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Reconstructing heads of insect pollinators with micro CT-3D

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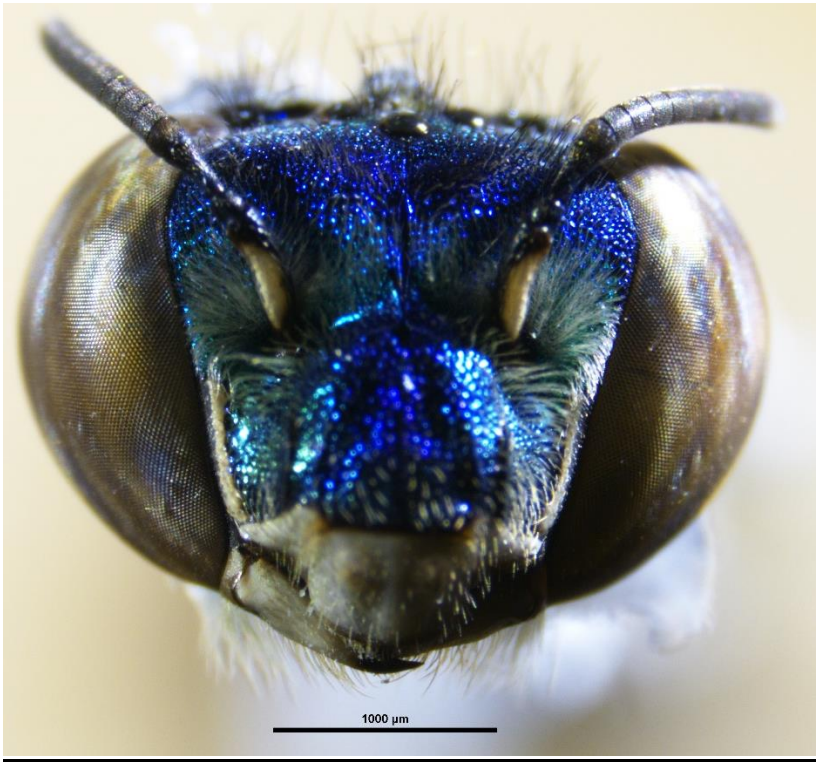

Handledare: Gavin Taylor, Emily Baird

Bees are mostly diurnal insects and rely on their vision to perform daily tasks. Their apposition compound eyes play a big part in regulating vision. To improve our knowledge of how vision functions, we will observe these apposition compound eyes using micro CT. The aim of this report was to compare two different methods of preparing and scanning the bees. The first method uses resin to embed the bee and was performed by our supervisor, in the second method the bees are dried through critical point drying and then glued onto metal pins. Scanning them in 3D will help us learn more about their eyes and other head features. A link has been found between head and eye size; the larger the bee, the bigger the eyes. The dried method can quickly prepare samples and perform quantitative analysis using micro CT. Results showed it to be a quick and non-invasive method to analyse exterior morphology. Information on exterior features of the compound eyes can provide future clues through analysing visual angles to identify where the eye sees. This provides an indication of different species visual capabilities and behaviours, and can then be used to design technological objects such as robots.

Introduction

Insects have advanced eyes despite their small eye and brain size. They depend on vision to accomplish daily tasks such as navigating and finding food. All insects possess compound eyes, which unlike human eyes possess multiple lenses. Indeed, many insects have compound eyes with thousands of individual lenses[1]. Each lens, combined with a photoreceptor called a rhabdom, as well as other associated structures, make up the ommatidia. Some insects, such as bees, possess apposition compound eyes meaning they have a convex structure where each ommatidia works as a single unit to provide a single pixel of visual information[1]. The eyes of bees have the potential to have a large visual field making them able to see a larger area helping their survival[1]. These eyes are especially adapted for daylight conditions since they have relatively small lenses with low sensitivity, although some insects with apposition eyes are nocturnal[2]. Other parts of their anatomy are therefore adapted to make up for this disadvantage[3]. Researchers are still unsure exactly how the compound eye functions as a whole[1]. The very small lenses that make up the apposition eyes have low sensitivity. Light diffraction is a factor that leads to decreased resolution[4]. This is not necessarily a disadvantage since low resolution vision has been found optimal for orientation towards visual landmarks[5]. Indeed, ants use skyline shape to navigate and the resolution cannot be too high for this to occur[5].

Bees possess multiple visual systems and have an additional visual system, ocelli. These are also called simple eyes and are important in most insects, their triangular shaped unit is usually placed on top of the bee's heads[6]. It has recently been found that ocelli play an important role in stabilizing the flight process **[6]**. They have also been shown to be of increased sensitivity in nocturnal insects, in order to make up for the disadvantage of the apposition compound eyes[3]. Insect heads and their specific features can be visualized through several methods, such as Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM), which are commonly used to examine

internal and external morphology[7]. There is now a new method that is starting to replace these two: X-ray Micro-CT, where the advantage is that it is a non-invasive method that provides a wide field of view[7]. If samples are scanned at high resolution, a considerable amount of data can be obtained and a quantitative image analysis where all data is processed, can be done[8, 9]. Our project aimed at finding a new method to easily prepare and scan samples of bee heads using micro-CT. We then aimed to observe the outside morphology, concentrating on the compound eyes where we then choose to measure the eye area and compared it to head size, both within and between species. This method was then compared to a previous method performed by our supervisor[3, 6, 10].

Method

Two different methods of preparing the bees prior to analyzing are compared in this project: the resin method and the dried method. About 100 individuals originating from around 30 different species were collected in Panama and Brazil in February 2016, prepared using the dried method and then several of these samples were scanned and compared with scanned samples from the resin method (Table 1). The scanned samples originated from 5 different species: *Nannotrigona testaceicornis*, *Melipona bicolor*, *Tetragona clavipes*, *Trigona spinipes* and *Euglossa cordata*. The first four are all stingless bee with *M. bicolor* having the particularity of being polygynous while *E. cordata* is a type of orchid bee.

Table 1: Species collected in February 2016 and prepared using dried method

Species	Gender	Origin	Number	Size
Exaerete frontalis	M	Panama	2	Big
Eulaema meriana	M	Panama	2	Big
Euglossa imperialis	M	Panama	6	Medium
Megalopta genalis	F	Panama	1	Medium
Apoica pallens	F	Panama	1	Medium
Eulaema nigrita	M	Panama	1	Big
Eulaema polychroma	M	Panama	1	Big
Scaptotrigona depilis	F	Brazil	4	Small
Plebeia remota	F	Brazil	8	Small
Scaptotrigona. bipuncta	F	Brazil	4	Small
Friesella schrottkyi	F	Brazil	4	Small
Plebeia pugnax	F	Brazil	4	Small
Bombus morio	F	Brazil	4	Big
Nannotrigona testaceicornis	F	Brazil	4	Small
Tetragonisca angustula	F	Brazil	4	Small
Halictidae sp.	F	Brazil	2	Small
Euglossa cordata	M	Brazil	4	Big
Eulaema cingulata	M	Brazil	2	Big
Plebeia droryana	F	Brazil	4	Small
Plebeia saqui	F	Brazil	4	Small
Tetragona clavipes	F	Brazil	4	Small
Trigona spinipes	F	Brazil	4	Small
Melipona bicolor	F	Brazil	4	Medium

Scaptotrigona depilis	M	Brazil	4	Small
Ptiloglossa	M	Brazil	2	Big
Melipona quadrifasciata	F	Brazil	4	Medium
Bombus terrestris	F	Brazil	2	Big

Size range: Small: 1,3- 2,7 mm head length Medium: 3,0-5,3 mm head length Big: 5,3-8,3 mm head length.

Resin method

Preparation: Bees were immobilized by chilling to 4˚C and the lower portions of their heads were cut off. Parts of the head are dissected away to allow for better chemical preservation of the interior. The bee heads were then fixed in a mixture of 3% paraformaldehyde, 2% glutaraldehyde, and 2% glucose in phosphate buffer (pH=7.3, 0.2M) for 2 hours followed by secondary fixation with 2% OsO_4 for one hour. Samples were then dehydrated using a dehydration series with ethanol concentrated at 70, 80, 95, 100% with an incubation of 10 minutes in each solution[6, 7, 10].
Mounting: The specimens were then immersed in acetone and then impregnated with liquid epoxy resin. They were mounted on top of Perspex cubes. Once dry, the external resin was peeled off[6].
Scanning: The samples were then scanned at an isotropic resolution of 2.44 microns using synchrotron X-ray micro-tomography at the Diamond Light Source located in Oxfordshire, United Kingdom.

Dried method

Preparation: The bees were immobilized and decapitated. Isolated head were then fixed for 2 days in primary fixation buffer as used in the previous method. They were subsequently put in 70% alcohol and refrigerated for 2 months. The samples were later dried using the dehydration series as listed in the resin method above[8]. The samples were cleaned using paintbrushes then put in an ultrasonic bath (Branson 1200) for 30 seconds to further remove dirty particles.
Critical point drying: the exchange of alcohol contained in the samples to liquid carbon dioxide. This is done by placing the samples into the machine (Bal-Tec CPD 030) which had been cooled to 9˚C, temperature at which the CO_2 is liquid. Five short steps with 2 minutes intervals followed by one 30 minutes interval were performed followed by three or four long 10 minutes intervals depending on sample size. Once no ethanol odor could be smelt, the samples were heated to 40˚C, which is slightly above CO_2's critical point of 31˚C[11].
Shrinkage Test: to verify that the drying steps did not cause a decrease in bee head size, a shrinkage test was performed. Five bumblebees (Koppert) were imaged with a microscope (Nikon SMZ18) and then cold anesthetized and their heads were dissected from the rest of the body. The samples were then fixated for 1.5 days in the first fixation buffer[8]. They were then transferred to 70% alcohol overnight. To achieve the maximal dehydration possible, the dehydration steps were performed and the samples were then left in 100% alcohol for one week. Critical point drying was then performed in the same way as listed above[11]. The bumblebees were scanned in the microscope where the head sizes were measured both prior to dehydration and after critical point drying was performed. Six different measurements were taken on each sample using ImageJ, an image processing program, to efficiently compare the bees. Each measurement of the dried samples was compared to those of the fresh samples to obtain a percentage. The average for the five bees was obtained for each of the six measurements (fig. 1).

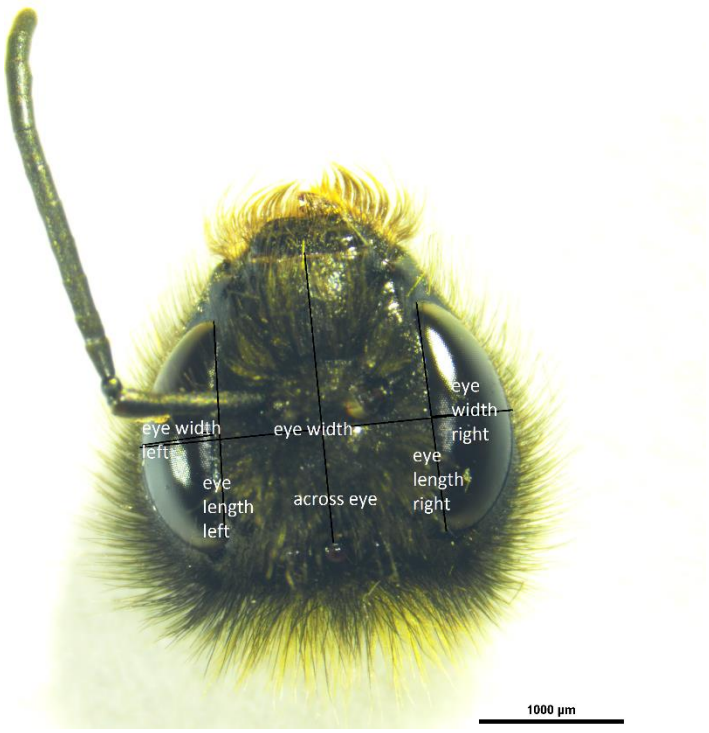

Figure 1 *indication of how measurements were taken for the shrinkage test, fresh bee number 3, high resolution. Scale: 1000 microns*

Mounting: The bees were mounted after drying onto a piece of paper glued onto either metal pins or, if the antennae were going below the level of the pin head, cut toothpicks to prevent X ray-shadowing from the metal.
Scanning: 4 Samples from the dried method were scanned using 3D X-ray microscope (Xradia520 Versa) with a resolution of around 5 micron depending on the samples and the images were processed in Amira 5.3.3., a software for 3D data visualization.

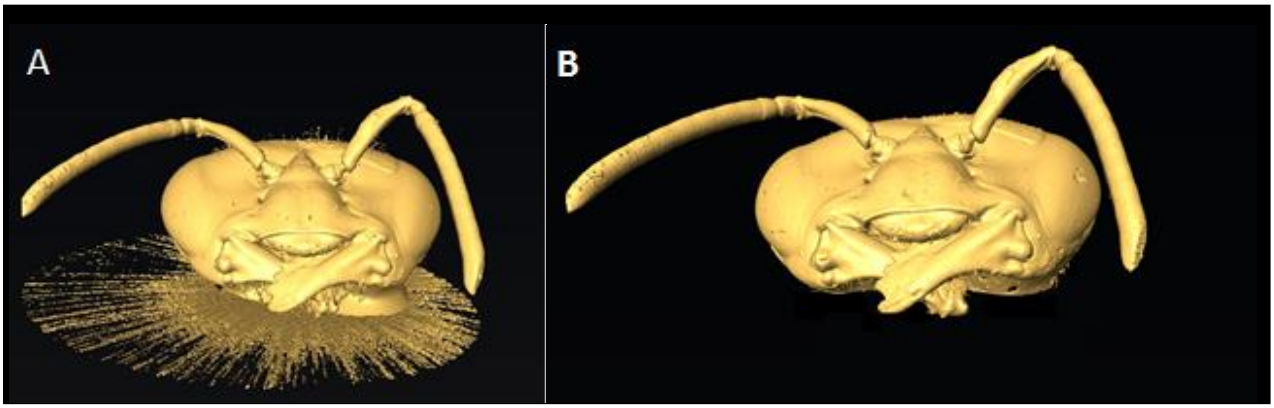

Figure 2 Isosurface showing glue removal process of *T.spinipes*, obtained in Amira A: Before B: After

Processing in Amira

Several steps were performed in Amira to clean samples and calculate different parameters. When the data is first obtained, it consists of a volume of grey scale values. To better visualize this we calculated the orthoslice and isosurface for the samples. The Orthoslice subsets a structured field by extracting a one slice plane. The isosurface is a 3D surface representing all voxels in the images together. For the dried method, some samples were slightly covered in glue due to the mounting process (fig.2A). Glue removal was performed using processing tools to remove the excess glue. Once the outside was clean (fig.2B) eye area and head size could be obtained for both methods. The eye area was selected individually using an area calculating tool while head width was measured. The square root of the eye area was then compared to the width of the head multiplied by 100 to calculate a final percentage.

Results & Discussion

Looking at the isosurfaces produced by the two methods (fig.3), we can observe some clear differences between the two methods. In the resin method the head is partly cut off, where, in this case, a part of the left eye was cut off. This makes it harder to get a clear view of the actual surface and therefore it also affects the calculation of the area. In the second method, the samples also possess the antennae giving the opportunity to analyze them and calculate a more accurate surface for the entire head. In the first method (fig.3A) the back contains some resin which could also affect surface calculation. In the second method, glue spots can occur which could make the surface inaccurate. In this particular sample (fig.3B), the glue spots were minimal and were easily removed using processing tools in Amira. For more substantial glue spots, such as ones on the top of the head, it is much harder to remove the entirety and can affect the total head area.
Since the resin samples were scanned in the Diamond Light Source they have a higher resolution than the ones sampled in the Xradia 520 versa. This makes it easier to have a better look at detailed features of the bee head.

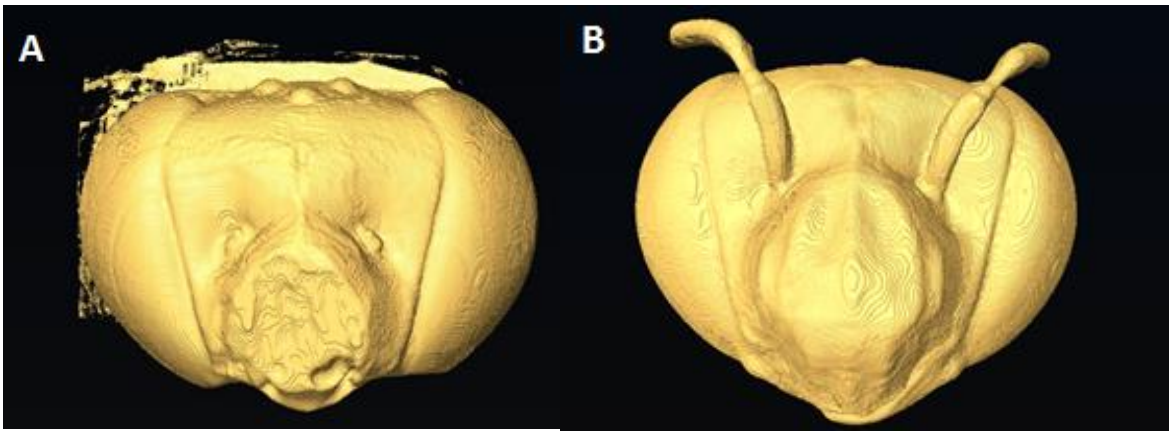

Figure 3 Isosurface of *E.cordata* obtained through 3D-imaging after Micro-CT, and Amira
A: Resin Method B: Dried method

If we now compare the orthoslices between the two methods (fig.4), the resin method (A) preserves the inside parts such as brain region and structure of the eye. In the dried method (B), the eye is fissured and the different brain parts are much harder to distinguish. This is probably due to the drying steps in the method, shrinking the inside features which consist of soft tissue. The resin

method has better preserved insides and in this case, were scanned at a higher resolution than the dried method. To improve the second method, the samples could be scanned in a synchrotron such as the one used for the resin method, which could increase resolution and show more details on the outside surface. The resin method preserves the soft tissues of the bee better and should therefore be used when wanting to analyze those parts, such as for segmenting the different sections of the eye. The dried method is optimal to get a fast and accurate outside surface as long as excess glue is removed. Several more features could be observed using this method, such as the ocelli and the antenna. These could then be measured in order to be compared both within the species and between different species. This would allow a better understanding of the role of each of a bees five eyes in their vision, which could be related to how they perform their daily tasks such as navigating and finding food.

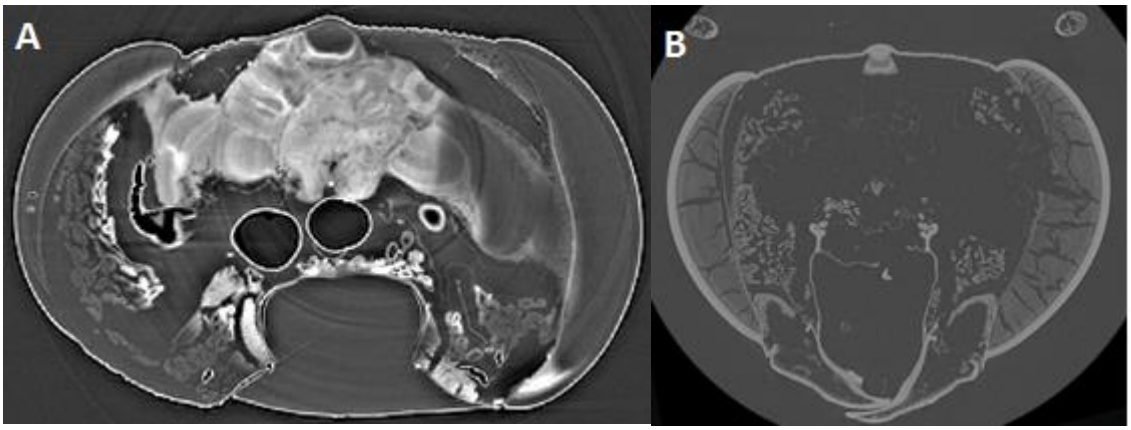

Figure 4 Orthoslice of *E.cordata* obtained through 3D-imaging after micro-CT, and Amira 5.3.3. A: Resin Method B: Dried method

Shrinkage test

Looking at these percentages (fig.5) we can see that for each measurement there is less than 5% change between fresh and dried. Some shrinkage might have been expected but for some measurements the percentage is above 100% meaning the dried samples would have increased in size. These slight changes in measurement seem to be due to human error since these are difficult to perform precisely. To verify this, the same measurement was measured 5 times on the same bee. As expected, the human error was of similar magnitude as the difference between the dried and fresh measurements, indicating that no substantial shrinkage occurred during preparing and drying steps.

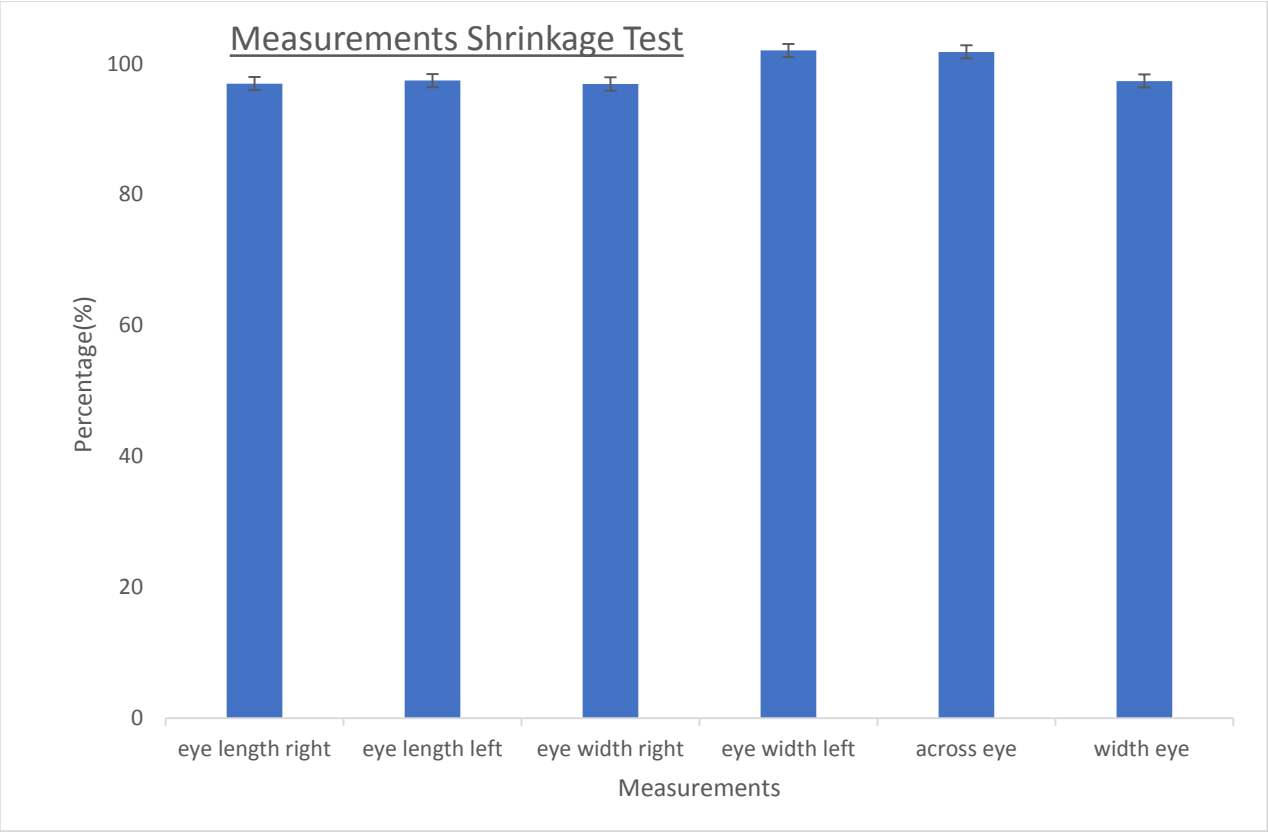

Figure 5 Change in percentage between fresh and dried bumblebees at different measurements during shrinkage test

Visually, the fresh and dried bumblebees look similar in size (fig.6). The only difference being that the eyes are clearly turned into a gray shade. This is probably due to inner shrinking of soft tissues as mentioned above and, as seen with the measurements (fig.5), this does not affect the outside surface of the head.

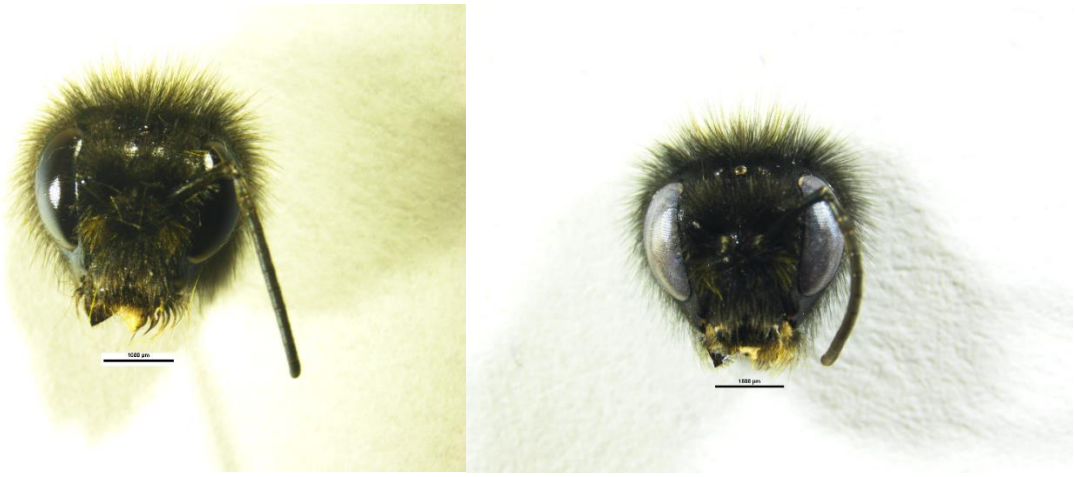

Figure 6 Bumblebees used for shrinkage test A: fresh bees B: dried bees, scale: 1000 microns

Head and eye size

The eye area/head width percentages are very similar in between the same species, indicating the different methods don't affect the size (fig.7). Comparing the different species, there is no striking difference although *E.cordata* have the biggest eyes compared to body size while *T.spinipes and N.testaceicornis* have the smallest. It seems that size of the eyes is linked to the total size, as *E.cordata* is the biggest species and *N.testaceicornis* the smallest. This increase in compound eye size according to head size has already been observed in several insects and this could also be the case in bees[5]. One factor to consider is that head width might not be the most accurate measurement to use as a comparison to area. In the future, total head area would be a better value to compare. In this case it was not possible to use this feature as the samples were of different types, as some had antennae, some parts of the head were cut off which would affect the final area. Glue or resin residues also affect total area.

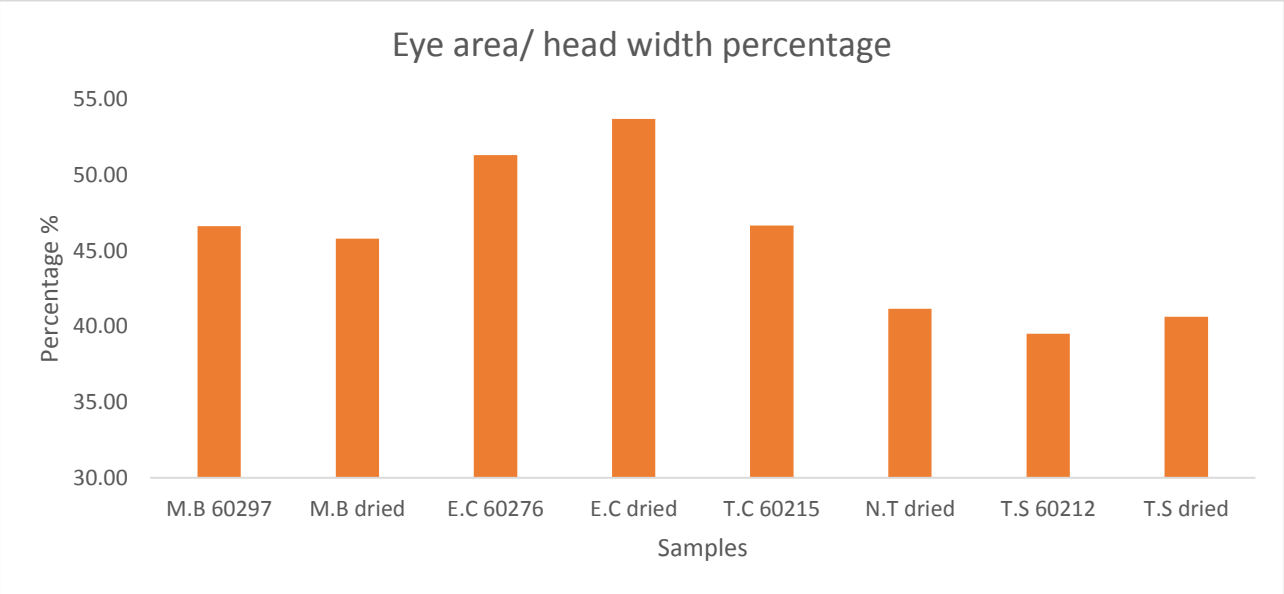

Figure 7 Eye area/head width for bee heads of different species and obtained through different methods

Conclusion

In this project, we have compared two different methods of preparing and scanning bee heads with the aim to better understand different features of the head. Our main focus has been on the apposition compound eyes that bees possess. The dried method has been shown to quickly and efficiently procure a good outside surface which would help us get a better understanding of these features. We have found a link between eye and head size, correlating with previous studies[5]. Indeed, the bigger the head size, the bigger the eye area. With higher resolution and a bigger sample size, the dried method shows promise to discovering more about compound eyes.

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