Standardization of the Compass Neuropils of the Australian Bogong Moth, *Agrotis infusa*.

Department of Biology, Lunds University

Liv de Vries
Supervisor: Stanley Heinze
MOBK01 Examination in bachelor's level Molecular biology. Ht 2015.

Abstract

Every summer the Australian Bogong moth migrates from southern Queensland to the Australian Alps in southern New South Wales. Other migrants such as the monarch butterfly use a time-compensated sun compass. However, the Bogong is nocturnal and only has access to skylight cues such as the moon, polarized light or the Milky Way. Since these are considerably unreliable only being visible short periods of time, another navigational mechanism could be a magnetic compass. In the Monarch the brain regions (neuropils) involved in navigation are the central complex, the lateral accessory lobes and the anterior optic tubercles; which together are called the 'compass neuropils'. As the brain anatomy is the structural basis for all information processing underlying orientation and navigation, it can be used as an access point to understand this extraordinary migratory behaviour. We therefore performed 3D-reconstruction of the compass neuropils of the Bogong moth brain. By generating a standardized version of the compass neuropils I have created a common frame of reference for registration of neuron morphologies and networks, as well as providing reference volumes that were compared to those of the monarch. The Bogong moth brain was found to contain all the compass neuropils, however there was a significant difference between the two species neuropil-volumes. Interestingly, all components of the central complex were larger in the Bogong, while the upper unit of the anterior optic tubercle was substantially larger in the Monarch.

The Bogong moth, *Agrotis infusa*, has a nocturnal lifestyle and migrates from the Darling Downs in southern Queensland to the Snowy Mountains in southern New South Wales where, in a single cave several hundred thousand of Bogong moths can aestivate, a form of hibernation in the summer during the dry period. After summer has passed the moths emerge again and migrate back to their breeding grounds in Southern Queensland. After laying eggs they die and the next generation repeats the migratory cycle. Hence, these moths find their way to the caves without any previous experience. How they manage this impressive migration is yet unknown (Common, 1954; Heinze, pers. comm.).

The strategy of long-distance migration to survive a seasonally fluctuating habitat has led to the evolution of complex navigational mechanisms (Merlin et al., 2011). The Monarch butterflies, *Danaus plexippus*, of North America have a similar migration to the Bogong moth, from eastern North America to central Mexico. However, the Monarch butterfly is diurnal and navigates using the sun and polarized light (Heinze & Reppert, 2012; Guerra et al. 2014). This navigation mechanism thus uses a time-compensated sun compass. The eyes senses directional diurnal light cues such as the horizontal position of the sun, polarization patterns or the skylight spectral gradient. This information is then integrated in the midbrain central complex area, after which it is time-compensated by the antennal circadian clocks. However, the monarch butterfly is still able to navigate when day light cues are absent suggesting an additional compass (Guerra

et al., 2014; Merlin et al., 2011)

The Bogong moth could potentially use navigational skylight cues such as the moon, the polarized light around the moon or the Milky Way. These cues are however unreliable in their visibility and are, when visible, only present during a short period of time during the night. Another more reliable cue would be a magnetic compass, which has been shown to be exploited by migrating monarch butterflies (Guerra et al. 2014). The study by Guerra et al. (2014) showed that the monarch has a light dependent inclination magnetic compass, which relies on ultraviolet-A/blue light between 380-420nm. For the monarch this compass is essential for keeping the directionality during migration when day light cues are absent. It may also act as a supplement to the sun compass (Guerra et al. 2014). The magnetic compass is seen in other long-distance migrants such as sea turtles and birds, utilizing the polarity, intensity and inclination angle of the earths magnetic field (Lohmann, 2007; Wiltschko & Wiltschko, 2005; Reppert et al., 2010). The information could either be used to establish geographic position relative to the destination, a map sense, or to maintain a constant heading, a compass sense (Reppert et al. 2010). A fundamental benefit of the magnetic compass, compared to the sun compass, is that there is no need for a continuous time compensation to keep on course throughout the migration (Merlin et al. 2011).

Although a lot is known about the behavioural aspects of animals' navigational abilities, very little insight has been gained with regards to the neural basis of migration. With their small and accessible brains, insects like the Bogong moth provide ideal models for investigating the networks and neurons underlying long distance migration. The general insect brain consists of optic lobes, antennal lobes, mushroom bodies and the central complex (CX), together with a number of smaller neuropils and large areas without defined boundaries i.e. unspecified neuropils. The optic lobes are involved in visual processing and contain the lamina, medulla and the lobula complex. Olfactory information is processed in the antennal lobes and is then relayed to the mushroom bodies. These play an essential role in olfactory learning and memory. The CX is a group of unpaired neuropils, consisting of the protocerebral bridge (PB), the central body and the paired noduli (NO). The NO seems to have a potential role in flight control as they appear to be only present in pterygote (winged) insects (Pfeiffer & Homberg, 2014). The central body is divided into an upper subunit, CBU, and a lower subunit, CBL. The CX receives indirect input mostly from the visual system but also mechanosensory- and, potentially, olfactory information. In the Monarch butterfly, as well as the desert locust, the CX is the main neuropil involved in migratory navigation, and its neurons are responsive to the direction of polarized light (Pfeiffer & Homberg, 2014). Despite the high degree of conservation across insects, there are several species-specific differences. For example, the PB appears as a discontinuous structure across the midline in lepidopteran insects, giving it a paired appearance. Several smaller neuropils, the anterior optic tubercles (AOTU) and the lateral accessory lobes (LAL), are tightly linked to the CX. Together with the LAL, two small regions, the gall (GA) and the bulb (BU), form the lateral complex. Even though poorly understood, these regions provide conserved input and output relays for information that enters the CX (via the BU) as well as information that leaves the CX (via the GA). Both regions are innervated by compass neurons in locusts and monarchs. In the monarch the GA has been referred to as the anterior loblet and the BU as the lateral triangle (LT). The AOTU can also be divided into several subunits a large upper unit (AOTU-UU) and the smaller nodular unit (AOTU-NO) and the lower unit (AOTU-LU). However, the monarch brain has an additional subunit called the strap (AOTU-SP) (Heinze & Reppert, 2012). Together the CX, AOTU and lateral complex make up the 'compass neuropils' in the monarch, (figure 1; Heinze et al., 2013).

As the function of a nervous system is closely linked to its architecture, a general understanding of the moths' brain anatomy is needed to explore this species' remarkable migratory behaviour and its underlying mechanism (el Jundi & Heinze, in press). This study

therefore aims to investigate the neural architecture of the Bogong moths' brain, particularly examining if the 'compass neuropils' are present and to what extent their internal structure resembles that of the monarch butterfly. To this aim I pursued the generation of an average-shape atlas of the compass neuropils of the Bogong moth. Additionally, I have quantitatively compared the Bogong moth data to the CX of the Monarch butterfly to identify potential subtler differences between the species in these highly conserved brain regions.

By means of 3D-imaging the brain can be studied in the X-, Y- and Z-dimensions. This provides a crucial advantage over 2D imaging, which calls for some sort of previous kno2wledge of the morphology since, there is no information about the Z-dimension of structures. A standardized brain can then be used as a frame of reference and be combined with individual neurons to deduce their roles in neural networks, e.g. the circuits underlying compass navigation (el Jundi & Heinze, in press). This work will provide the foundation of analysing the neural networks underlying the remarkable migratory abilities of the Bogong moths. Furthermore, it could be used in mapping of gene expression, neurotransmitter expression and more precise targeting during electrophysiological recordings (Heinze & Reppert, 2012).

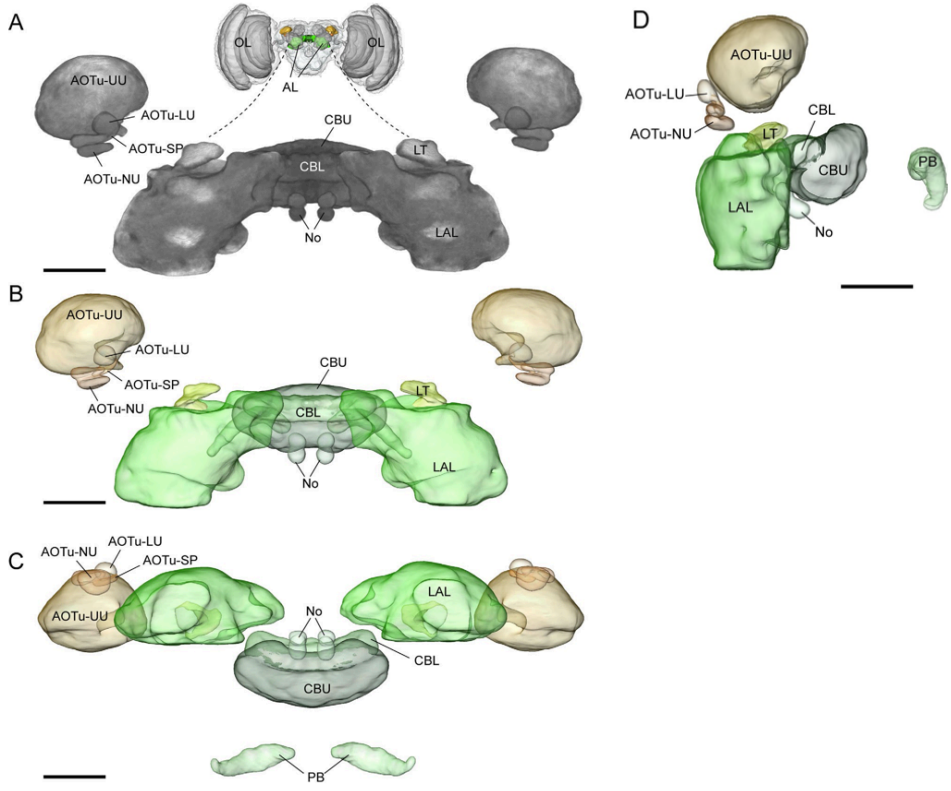

Figure 1. The standardized Monarch compass neuropils. Averaged from 10 individual Monarch brains. A, shows a frontal view of the image stack results from the iterative shape averaging protocol. B-D, are surface models based on whole-mount anti-synapsin staining, frontal view; C, ventral view D, side view. The abreviations used are: OL (optic lobe), CBU (central body upper unit), CBL (Central body lower unit), PB (protocerebral bridge), NO (noduli), LAL (lateral accessory lobe), LT (lateral triangle), AOTu-UU (anterior optic tubercles upper unit), AOTu-NU (anterior optic tubercles nodular unit), AOTu-LU (anterior optic tubercles lower unit) and the AOTu-SP (anterior optic tubercles strap). Scale bar = 100 μm (Heinze et al., 2013).

Materials and Methods

The aim of this project was to generate an average-shaped ("standard") 3D model of the Bogong moth compass neuropils. This process consisted of three major parts; histology, confocal imaging and image processing. The histology in turn can be divided into six steps: dissection, fixation, permeabilization, antibody incubation, dehydration/clearing and embedding in mounting medium. For this project whole-mount preparations were used, meaning the whole moth brain was processed as is, without the need to physically section the samples. Whole-mount preparations have not been routinely used for immunohistochemical staining until recently, since tissue has to be made permeable to the antibodies to avoid gradients, while still preserving the tissue integrity. The fixation protocol plays an essential role and has to be carefully optimized. The staining process also becomes considerably slow. The incubation time for the primary antibodies is six days and five days for the secondary antibodies. These methods are based on the protocol in "Insect brain atlases chapter 3" (el Jundi and Heinze, in press, 2015), Heinze & Reppert (2012), as well as Heinze et al. (2013). All concentrations mentioned below are calculated using a volume of 500 μl per brain.

Animals

The animals used in this project were collected either in early January or in late October in caves near the peak of South Ramshead mountain, in Kosciuszko National Park, NSW, Australia. Animals from 2013 to 2015 were used with several people involved in the collection; among others: Eric Warrant, Anna Stöckl, Kristina Brauburger, Ken Green and Stanley Heinze. Moths were in an aestivating state during collection and were kept in this state after transport back to Lund. In Lund they were kept at 6° C at night and 10° C during the day. They had approximately 16 hours of daylight and eight hours of night. Illumination was dim during the day and off during the night. The animals were used up to five months after capture.

Immunocytochemistry in whole mount preparations

The histology began with the dissection of the moth. The head was cut off and mounted in a wax-filled petri dish. The brain was then taken out and fixed in a standardized fixative, consisting of a 1% formaldehyde/zinc-chloride solution, over night in the fridge (according to Stöckl & Heinze, 2015). The zinc is added to the formaldehyde fixative to increase permeability, compensating for the fact that formaldehyde fixation reduces the tissues permeability, which may cause a gradient in staining intensity.

The tissue was then extensively washed (8x20min) in Hepes-buffered saline (HBS). During the first washing steps the retina, as well as left-over trachea, were removed. This is also done to ease antibody diffusion into the tissue. The brain was then bleached with 10% H_2O_2 in Tris/HCl buffer for six hours to increase visibility (Stöckl & Heinze, 2015). After an additional washing step with Tris/HCl buffer (3x10min) the tissue was made permeable to the antibodies by incubation in a solution of methanol and dimethylsulfoxide (DMSO) (80:20) for 70 min. Following a Tris/HCl buffer wash (3x10min) the moth brains were preincubated with 5% normal goat serum (NGS) in 0.01M phosphate-buffered saline, containing 0.3% TritonX-100 (PBT) in the fridge at 4° C over night. This preincubation or blocking step reduces unspecific binding of antibodies to non-target epitopes, thus increasing signal to noise ratio.

The following day the brains were incubated with a solution of 1% NGS, (1:25) primary mouse-derived antibodies against synapsin, a presynaptic protein, in PBT for six days in the fridge. The brains were then rinsed in PBT (8x20min) to wash out excess antibodies and then transferred to a secondary antibody, goat anti mouse, solution (GAM-CY5 1:300, 1% NGS in PBT). The secondary antibody is fluorescence labelled with CY-5, which absorbs light at around 633nm and emits at wavelengths in the near infrared. The samples were incubated in

this solution for five days in the fridge. After two subsequent washing steps with PBT (6x20min) and 0.1M phosphate-buffered saline (PBS) (2x30min) the samples are finally dehydrated with an ethanol series of increasing concentrations (50, 70, 90, 96, 2x 99.8%, incubation time of 15 minutes per concentration). Thereafter, the brains were submerged in a solution of methyl salicylate and 99.8% ethanol (1:1) for 15min, after which they were immersed in pure methyl salicylate for 60 min. Methyl salicylate makes the brain transparent and enables visualization of structures within the brain using the microscope. The brains are then embedded in Permount between two coverslips using a stack of eight plastic reinforcement rings as spacers. A portion of the ring was removed in the last two spacers to allow excess embedding medium to flow out. The samples were then left to dry and equilibrate for three days.

Confocal Imaging

Using a confocal microscope (Zeiss LSM 510) the sample was scanned in several imagestacks, each image a virtual slice of the brain (distance between slices 1μm). This was done using a laser, which stimulates the CY-5 molecules to emit light, making the neuropil structures visible in the microscope. The objective used possessed 25x magnification with a numerical aperture of 0.8 (Zeiss; long-distance objective). To cover the neuropils of interest, two to four image stacks were generated. Lastly, these image stacks were aligned, merged and resampled to a voxel (volume pixel) size of 1x1x1 μm using the software Fiji or Amira 5.2.

Three-dimensional Reconstructions

A label field was created for each final image generated (aligned, merged and resampled) in which voxels were assigned a neuropil identity in the software Amira 5.2. Only the neuropil boundaries of a couple of crucial optical sections in each spatial plane (x-y, x-z, y-z) were labelled. This process was repeated for all relevant neuropils in the image stack, thereby generating a scaffold for each brain region. The whole structure of each neuropil was then interpolated using the Amira wrapping function, creating a 3D surface of the neuropil. Following completion of the label field, a triangulated surface model was automatically obtained (el Jundi and Heinze, in press, 2015). The neuropils reconstructed were: CBU, CBL, NO, PB, LAL, GA, BU, AOTU-UU, AOTU-LU and the AOTU-NU. As in the monarch, the PB of the Bogong has a paired appearance. The colour code used for the compass neuropils in Heinze & Reppert (2012) was used as a template.

Volumetric Analysis

The volume data from each neuropil were obtained from the label field using the tissue statistics tool in Amira 5.2. this data was then used to analyse the variability between individuals and hemispheres. The data from the left and right hemispheres were summed and normalised to the total volume of each brain, creating relative volume data that could be compared to the Monarch butterfly (Heinze & Reppert, 2012). As the neuropil volume of each hemisphere was summed, intrahemispheric variability was eliminated and the comparison analysis was simplified. The comparison between the two species was done using non parametric statistics, a Mann-Whitney U test, giving a conservative estimate of the differences. Each neuropil was compared side by side. This test was chosen since the monarch data was not entirely normally distributed as well as due to the overall small sample sizes. A paired Mann-Whitney U test was used to compare the right and left hemisphere. The means and standard deviations of each neuropil were calculated and displayed together with the absolute values of each individual brain both for the summed relative volumes and the relative volumes of each hemisphere (Heinze et al. 2013). A regression analysis, showing the allometric relations of neuropils, was also done for the Bogong. Plotting the volume of each individual brain for each neuropil against the volume of

the remaining compass neuropils (Ott & Rogers, 2010). All statistical analyses were done in Graphpad-Prism6.0 software. The GA was not reconstructed separately in the Monarch; therefore, the Bogong GA volume was added to the LAL for all direct comparisons. Additionally, as the AOTU-SP was only present in the Monarch, its volume was added to the AOTu-LU.

Standardization of Compass Neuropils

For the final standardization of the 'compass neuropils' nine compass neuropils were reconstructed. The standardization was done using the Computational Morphometry toolkit (CMTK) implemented in the Iterative Shape Averaging (ISA) protocol (el Jundi & Heinze, 2015), which utilizes grey values of the image data to obtain information about the edges of the neuropils. By eliminating all image data more than 30 voxels away from the labelled neuropils, thus eliminating artificial edges created by the boundaries of the image stacks, a "cut-out" image stack was generated using the arithmic tool in Amira. This image stack was then run through the ISA protocol; all ISA computations were performed on a Linux cluster based at the HRZ-Marburg. This is still ongoing and the final results have not yet been obtained.

In general terms, the ISA protocol entails choosing a reference brain amongst the nine reconstructed that is closest to the population average in regards to the volume of the neuropils. This is needed since the reference brain strongly influences the final volumes of the neuropils in the ISA standard. The remaining brains are then registered onto the reference brain by means of affine registration (9 degrees of freedom), which compensates for the rotation, size and position between different image stacks. The resulting average brain was then used as the reference for the elastic registration, which revises shape differences between brains. This is then repeated four times, each round using the previously generated averaged image stack as a template. All registrations give rise to transformation parameters which were applied to each of the nine label fields, creating the final average/standardized surface reconstruction of the Bogong compass neuropils.

Results

Nine reconstructed compass neuropils were used to create the final standardization; representative examples can be seen in figure 2. Figure 2 also displays the size and shape differences between individuals. This is most apparent in the smaller neuropils e.g. the BU and GU, as well as in the relative placement and size of the AOTU subunits. Of the nine brains used, six were reconstructed during this project and added to the ones reconstructed by Stanley Heinze. The moths used were from a population spanning the years 2013-2015, of the six moths used in this project five were females and one male. All the Bogongs reconstructed during this study possesed all compass neuropils, two unpaired (CBU, CBL) and eight paired. In the Bogong as in the monarch the PB is a paired structure (figure 2). The brains are named after the date they where dissected with a lower case letter according to order of dissection.

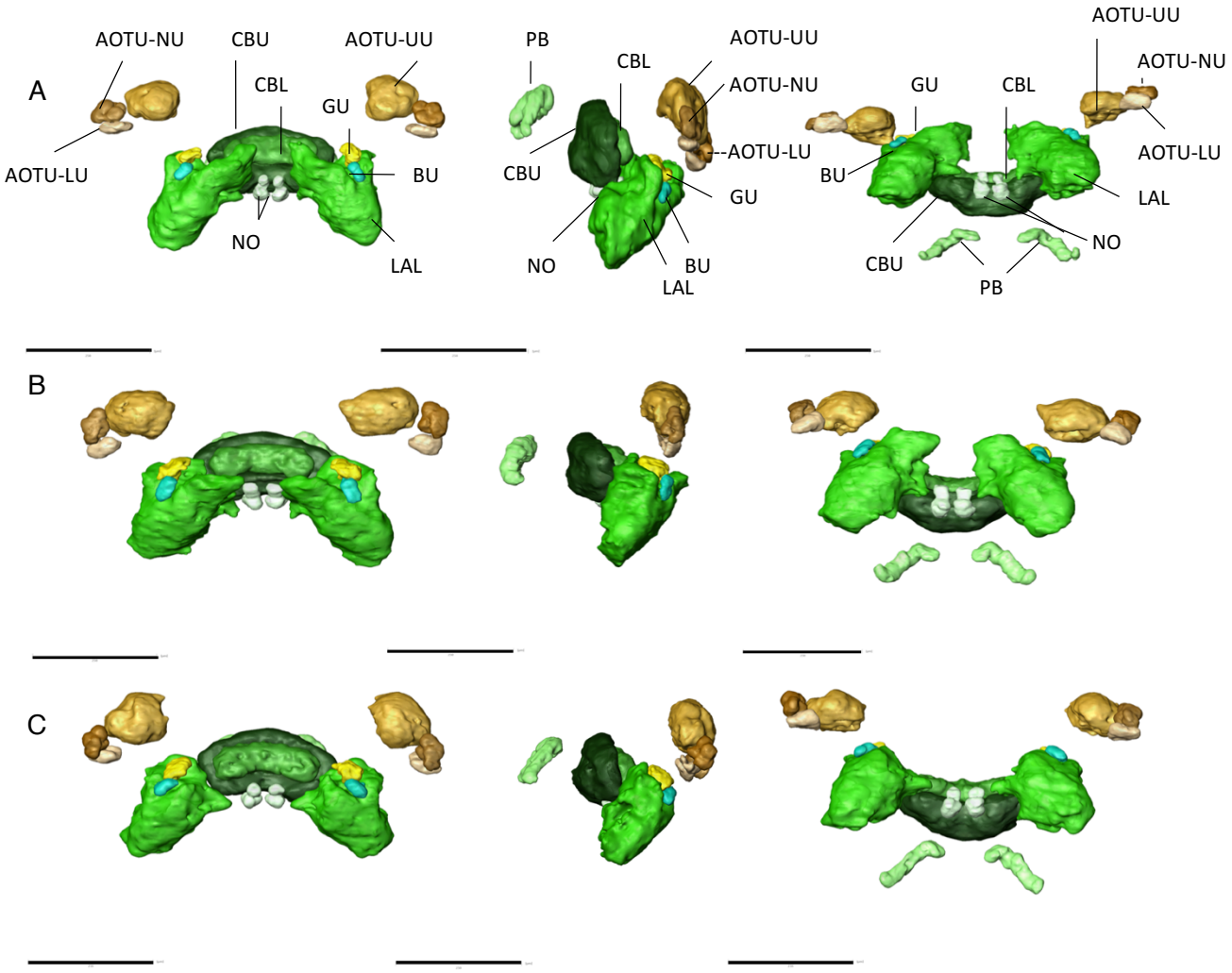

Figure 2. The 3D reconstructed compass Neuropils of three of the Bogong moth used for standardization. Shown are representative reconstructions of three out of six brains. Based on whole-mount anti-synapsin staining and reconstructed using labelfield and SurfGen modules in Amira 5.2. Colour code from Heinze & Reppert (2012). From left to right: frontal view, side view and ventral view. The brains showed are **A)**140310f **B)**150407b **C)**150413b. Scale bar = 250μm.

The relative volume distribution between the reconstructed compass neuropils for eight of the individual brains show some variability between the left and right hemisphere as well as between the different individual brains (figure 4, A & B). However, there is no significant difference between the right and left hemisphere. The summed volumes of the left and right hemispheres show less variability than each hemisphere on its own (figure 3, A & B). Both figures show larger variability in the small neuropils than the large. The regression analyses showed that each neuropil is propotional to the total volume, hence the larger the brain the larger each neuropil becomes (see appendix).

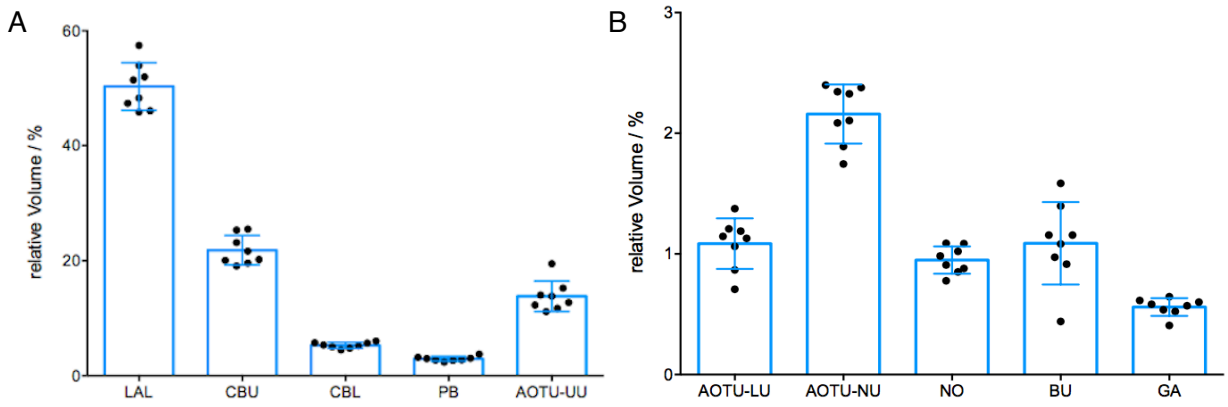

Figure 3. The summed relative volumes of the compass neuropils of the Bogong moth. In A and B the left and right relative volumes have been summed for each compass neuropil. The black markers show the individual values of each individual brain. The error bars show the standard deviation and the bar the mean value of each neuropil for the Bogong moth. A, Summed relative volumes of the large neuropils. B, Small neuropils.

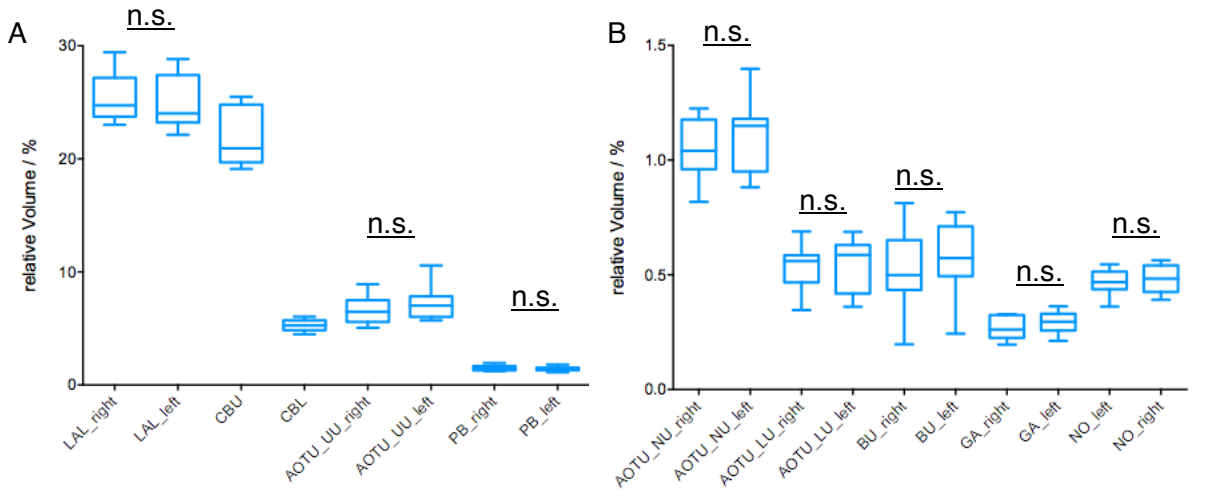

Figure 4. The relative volumes of the compass neuropils of the Bogong moth. In A and B the left and right neuropils relative volume is shown. They display the variability between individuals as well as between the left and right hemisphere. The boxes are standard deviations, whiskers the data range and the lines displays the means. Figure 4A shows the relative volumes for the large neuropils and B shows the small neuropils. No statistical differences between corresponding neuropils on the right and left hemisphere were found (indicated by n.s.).

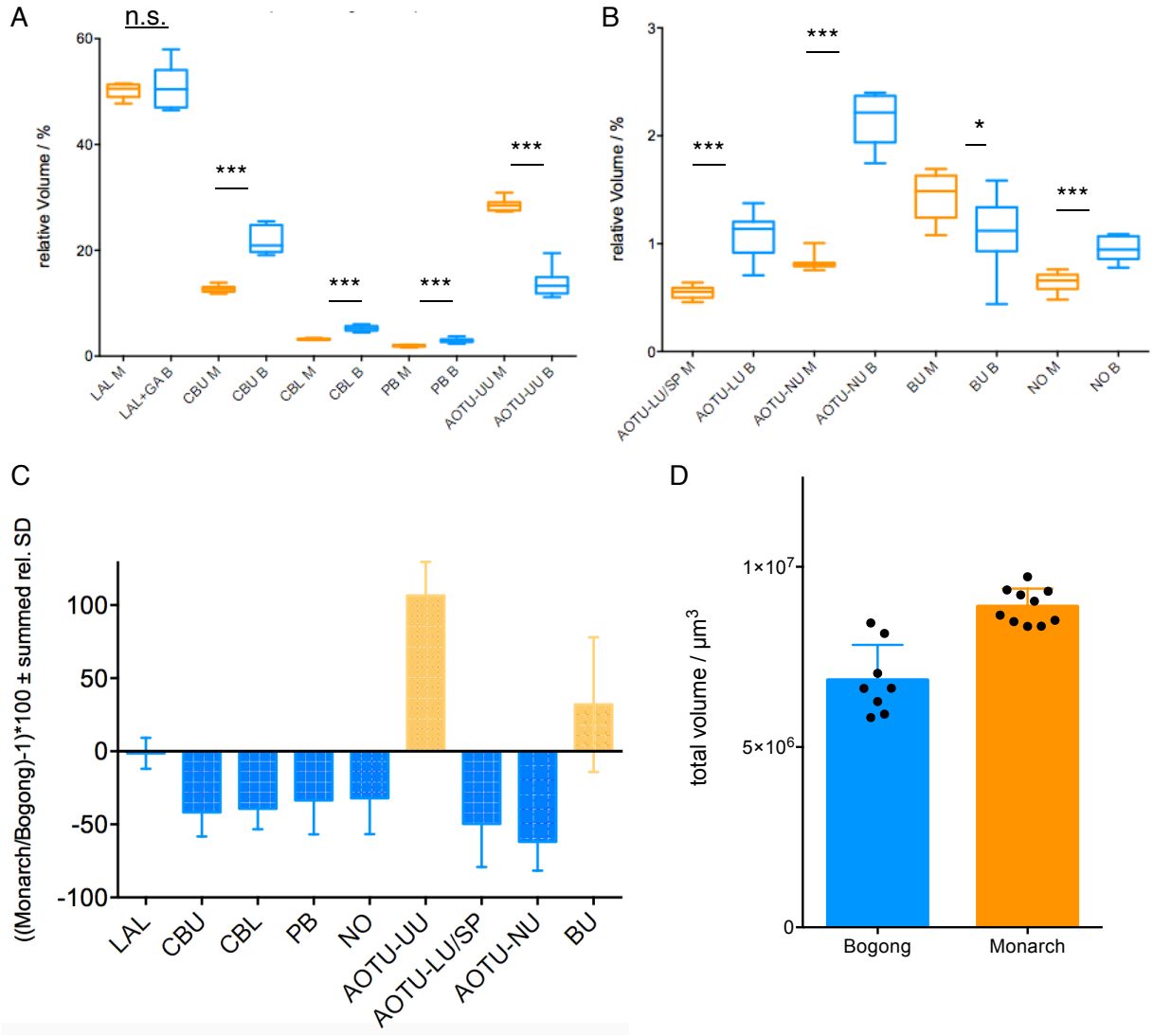

Figure 5. A comparison between relative volumes of the Bogong and the Monarch compass neuropils. In A and B the boxes are standard deviations, whiskers the data range and the line displays the mean. Data from eight Bogongs and ten Monarchs were compared. Significant values are marked by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Non-parametric Mann-Whitney U test were used. A shows the relative volumes for the large neuropils and B shows the small neuropils. The ratio between the two species in % are shown in C. Positive values indicate the monarch neuropil is larger. Negative values indicate the monarch neuropil is smaller. The error bars are the combined relative errors of both species for each neuropil. D, shows the total volume of the compass neuropils for Bogong moth and the Monarch butterfly. The black markers show the individual values of each individual brain. The error bars show the standard deviation and the bar the mean value.

The Mann-Whitney U test showed that all neuropils, except the LAL ($P = 0.8144$), were significantly different ($P < 0.05$, two-tailed) in volume (fig 5, A & B). Excluding the BU ($P = 0.0205$) all other neuropils had a P-value of less than 0.0001. Data from ten Monarchs and eight Bogongs were used. All the data from the Bogong and Monarch were first normalised to be able to make an accurate comparison since the monarch is larger in size and has a larger brain so the absolute values would automatically be larger than the Bogongs (fig 5, D). Figure 5 (A

& B), also shows the data range for each species which demonstrates a wider spread for the Bogong population. The Bogong CBU, CBL, PB, AOTU-LU, AOTU-NU and NO are significantly larger than the corresponding Monarch neuropils. These neuropils being nearly 50% smaller in the Monarch. While the AOTU-UU and the BU are larger in the Monarch, the AOTU-UU approximately twice the relative size (figure 5, C). There is however no significant difference between the LAL's of the two species. For all comparisons between the two species the volume of the GA was added to the Bogong LAL since there the corresponding structure (anterior loblet) was included in the LAL reconstruction in the Monarch. Additionally, the Monarch AOTU-SP was added to the lower unit since it is not present in the Bogong.

Since the standard is still being computed it is here represented by the chosen reference brain (fig 6). However, figure 6 still displays the difference between species shown in figure 5. Additionally, the difference between individual reconstructions and the standard reconstruction can be appreciated. The most obvious difference is the smoothness of the Monarch standard.

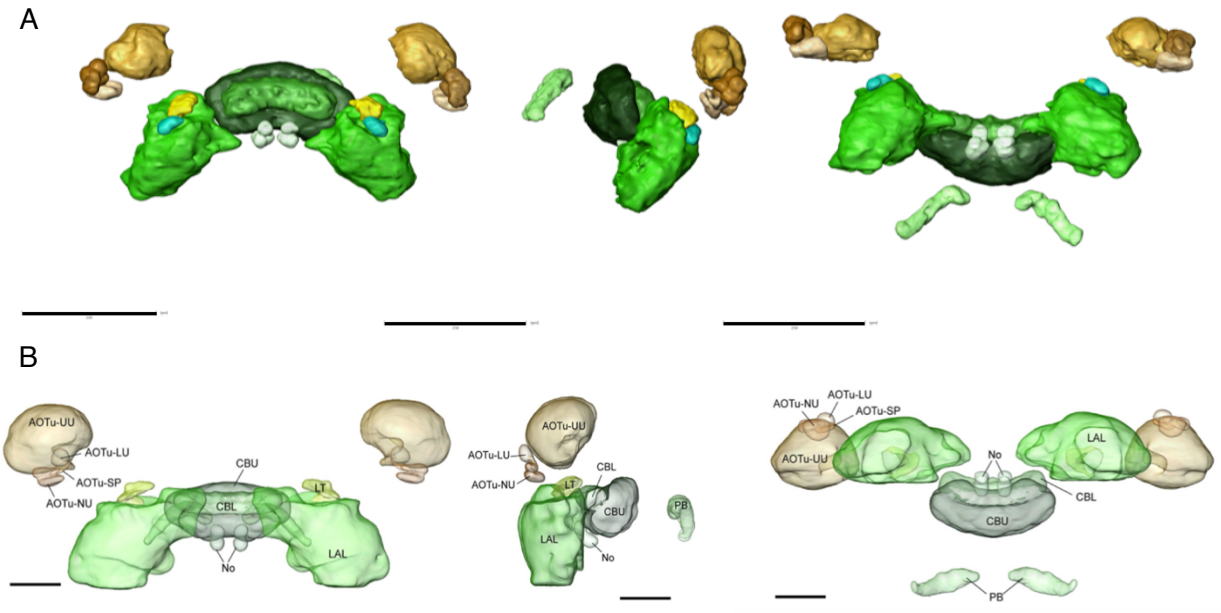

Figure 6. The standardized compass neuropils of the Bogong moth (as the standard is still being computed, it is represented by the chosen reference brain) compaired to the Monarch standard. A, displays the Bogong standard. Based on whole-mount anti-synapsin stainings of nine Bogong brains, reconstructed using labelfield and SurfGen in Amira 5.2. Standardized using the CMTK (ISA) protocol. From left to right: frontal, side and ventral view. Scale bar = 250μm. B, shows the standard generated by 10 individual monarch brains. From left to right: frontal, side and ventral view. Scale bar = 100 μm (Heinze et al., 2013). Colour codes from Heinze & Reppert (2012).

Discussion

In this study I used 3D reconstructions based on anti-synapsin staining's in whole-mount preparations to generate a standardized atlas of the compass neuropils of the Bogong moth brain. There have not been any earlier published attempts to standardize the compass neuropils of the Bogong. The main reason for this anatomical study was to examine the structural basis of the navigational mechanism that enables the moths' incredible migratory behaviour. the protocol followed in this project is the same used to acquire the Monarch data (Heinze et al.

2013). The disadvantages with this protocol is the risk of staining gradient, development of air bubbles from the embedding medium as well as some shrinkage of the tissue from the dehydration step which has to be taken in consideration when viewing the final 3D image. However, since this protocol was used to obtain both data sets it should not affect relative volume comparison between the two species or the overall shape of neuropils. Possible improvements to the study would be to collect data from a more homogeneous population, as well as having a more even ratio between males and females used for the standardization. A potential source of error was that the Bogong brains appeared to be more sensitive to distortion during the histology protocol than other species, which could have affected the final product. This can be resolved by having a larger population. The staining quality of the brains showed some variability. Thus the staining protocol could be optimized for more consistent staining, since the staining quality effects the labelling process. The labelling process is made reproducible by labelling key sections of the neuropil, for example the anterior and ventral bounderies as well as keeping a near consistent number of labelled sections for each neuropil (three to five sections in each spatial plane). However, there will be some variability between individual labelling's due to the human factor. This variability would also be reduced by an increased sample size.

As expected, the Bogong moth brain includes all major compass neuropils seen in the monarch: the CX, AOTU's and the LAL's. The Bogong moths' CX contained all four components (CBU, CBL, NO, PB) and like the monarch the PB unit of the Bogong is a paired structure. Similarly, the LAL contained the BU as well as the GA. The Bogong had three subunits of the AOTU, the upper, lower and the nodular unit. This is consistent with the Heinze & Reppert´s (2012) suggestion that the AOTU-SP is unique to the monarch. The regression study of the neuropil volume indicates that the size of the neuropils scale with the total volume meaning the bigger the brain the bigger the neuropil.

There was no significant difference between the two hemispheres and as in the summed relative volumes the smaller neuropils are more variable. There was however a significant volume difference between all the neuropils of the two species, except the LAL's. The spread of the data was larger in the Bogong, probably a result of the less homogeneous population since the data is collected during a period of three years. In general, the Bogongs neuropils were larger than the monarchs, except for AOTU-UU and the BU. The CX of the Bogong was larger while the AOTU-UU was substantially larger in the Monarch. What these differences in volume indicate for the navigational mechanism is unknown and is a potential subject for further studies. However, in bees the AOTU-UU is involved in colour vision, if it has the same function in butterflies and moths this would explain the size difference (Mota et al., 2011). The Bogong moth is nocturnal and therefore enhanced colour vision would not be essential for survival or navigation. An explanation for the larger smaller subunits and the components of CX in the Bogong could be that these structures play a more important role in the moth or that the larger AOTU-UU in the Monarch distorts the other neuropils, making them relatively smaller. A potential solution would be to normalize the data without the AOTU-UU and see if there still is a similar trend or if then all neuropils are the same. The lack of significant difference in LAL volume might be due to a greater conservation of the structure and size of this neuropil. One brain was deformed and may have caused some variability. Yet in figure 3 the data doesn't stand out amongst the other neuropil volumes.

To conclude, this standardization provides a framework to map expression patterns of neuro-chemical substances, gene expression patterns, neuron networks as well as individual neurons. All of which will aid in the further understanding of the potential magnetic compass based navigational system of the Bogong.

Acknowledgment

I would like to thank my supervisor Stanley Heinze for all the advice and technical support through out this project. Andrea Adden for assistance with laboratory work and dissections. The University of Marburg for providing the tools and preforming the standardization.

References

el Jundi, B. & Heinze, S. 2015. Three-dimensional atlases of insect brains. Neurohistology and Imaging: Basic techniques. Pelc R, Doucette JR, Walz W (editors). Springer (in press).

Guerra, P.A. Gegear, R.J. & Reppert, S.M. 2014. A magnetic compass aids monarch butterfly migration. *Nature Communications*. 5: 4164. DOI: 10.1038/ncomms5164

Heinze, S. & Reppert, S.M. 2012. Anatomical Basis of Sun Compass Navigation I: The General Layout of the Monarch Butterfly Brain. *The Journal of Comparative Neurology* – Research in Systems Neuroscience. 520:1599-1628.

Heinze, S., Florman, J., Asokaraj, S., el Jundi, B. & Reppert, S.M. 2013. Anatomical Basis of Sun Compass Navigation II: The Neuronal Composition of the Central Complex of the Monarch Butterfly. *The Journal of Comparative Neurobiology*. 521: 267-298.

IFB Common. 1954. A study of the ecology of the adult bogong moth, Agrotis Infusa (Boisd) (Lepidoptera: Noctuidae), with special reference to its behaviour during migration and aestivation. *Australian Journal of Zoology*. 2: 223-263.

Lohmann, K.J. 2007. Sea Turtles: Navigating with Magnetism. *Current Biology*. 17: R102-104. DOI: 10.1016/j.cub.2007.01.023

Merlin, C., Heinze, S. & Reppert, S.M. 2011. Unraveling navigational strategies in migratory insects. *Current opinion in Neurobiology*. 22: 1-9. DOI: 10.1016/j.conb.2011.11.009

Mota, T., Yamagata, N., Giurfa, M., Gronenberg, W. & Sandoz, J.C. 2011. Neural organization and visual processing in the anterior optic tubercle of the honeybee brain. *The Journal of neuroscience*. 31(32):11443-56. DOI: 10.1523/JNEUROSCI.0995-11.2011.

Ott, S.R. & Rogers, S.M. 2010. Gregarious desert locusts have substantially larger brains with altered proportions compared with the solitarious phase. *Proceedings of the Royal Society B: Biological Sciences*. 277: 3087-3096. DOI: 10.1098/rspb.2010.0694

Pfeiffer, K. & Homberg, U. 2014. Organization and Functional Roles of the Central Complex in the Insect Brain. *Annual Review of Entomology*. 59:165-184. DOI: 10.1146/annurev-ento-011613-162031

Reppert, S.M., Gegear, R.J. & Merlin, C. 2010. Navigational mechanisms of migrating monarch butterflies. *Trends in Neuroscience*. 33: 399-406. DOI: 10.1016/j.tins.2010.04.004

Stöckl, A.L. & Heinze, S. 2015. A clearer view of the insect brain-combining bleaching with standard whole-mount immunocytochemistry allows confocal imaging of pigment-covered brain areas for 3D reconstruction. *Frontiers in Neuroanatomy*.9: 121. DOI: 10.3389/fnana.2015.00121

Wiltschko, W. & Wiltschko, R. 2005. Magnetic orientation and magnetoreception in birds and other animals. *Journal of Comparative Physiology A*. 191: 675-693. DOI 10.1007/s00359-005-0627-7

Personal communication

Heinze, S. <stanley.heinze@biol.lu.se>

Appendix

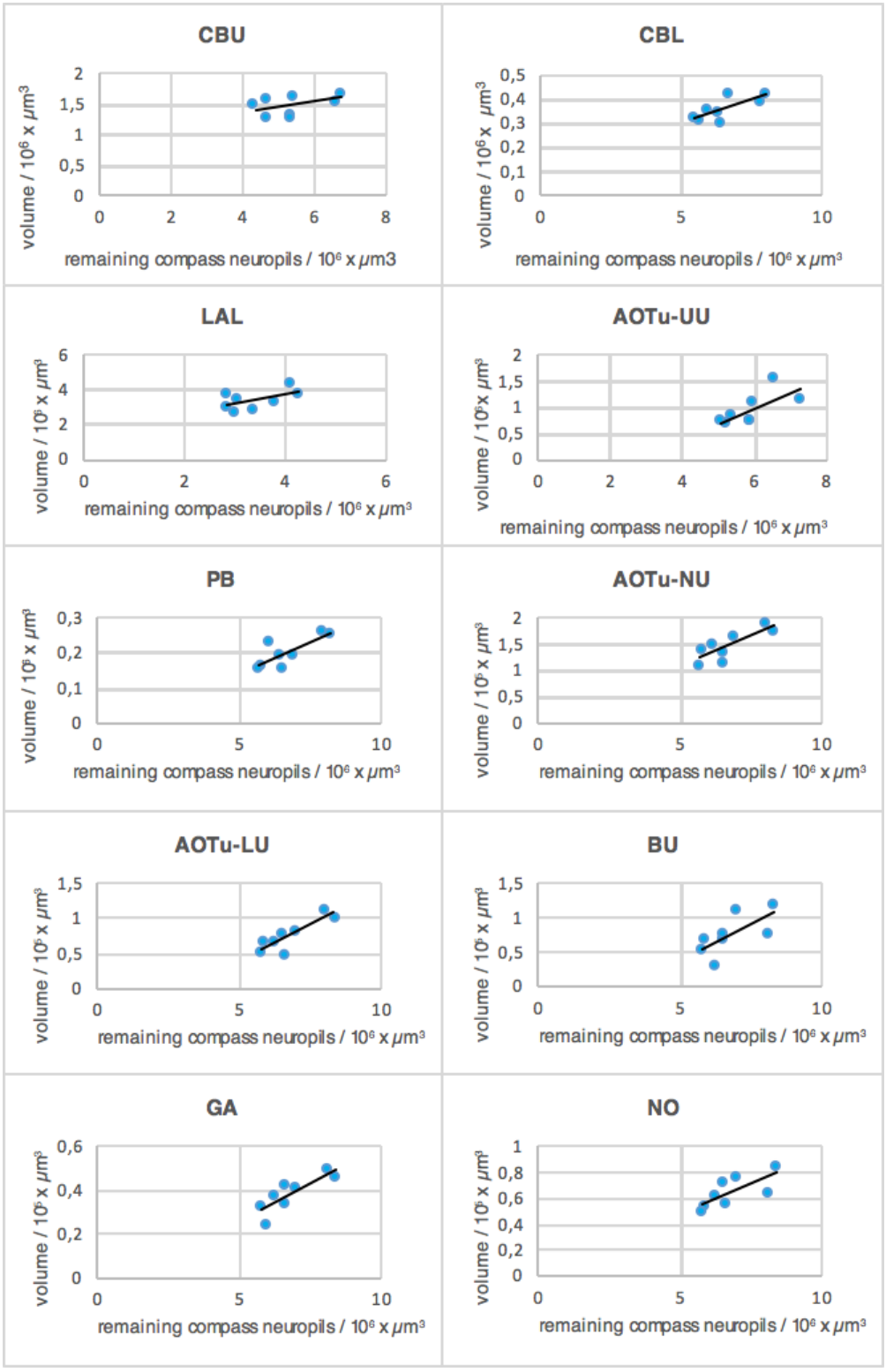

Figure A. The relationship between each neuropil and the remaining compass neuropils. The blue dots are the data points of each individual brain, the regression line in black shows the relationship between that neuropil volume and the volume of the remaining compass neuropils.