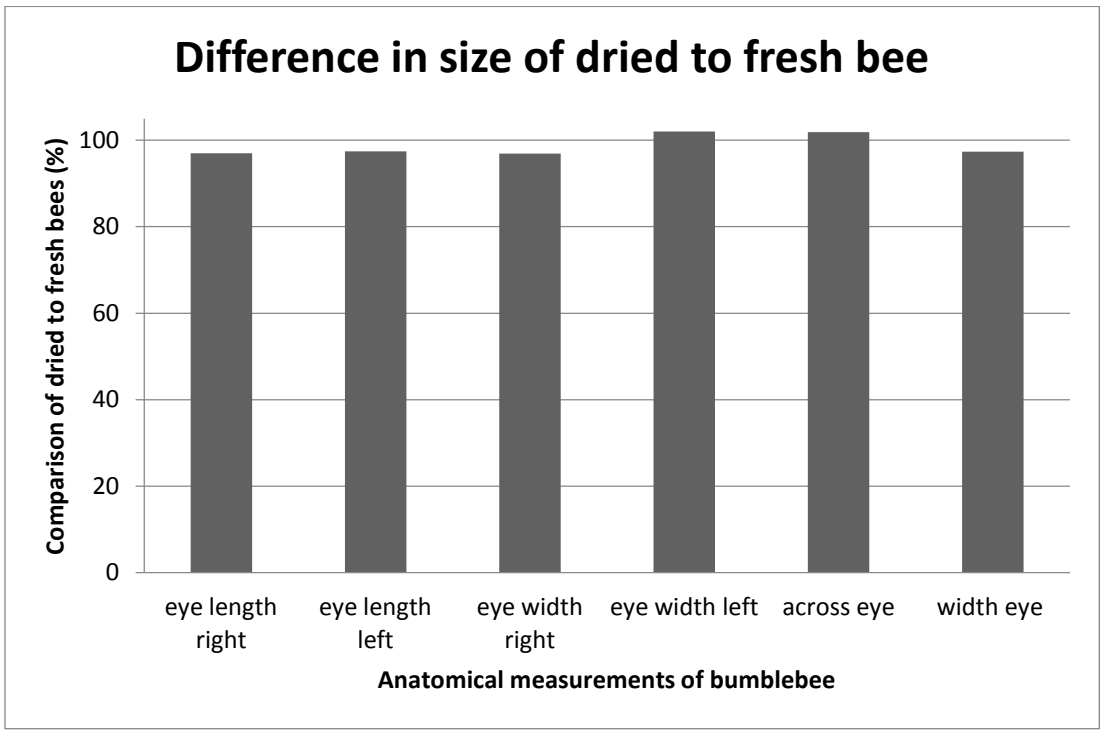

Figure 2. Bars made from the measurements in percentage of the dried bee compared to the fresh bee of *B. Terrestris*.

When comparing the fresh bee to the dried sample, one can see that there is a slight difference, however not in shape (except for the antenna that has been slightly bent during the procedure, seen with the dried bee) but in the colour of the eyes (**Figure 3**). The fresh bee show black facets and the dried bee exhibits grey like colour. This difference in the colour of the facets is indicating that externally, little has altered, but instead the internal structure of the facets have transformed. This transformation is due to that in the fresh bee, the facets and their ommatidia are absorbing the light displaying the black colour. After the dehydration and critical point drying, the ommatidia is no longer absorbing light, but instead the internal structure are exposed, such as the brain, thus giving rise to the grey colour switch from the original black appearance. Except for the difference in eye colour, the pictures were taken with different light conditions, thus the slight disparity in brightness is seen.

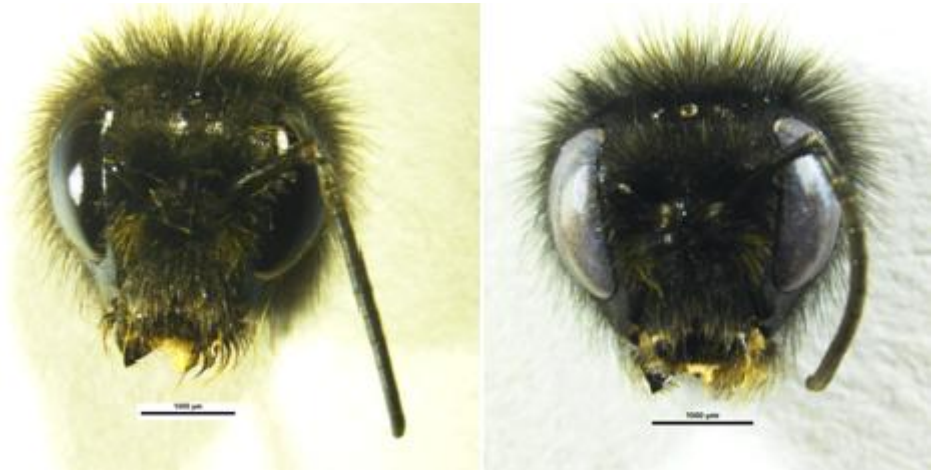

Figure 3. Comparison of appearance between fresh bee (shown to the left) and dried bee of *B. Terrestris* after dehydration and critical point drying (shown to the right). The small black scale seen is indicating the length of 1000 µm. Pictures taken by stereomicroscopy, Nikon SMZ18.

Comparing the methods

When comparing what the samples looked like on the external structure from the beginning without any removal of glue, paper or resin, one can clearly see a huge difference (**Figure 4**). The isosurfaces shown are from the same species of *E. Cordata*, but taken from different individuals. By looking at the head having preparations from the old method (**A**), the resin is shown at the back of the head. The frontal part have also been cut off somehow during the preparing procedure as well no antenna are attached to the head. However, by the new method (**B**), the head surface seems intact with having only a few dust particles or small glue remains attached to it. Thus by comparing from these two heads, there is a clear distinction showing the new method being superior.

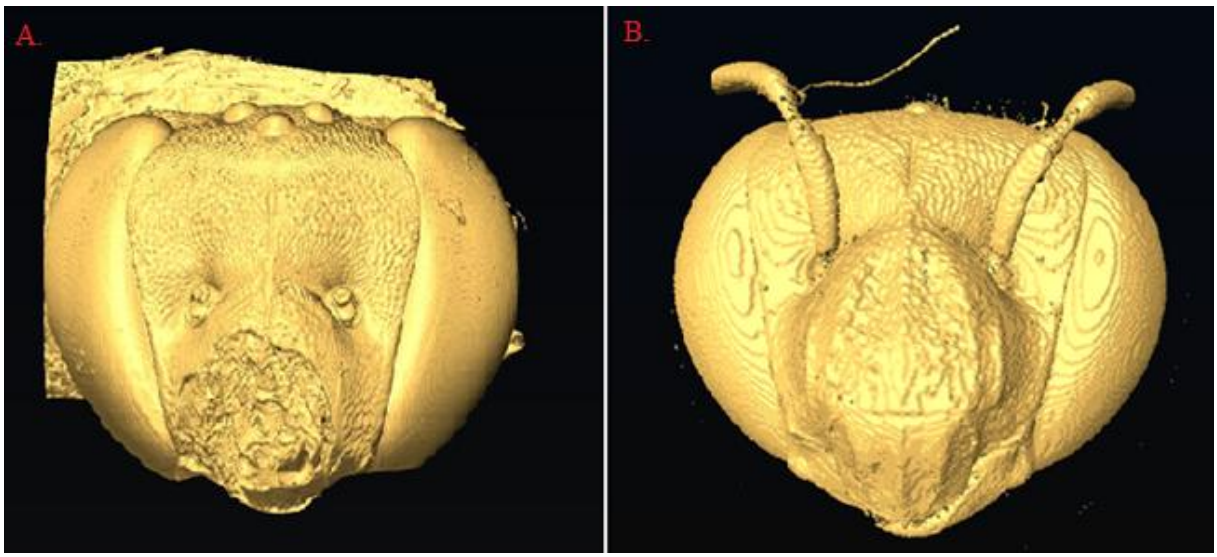

Figure 4. Isosurface displaying the external structure of *E. Cordata* before cleaning in Amira (version 5.3) of both the old method (A) and the new method (B).

When analyzing the heads from the different methods after being cleaned by the programme Amira, the samples have a much finer surface (**Figure 5**). There is still however some resin attached to the back of the *E. Cordata* sample from the old method which could be adjusted with having more time and remove if one so desires. By comparing the isosurfaces of *E. Cordata* before (**Figure 4**) and after the clean up (Figure 5), the surface is further more refined due to up sampling of the resolution, thus displaying more clear structures.

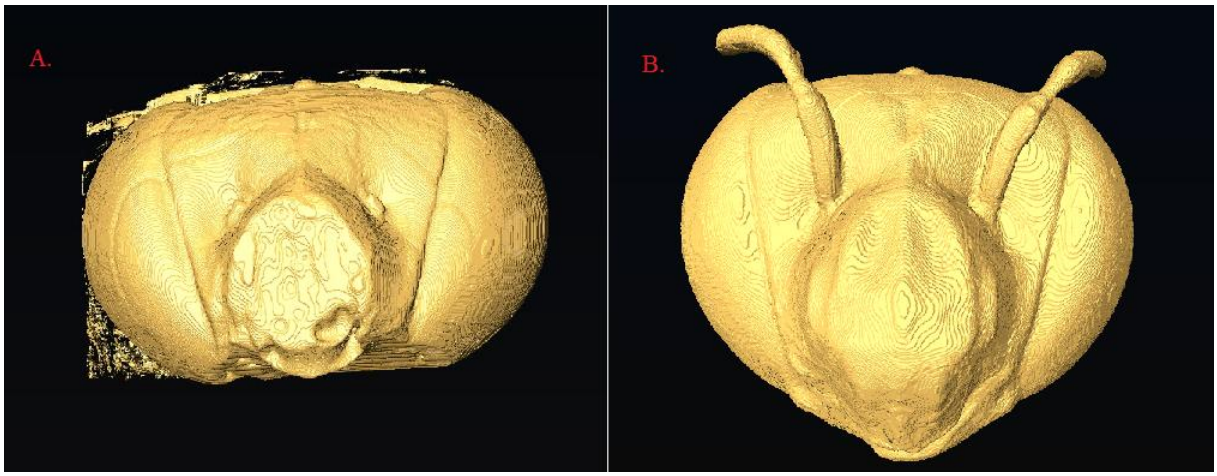

Figure 5. Isosurface displaying the external structure of *E. Cordata* heads after cleaning in Amira (version 5.3) of both the old method (A) and the new method (B).

By analyzing the orthoslice, the internal structure can be seen and examined (**Figure 6**). The orthoslice is one of the virtual digital slices by the microCT imaging, in this case the *E. Cordata*. When looking at the bee's internal structure prepared by the old method, it seems rather good. But as already mentioned, the antennas were somehow removed and is therefore not visible in the orthoslice. The mandible is not visible either since it was lost during the preparations. However the eye structure and some of the brain seems rather intact, suggesting that the method can be useful when analyzing the internal structure. By comparing to the new method, both the antennas, shown as the two dots over the head structure, as well the mandible are well and still attached to the bee head. However, the eyes seem to have been perished as well as the brain is not intact anymore. Thus one might suggest that the new method is not ideal when trying to preserve the internal structure of a bee head.

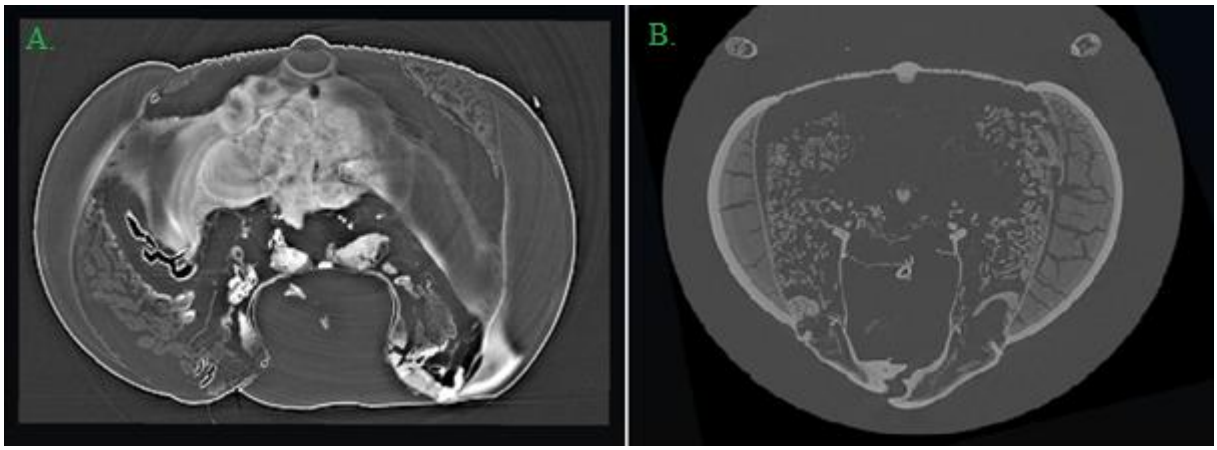

Figure 6. Orthoslice of *E. Cordata* from Amira (version 5.3) displaying the internal structure of the old method (A) and the new method (B).

Pictures of the isosurfaces from *M. Bicolor*, one of the four other bee species that were scanned through microCT, are shown in **Figure 7**. The figure is demonstrating how the sample looks, with the new method used, before and after clean up by Amira. As seen with the previous bee head of *E. Cordata*, the antenna as well the mandible is still attached. Thus, the new method seems fairly reliable when it comes to preserving the external structure of the sample of interest.

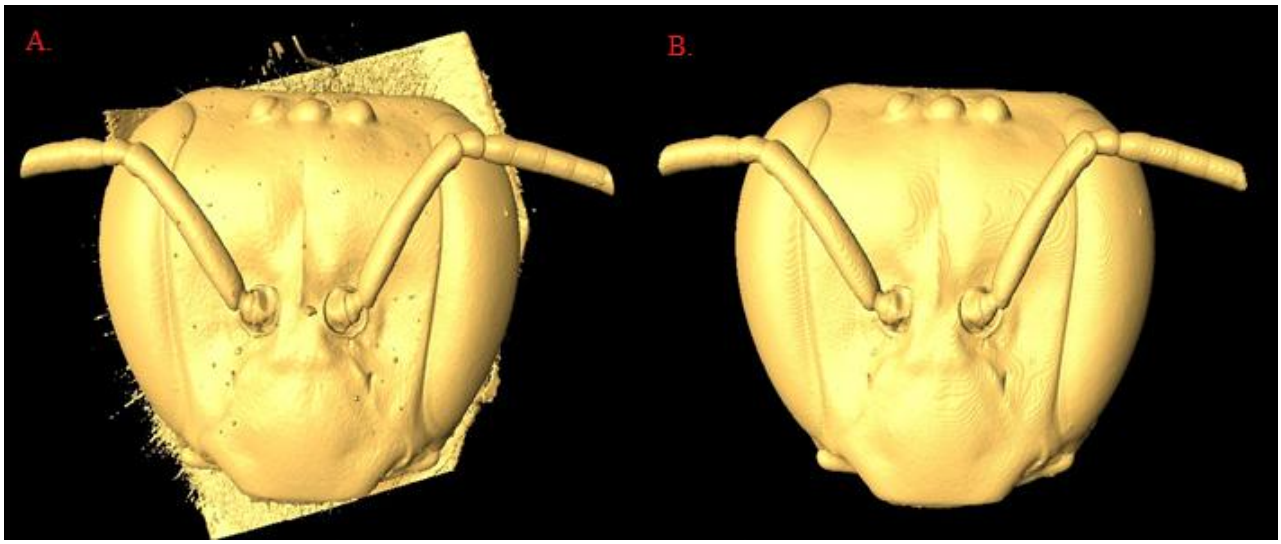

Figure 7. Pictures taken from above of isosurfaces displaying the external structures in Amira (version 5.3) of the bee species *M. Bicolor,* before cleaning of the isosurface (A) as well after the cleaning (B).

Comparing eye to head ratio

When studying the isosurfaces of the different bee species eyes, the eye area was compared relative to the head area. Measuring the eye size was done in Amira by a custom made script. When the head area was to be determined, some issues arose. Since not all the samples had antenna still attached to their heads, and since some of the glue or resin was still there after the clean up, the head area was hard to define and the values were unreliable. Thus, the value of the head area was determined by measuring the eye to eye width in Amira, in a three dimensional line. And so, only the relative percentage was compared between the bee species, by calculating the square root of the eye area and dividing with the head width. The following values was obtained in **Figure 8**, where the raw data is shown in **Table S1**. As seen, the *E. Cordata* has the biggest eye to head ratio of both the old and the new method lying over 50%, as well having the overall biggest head compared to the others. The *M. Bicolor* samples lies just beneath, with having the secondary biggest head size. *T. Spinipes* as well *N. Testaveicornis* has the lowest eye to head ratio with having smaller heads. The sample that is questioning the trend, where the bigger head results in bigger eyes in relation to the overall head size, is the *Tetragona Clavipes* from the old method, with being even smaller than *T. Spinipes*. This can be due to many factors, one possible "error" being the measurement of the head width. Why? Because some species have a longer head length across, instead in the width of the head. Thus, it might be hard to draw any distinct conclusions regarding the head size.

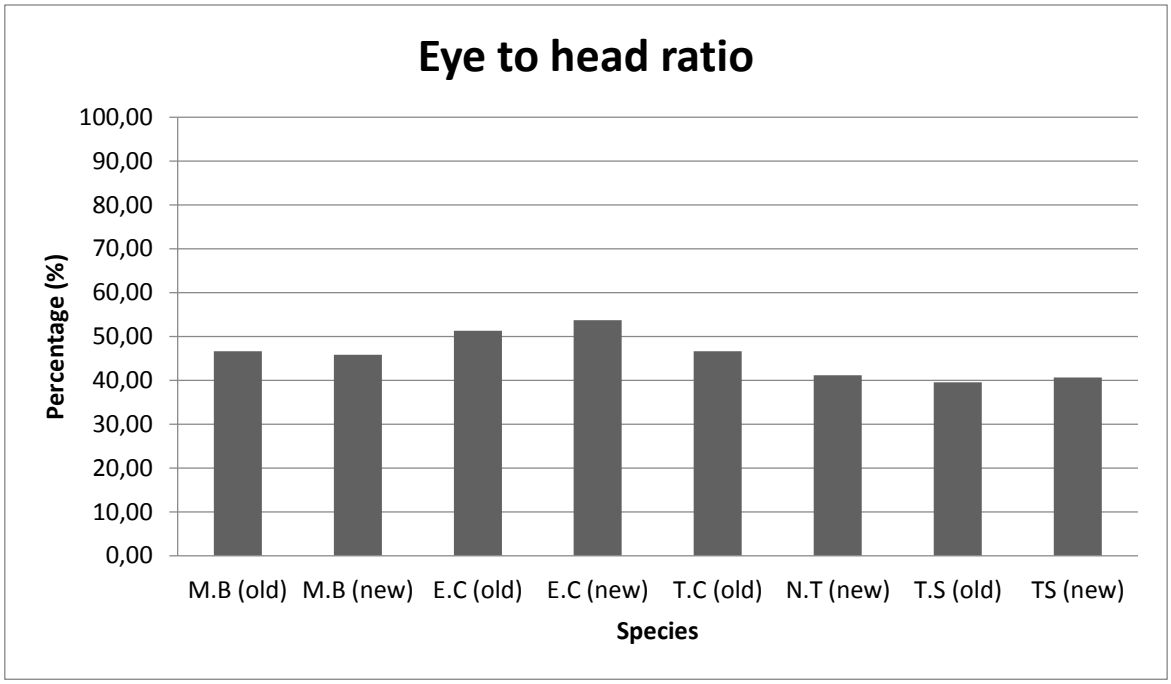

Figure 8. Bar exhibiting bee species of *Euglossa Cordata* (E.C)*, Melipona Bicolor* (M.B)*, Nannotrigona Testaceicornis* (N.T)*, Tetragona Clavipes* (T.C) and *Trigona Spinipes* (T.S) from both the old and the new method. The eye to head ratio is analyzed in percentage.

Why the eye to head ration is interesting to discuss is due to that depending on where and in what habitat the bee species of interest is living, the shape of the eye and size have through evolution adapted to having their own favourable niche. With this information provided, one can suggest what features in an eye is important to possess under certain environmental conditions. Whether it is dark and only a small portion of light can be absorbed each day as in a dense rainforest, or if there is an excess of light in a sparse forest, the eyes will have a certain type of characteristics. A recent study on compound eyes of the wood ant (*Formica rufa*) suggests that the scaling of the diameter of the facets can either lead to an increase or a decrease in size, at same or different rates, within the same species [10]. Thus, there is an adaption with the specific environment the species encounter, as the eyes further develops depending on the context of the habitat. Whether it is low resolution which is useful for detecting landmarks, or a higher resolution to have good near vision but being poor at finding landmarks, one can understand what is important for a bee to see in a tropical environment. Another study found that the ants who were active during the day had much smaller facet sizes, but compensates this with having an augmented number of facets for better sampling resolution [11]. However, this might not actually change the eye size. Thus, one cannot easily draw any conclusions to know what eye to head ratio makes a good eye depending on the environment it encounters. Further studies needs to be done on the bee species we have analyzed to draw any conclusions regarding their eye to head ratio, as for the number of ommatidias or the facet size and what this does to the overall eye size. As well comparing the whole head area instead of just the width, which in our case was not viable due to the loss of antenna in some specimens, cut off parts as the mandible and unwanted glue or resin still attached to the bee head.

The main conclusion to draw from this comparative study, is that the new method compared to the old method is definitely a better one to use if one wants to study the external structure of a bee's head. If one wants to go more into detail of the internal structure, the new method is not the most reliable and the old method might seem better to use in this case.

Author's note

The overall guidelines and layout for this article is based on the journal Nature.

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Supplemental Information

Table S1. Table showing the head width length and the eye length measured with Amira (version 5.3) to obtain the eye to head ratio.

	Head width	eye area	square root eye	eye/head ratio(%)
M.B (old)	3349.28	2438335.00	1561.52	46.62
M.B (new)	3566.55	2668567.00	1633.57	45.80
E.C (old)	4342.74	4967083.00	2228.70	51.32
E.C (new)	4741.85	6484799.00	2546.53	53.70
T.C (old)	2453.85	1310821.00	1144.91	46.66
N.T (new)	1804.21	551871.00	742.88	41.17
T.S (old)	2795.49	1220380.00	1104.71	39.52
T.S (new)	2715.93	1218397.00	1103.81	40.64