CORRELATION OF NEURON SIZE AND NUMBER WITH BRAIN SIZE IN BUMBLEBEES

By

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Abstract

Over the past several decades, cell size and its resulting effects on tissue and organ function, as well as on its overall ability of the animal to perform complex tasks, has been studied extensively. Neuronal size (diameter of individual neurons) could have an influence on intelligence, brain capacity, and ability to perform complex behavioral tasks. Furthermore, there appears to be an increase in number of neurons with an increase in brain size in vertebrates. In insects, increased neuron number has also been correlated with more complex behavior. In this thesis, I test the hypothesis that the neuronal number and/or neuronal size correlate with the brain size using an insect model. This may help elucidate the apparent positive correlation between brain size and intelligence.

To achieve this goal, I used a species of bumblebee, *Bombus impatiens.* Bumblebee workers vary extensively in brain and body size and weight, therefore allowing comparison between individuals of the same species. Workers within a colony differ in size and the amount of work a worker does depends on their body size. Larger sized workers have more foraging capability than smaller sized workers and foraging requires a more demanding sensory integration and memory capacity. In my study, it was found that brain volume was positively correlated with bee body size. Three cell body regions of the brain were further analyzed: inside of the mushroom body calyces, a cell body region next to the lobula, and cell bodies associated with the antennal lobe. No significant correlations between neuron number per unit of volume (neuron density) and brain volume were found. Assuming similar neuronal density in large and small brains, increased brain size is thus correlated with an overall increased neuron number.

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Introduction: Neuron size, number of neurons, brain volume, and its consequences on brain capacity.

Brain Capacity

In general, primates and elephants are assumed to be more intelligent than other mammals such as lions, dogs or rabbits; human beings and great apes more than monkeys, and human beings more than great apes (Roth, 2005). Intelligence has been difficult to define because it is a complex phenomenon with a collection of context specific abilities. Even though there has not been a single universally accepted definition of intelligence, many ecologists and psychologists believe that in vertebrates, intelligence can be measured by observing how they use their problem solving skills in the wild (Roth, 2005). In vertebrates, intelligence is described by Jerison (1985) as a measure of capacity of the brain to process information and that is revealed by differences among species in their ability to integrate sensory information and construct perceptual variants. In general, intelligence in insects is defined as the ability to engage not only in work, but the ability to constantly monitor the physical and social environment for signs of environmental changes that might indicate them to adjust their occupation (division of labor of the insects) or completely switch to another occupation (Franks, 2002). In the past, people have been correlating interspecies variations such as body size and brain size. Roth (2005) suggested the amount of cerebral cortex volume as a possible predictor of intelligence, while Jerison (1985) deemed brain size to be an estimator of intelligence. These studies have a defect because different species of vertebrates and insects differ in their brain structure, ecological strategies, environmental adaptations, and behaviors, and it is difficult to come to a conclusion regarding the differences in brain organization (later leading to variation in intelligence). Thus, it is beneficial to look within a species to reduce any confounding factors. However, within species, the biological phenomenon responsible for variations in intelligence is not completely understood and the above studies are not enough to come to a conclusion. To explain the physical causes of the brain size variations (then leading to intelligence), neuron number and neuron size in relation to brain size were studied in this experiment.

Brain and Overall Body Size

In nature, larger body individuals of a species have larger brain sizes, given the example of dogs. This has been observed between species (vertebrates and insects) where brain volume positively correlates with body size ($R^2 > 0.5$) (Chittka *et al*., 2009; Dunbar, 1998; Jerison, 1985). The correlation has also been detected within a single species (bumblebees) in Mares *et al*., (2005). This relation is called the brain-body allometric function (Martin, 1983). Furthermore, increased brain size correlates with increased intelligence/brain capacity, better learning ability, and complex behaviors. Sternberg, 2012 demonstrated increased measured value of intelligence and brain size in humans while Deaner *et al.*, (2007) showed increased cognitive ability with increased brain size in non-human primates. Additionally, increased brain size correlates with specific, complex types of behavior, which might correspond to the level of intelligence (Reader, *et al*., 2002). Among Scarabaeid beetles, members of the genus *Scarabaeus* feature larger brains and more complex behavior (building breeding-pills from dung) while members of the genus *Aphodius* feature smaller brains with less complex behavior (just placing their eggs in the dung) during their egg-laying season (Rensch, 1956). The same is also true for carrion beetles (*Silphidae)*, in which large members of the species *Necrophorus* (large burying beetles) (33mm long) show complex breeding instincts (by digging into the carcass), whereas the small members of the related genus *Catops* (only 3mm long) show nothing like this (Rensch, 1956). Larger ants perform more complex tasks (as measured by the variety of tasks performed) than the smaller ants (Cole, 1985). Larger brains have also been inferred to have more computational capacity. Larger flies (with larger brains) have larger photoreceptors with neuronal membranes that allow faster rates and more volume of information processing than the smaller flies (Chittka, *et al*., 2009). When comparing pair-bonding species to non-pair-bonding species in non-primate mammalian taxa, pair-bonding species with larger brains manage polygamous mating relationships, which uses more cognitive skills (Dunbar, *et al*., 2007; Gittleman, 1986). The reason is that the cognitive demands (for example, forming of social bonds to prevent the disintegration of the group under the pressures of predation risk) of pair bonding species triggered the initial evolution of larger brains and thus more cognitive skills (Dunbar, *et al*., 2007). These studies suggest that the complexity of their behaviors is based on brain capacity to perform tasks (intelligence).

Neuron Size

There are numerous potential reasons for different brain sizes that might lead to difference in intelligence and behavior. Most notable among them is neuronal size and there have been studies (see below) showing that increase in brain size correlates with an increase in neuron size. It has been demonstrated that larger species such as elephants and whales show an increase in neuronal size proportional to their body size while smaller species such as birds have smaller sized neurons also in proportion to their smaller body size (Szarski, 1976). Kozlowski, (2010) found that Purkinje and neuronal cell sizes were positively correlated with body mass of birds and mammals while in (vertebrates: Jerison 1973) and (insects: Chittka, *et al*., 2009), neuronal size was positively correlated with larger brains. Additionally, an increase in neuron size could correlate positively with learning ability, intelligence and brain capacity. An increase in the size of neurons in one part of the sensory system influences processing in higher order centers, which in turn affect behavior and learning ability (Chittka, *et al*., 2009). Larger neurons may process higher frequency information and they give rise to dendrites and axons with increased axon diameters capable of supporting increased rates of vesicle release (Chittka, *et al*., 2009). This suggests there might be correlation between larger brains and larger neurons possibly showing an increase in intelligence between species (Jerison, 1985). However, within a single species, such as bumblebees, it is uncertain whether increased brain size is due to increased neuron size. Further studies need to be made.

Neuron Number

Another potential reason of variation in brain size could be the number of neurons. Larger species with larger brains have been found to have a higher number of brain cells (Szarski, 1976). This correlation has been demonstrated in the Figure 1 (reproduced from Braitenberg, 2001) and Roth, (2005) where larger animals with larger brains (with more weight) have higher number of neurons when comparing many different species. Furthermore, an increase in the number of neurons could affect intelligence, learning ability, and behaviors. For example, when compared to vertebrates, insects have a smaller number of neurons in their central nervous system underlying flight control (Jerison, 1973), but they also employ fewer muscles and motor neurons and they rely on different mechanisms for flying when compared to vertebrates.

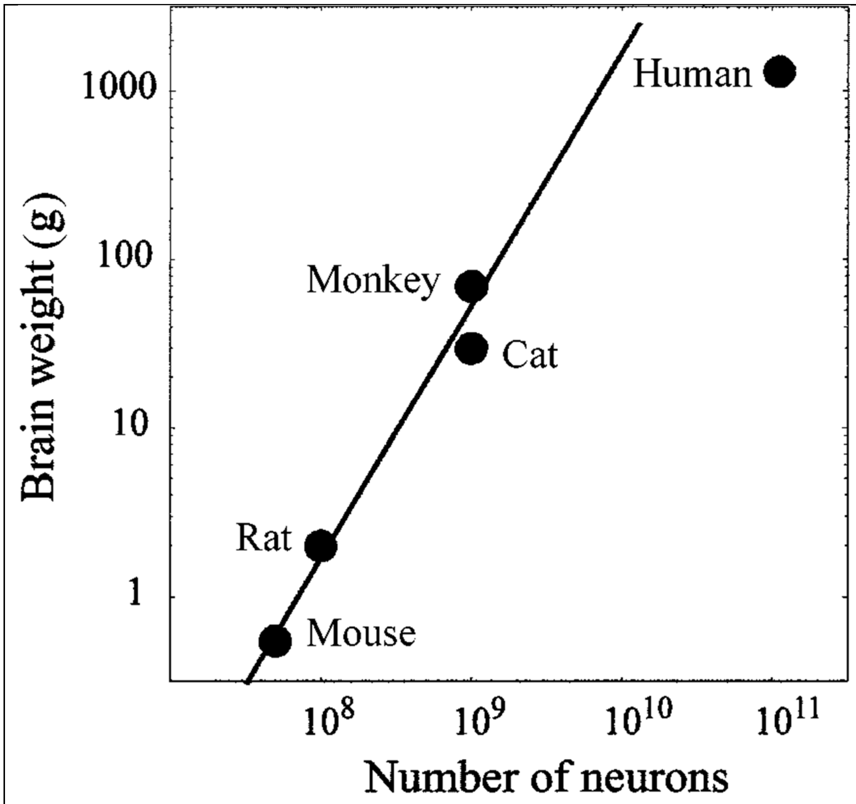

Figure 1: Brain weight and neuron number. Brain weight has a strong positive correlation with the number of neurons for five different mammals. The number of neurons were obtained from the neuronal density and using the volume of the cortex (Braitenberg, 2001).

In insects, such as different fly species with different brain sizes, an increase in neuron numbers contributes to higher temporal and spatial resolution in vision, information processing, and intelligence (Chittka, *et al.*, 2009). However, it is still not clear how this variation in neuronal number affects brain function within a species.

Bumblebee As A Model

In general, previous research has compared the brains of related species that differ in body and brain size. However, species differ in their brain structure, ecological strategies, environmental adaptations, and behaviors, and it is difficult to come to a conclusion regarding the differences in brain organization. These confounding factors get reduced when only a single species is used. A species that is particularly suited for this kind of study is a bumblebee (*Bombus impatiens)*. This is because individuals differ considerably in brain and body size (but closely related in all other aspects) as described by Garófalo, 1978 and thus allow for intra-species comparisons with great statistical power. This is based on the finding of strong positive correlation between brain and body size (Jerison, 1985). In my experiment, *Bombus impatiens* species individuals were studied for the purpose of looking at variations in body size and corresponding brain size to investigate whether this brain-body size trend is reflected in bumblebees. Thus, *Bombus impatiens* is an excellent model for examining variations in brain size, neuron size, and neuron number between individuals of the same species.

Bumblebees are social insects that form colonies with a single queen. The bumblebee body can be divided into three sections: the head, thorax, and abdomen. The head contains the eyes, mouthparts, and antenna. The thorax includes wings, wing muscles, and legs. The abdomen contains the digestive and reproductive organs and sting. Figure 2 illustrates the basic anatomy of a bumblebee.

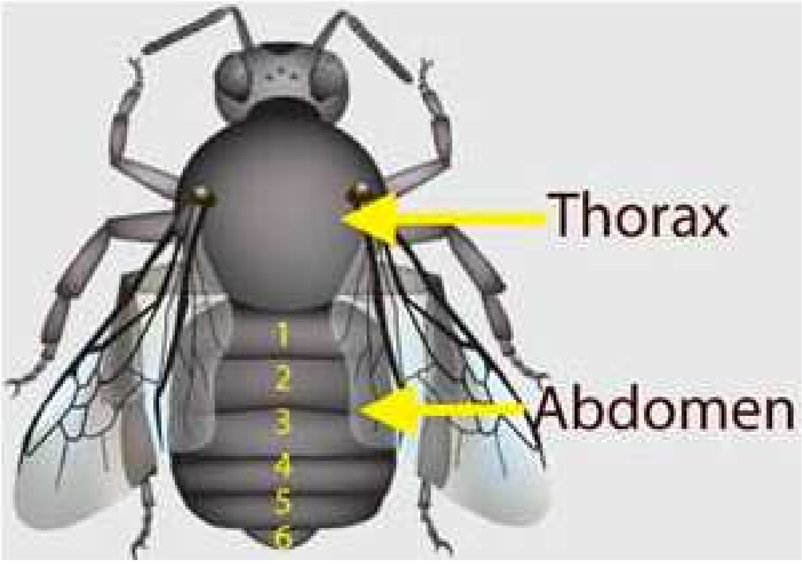

Figure 2: Basic anatomy of the bumblebee: The head contains the eyes, mouthparts, and antenna. The thorax includes wings, wing muscles, and legs. The abdomen contains the digestive and reproductive organs and sting. (Xerces, 2016)

The typical insect brain consists of optic lobes (lamina, medulla and lobula), antennal lobes, central body, and mushroom bodies containing calyx, peduncle, vertical, and medial lobes (Figure 3). Optic lobes process the information provided by the receptor surfaces of eyes (Fischbach *et al*., 1989) while the antennal lobes receive the signals from the olfactory sensory neurons (Laissue, 1999). The central body is involved in the coordination of motor control (Strauss, 2002). The mushroom bodies function in learning, memory, olfactory processing, and are involved in more complex tasks (Strausfeld and Li 1998; Zars *et al*., 2000). In my experiment, three specific regions of the *Bombus impatiens* brain were examined: the mushroom body (calyx), antennal lobe (includes olfactory sensory neurons) and lobula region (optical lobe). In bumblebees, these represent small, medium, and larger neurons and examination of these regions would provide insights in to the brain composition.

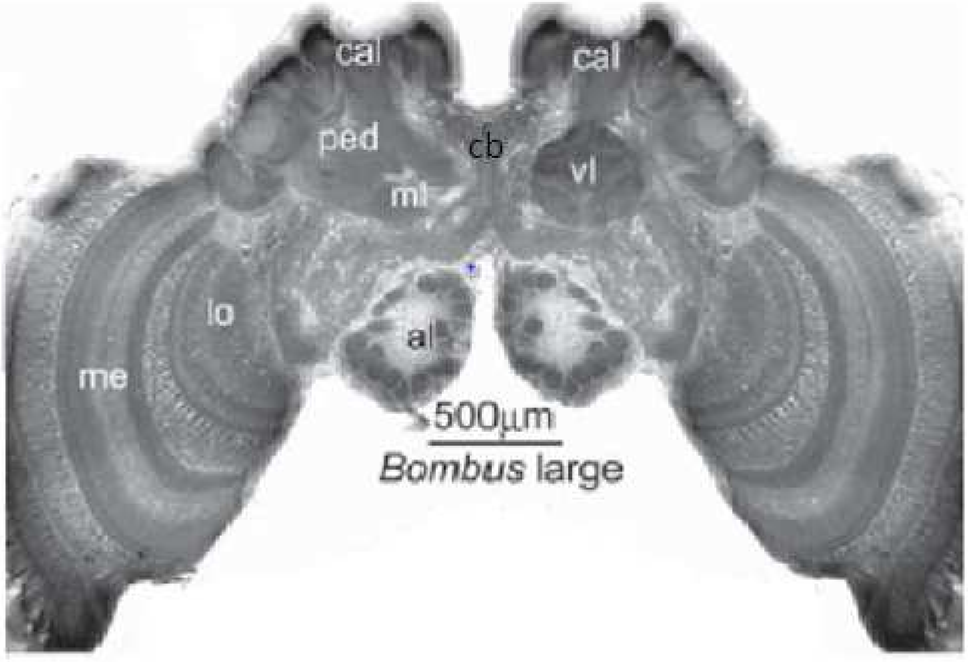

Figure 3: Brain composition of bumblebee brain that this study has focused on: The bumblebee brain is mostly composed of optic lobes: medulla (me) and lobula (lo), antennal lobes (al), central body (cb) and the mushroom bodies comprising the calyx (cal), the peduncle (ped), the vertical (vl) and the medial lobe (ml). (Mares *et al*., 2005)

The most important question in this project is whether larger brains are composed of larger neurons, comprise a higher number of neurons or both. Figure 4 illustrates possible outcomes of the study with: 4A) larger brains having a higher density of neuron bodies (a greater number of neurons per unit area) than the smaller brains; 4B) larger brains have the same density of neuron cell bodies (same number of neurons per unit area but more neurons in their overall larger total volume) as smaller brains; 4C) larger brains having a lower density of neuronal cell bodies (fewer neurons per unit area) than the smaller brains. Some other possible outcomes are that: 4D) larger brains have larger sized neurons than the smaller brains 4E) larger brains having same-sized neurons as the smaller brains 4F) larger brains having smaller sized neurons than the smaller sized brains. Not illustrated: both correlations observed. Possibilities of 4A-4C and 4D-4F are not mutually exclusive. Thus, it could be possible that larger sized brains can both have larger sized neurons and higher number of neurons (with less inter-neuron space as a result) in comparison to smaller sized brains. This is because larger brains could possibly have more synapses and thus the brain would have more interconnections with more neurons and increased cell size. It is also possible that different rules might apply to different brain regions.

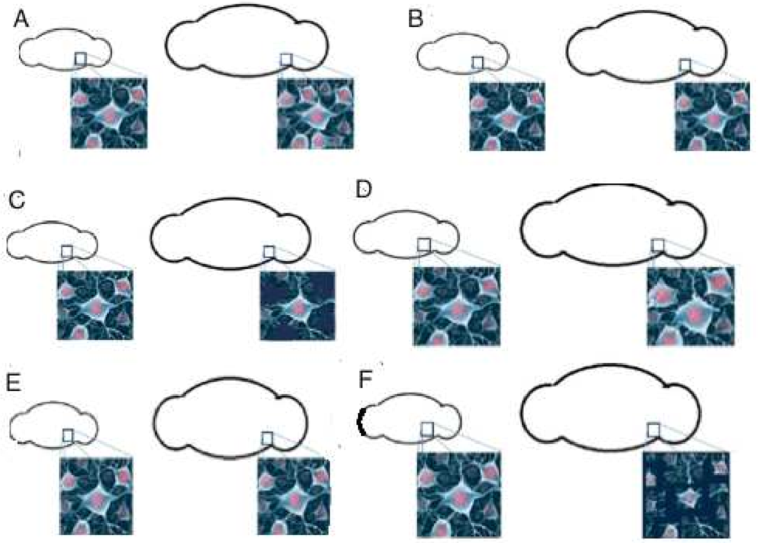

Figure 4: Possible outcomes of the study: 4A) Larger brains having higher density of neuron bodies than the smaller brains 4B) Larger brains having the same density of neuron bodies as smaller brains 4C) Larger brains having a smaller density of neurons bodies than the smaller brains. 4D) Larger brains have larger sized neurons than the smaller brains 4E) Larger brains having same-sized neurons as the smaller brains 4F) Larger brains having smaller sized neurons than the smaller sized brains.

Materials/Methods:

Bumblebee Stocks and Maintenance

Bumblebee colonies (*Bombus impatiens*) were purchased from Koppert Biological Systems (Romulus, MI, USA) and maintained in the laboratory with access to sugar, pollen, and water. Different sized bumblebees ranging from 1.94-4.47 mm head width and 0.02-0.13g body weight were used for analysis. All the bees analyzed were female workers without any age bias.

Section Preparation

Bumblebees were anesthetized by placing them in an ice-cold container. The abdomens were discarded as they store nectar or sugar water and the weight of abdomen varies based on how much nectar the bees drink. Then bumblebees without an abdomen were weighed because they are a less variable proxy for body size. Head width (from left eye outer margin to the right one) was measured by the use of calipers under stereomicroscope control and the heads were separated from their bodies.

The heads were glued in a small container using a wax-rosin mixture and the head capsule was cut open at the front. The brains were then dissected out under a microscope and fixed in 4% phosphate buffered formaldehyde at pH 6.8 for 3 hours. They were washed 3x and stained with Cajal's Block reduced silver to count cell bodies. Briefly, to stain by block silver method, the brains were fixed in ammonium ethanol (98% EtOH+2% Ammonia in H_2O) for 1-2 days. The brains were washed in H_2O overnight and impregnated in 4% $AgNO_3$ at 37°C in the dark for 6 days, washed with H_2O for 1 min and developed in 4% pyrogallol for 6 hours and washed in H_2O.

The processed and fixed brains were washed for 4h, dehydrated, plastic embedded in Spurr's epoxy mix, polymerized at 65°C and sectioned using a microtone. 6-8µ sections were mounted on microscopic slides using cytoseal 60 medium and were examined. A total of 17 bees with intact sections were used for the final analysis.

Brain Volume Estimation

The brain slides were traced using a drawing tube (Figure 5A) (Hodges, 1989) attached to the microscope using a 20X lens. For each brain, depending on the size, every fourth or sixth section was drawn on a sheet of paper. For each section, only half of the brain slice was drawn. These sections were used to calculate the volumes of the brain.

Figure 5A: Drawing Tube attached to the Microscope (Hodges, 1989)

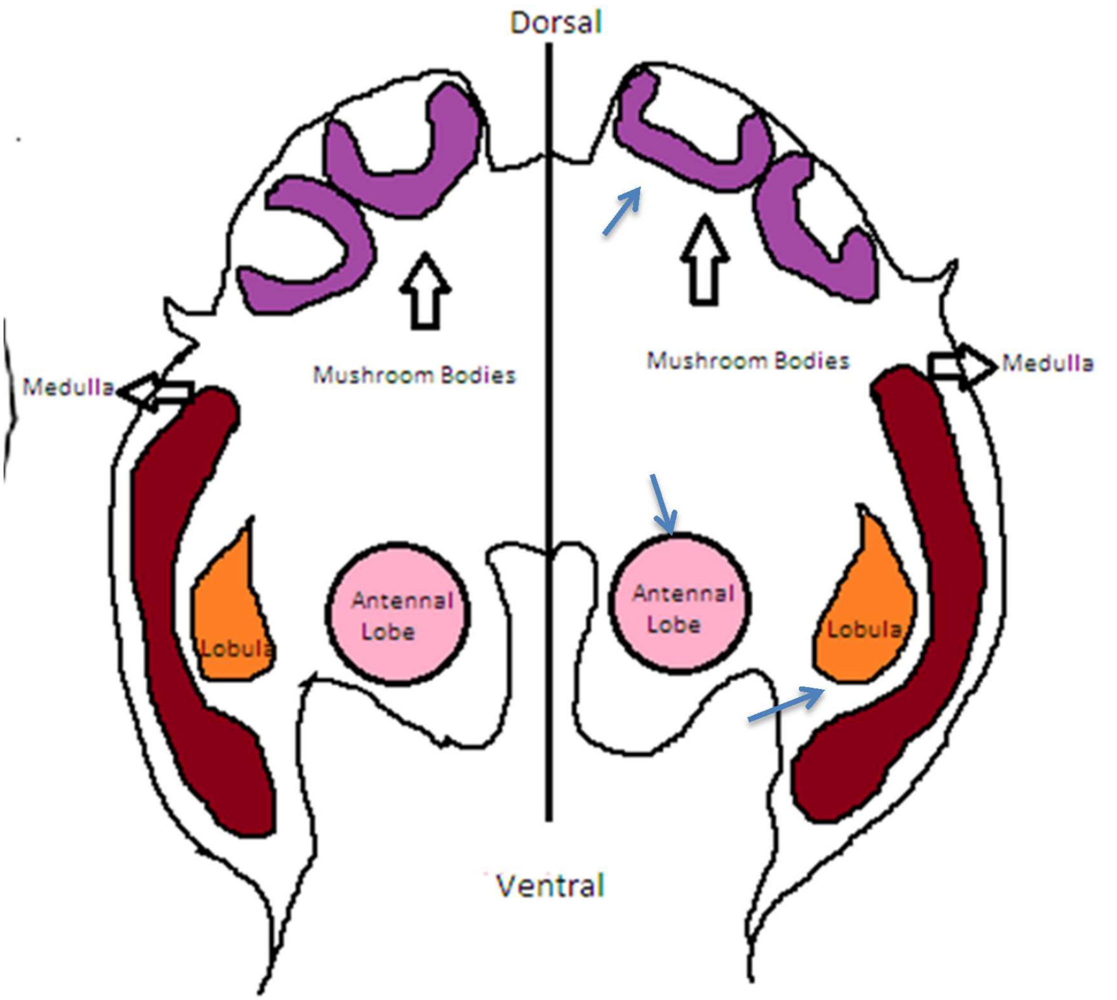

Figure 5B: The bumblebee brain drawing. Purple figures represent mushroom bodies (calyx region), pink round figures represent antennal lobe regions, orange figures represent lobula regions, and brown figures represent medulla regions. Blue arrows point to the three regions where cells were counted.

The traced drawings were scanned. Using the latest version of Adobe Photoshop, the areas of the sections were measured using the pixel count function: the midline was clearly marked and all of the brain section area was filled with a color from the midline up to the end border of the brain but not including the lamina (Figure 6). This was because the lamina (distal to the medulla) was often damaged during dissection. Then, via the “zoom in” feature in Photoshop, 100% zoom size was used. Using the “histogram” function, the actual pixels were obtained. Next, a calibration scale was used using the 20X lens to convert the pixels into um^2. That value was then multiplied by section thickness to get the (half) volume. Then the value was multiplied by 2 because only half of the brain slices were used to calculate the final volume of the brain.

Density of Neurons

To measure the density of neuronal cell bodies in the brain, three regions of the brain were chosen. The three areas were inside the calyx cups of the mushroom bodies, cell body region between the medulla and lobula, and around the antennal lobe region (Figure 5B). These regions were selected because they represent small (calyx), medium (lobula region) and large neurons (antennal lobe). In insect brains, the cell bodies are in the peripheral region and the dendrites and axons are in the central area. In the Figure 5B, a bumblebee brain drawing shows the three areas this study focused on. For these sections, every second or third section per area was used in this data for a total of 5-6 sections per brain. The primary magnification used for counting cell bodies was 40X. The cell bodies were counted using camera lucida (microscope) for only the selected sections. The traced drawings were scanned and filled in with color (see Figure 6). Using the histogram, the actual pixels (using a calibration scale where particular pixels were calibrated with per micron (area)) were converted into area value. Then the area value was multiplied by section thickness to get the volume. This was done to calculate the neuron density (cell bodies/unit of volume) for each region of the brain.

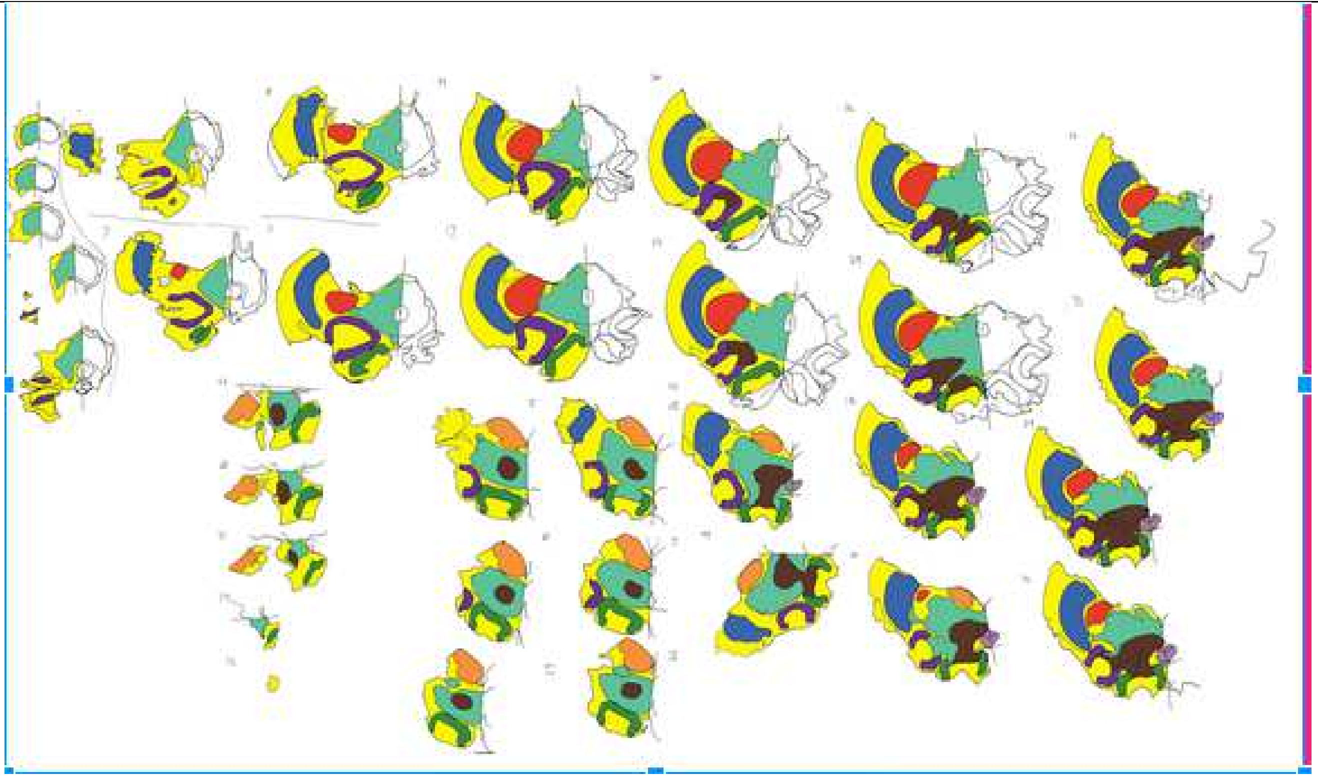

Figure 6: Adobe Photoshop Section Drawings: Different colors representing different areas of the brains.

Cell/Neuron Size

The neurons were analyzed using 40X lens, traced to measure the cell diameter only in the calyx region. The average and standard deviation were calculated from 50 cells per bee in the calyces region for 17 bees (experiments).

Data Analysis Calculation

The brain volumes with head width and weight without abdomen were correlated onto a linear regression with a slope and R^2 regression. For different regions of the brain (calyx, lobula region, and antennal lobe region), density of neuron cell bodies was calculated. Then the density was correlated along with brain volumes. This value would allow me to determine whether particular areas differ in their number of neurons. Last, for determining cell size effect, cell diameter (calyx region only) average was calculated along with standard deviation. The averages and respective brain volumes were correlated with a slope and R^2. ANOVA (p-value) was calculated for all the correlations to determine how true the data is.

Results

Correlation between Brain Volume and Body Size

Bumblebees show extensive differences in body and brain size. Figure 7 A-B show a large and a small bee.

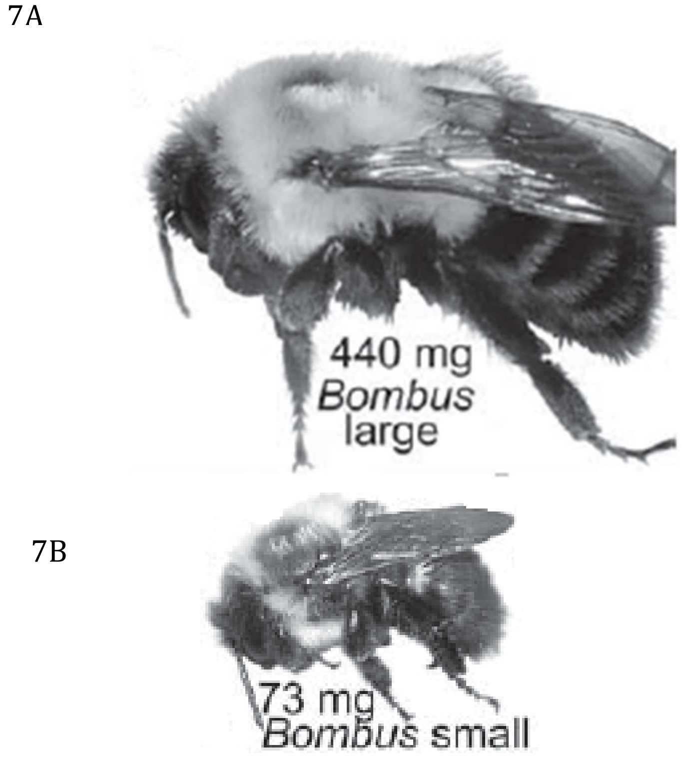

Figure 7: Bumblebees sizes. 7A: Larger bee with larger brain (440 mg *Bombus* large). 7B: Smaller bee with smaller brain (73mg *Bombus* small). (Mares *et al*., 2005)

The first goal was to investigate the brain and body size trend (using head width and body weight) in bumblebees as reported by Mares *et al.,* (2005). As is generally the case in insects, head width and weight without abdomen increased with increased brain volume. Head width and weight without abdomen correlated positively with brain volume in bumblebees respectively (Figure 8A, linear regression: $y = 1.6102x + 2.287$; $R^2 = 0.55154$, n= 17) (Figure 8B, linear regression: $y = 0.0932x + 0.0031$;$R^2 = 0.6661$ n= 17) with a 2 fold difference between the larger workers and smaller workers head width and 2 fold difference in regards to weight without abdomen. The maximum brain volume was 1.53mm^3 and the minimum brain volume was 0.441mm^3 showing the range of a factor of almost 4. The mean brain volume was 0.902mm^3.

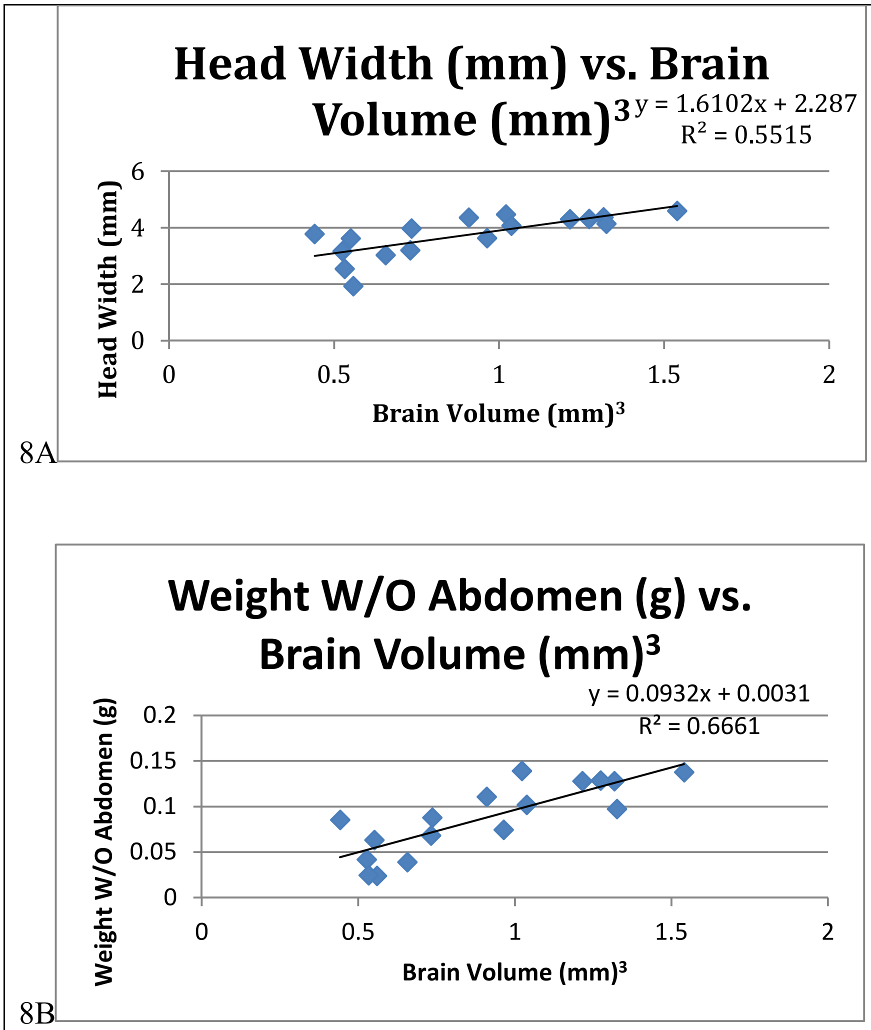

Figure 8: Correlation between brain volume, head width, and body weight. A: Correlation between brain volume and head width of bumblebees. $y = 1.6102x + 2.287$; $R^2 = 0.55154$. **B:** Correlation between brain volume and weight w/o abdomen. $y = 0.0932x + 0.0031$; $R^2 = 0.6661$.

Cell body/Brain Region Volume (Density)

In order to examine the density of neurons in all three regions of the brain (calyx, lobula, and antennal lobe), the number of cell bodies/unit of volume of a given brain region were plotted along with brain volume. This value determines whether particular areas differ in their number of neurons.

Cell body density for the calyces region was slightly positively correlated (no correlation) with brain volume (Figure 9A, linear regression: $y = 15.842x + 11.336$; $R^2 = 0.1356$; n= 17). Density for the lobula region was slightly positively correlated (no correlation) with brain volume (Figure 9B, linear regression: $y = 3.8501x + 18.444$; $R^2 = 0.01247$; n= 17). Density for antennal lobe region was slightly negatively correlated (no correlation) with brain volume (Figure 9C, linear regression: $3.8501x + 18.444$; $R^2 = 0.01247$; n= 17). The correlations were not significant for all three graphs because of the very low R square values. The P-values (0.21-calyces, 0.20 –lobula region and 0.21-antennal lobe region) were greater than .05 and this suggests there was no significance.

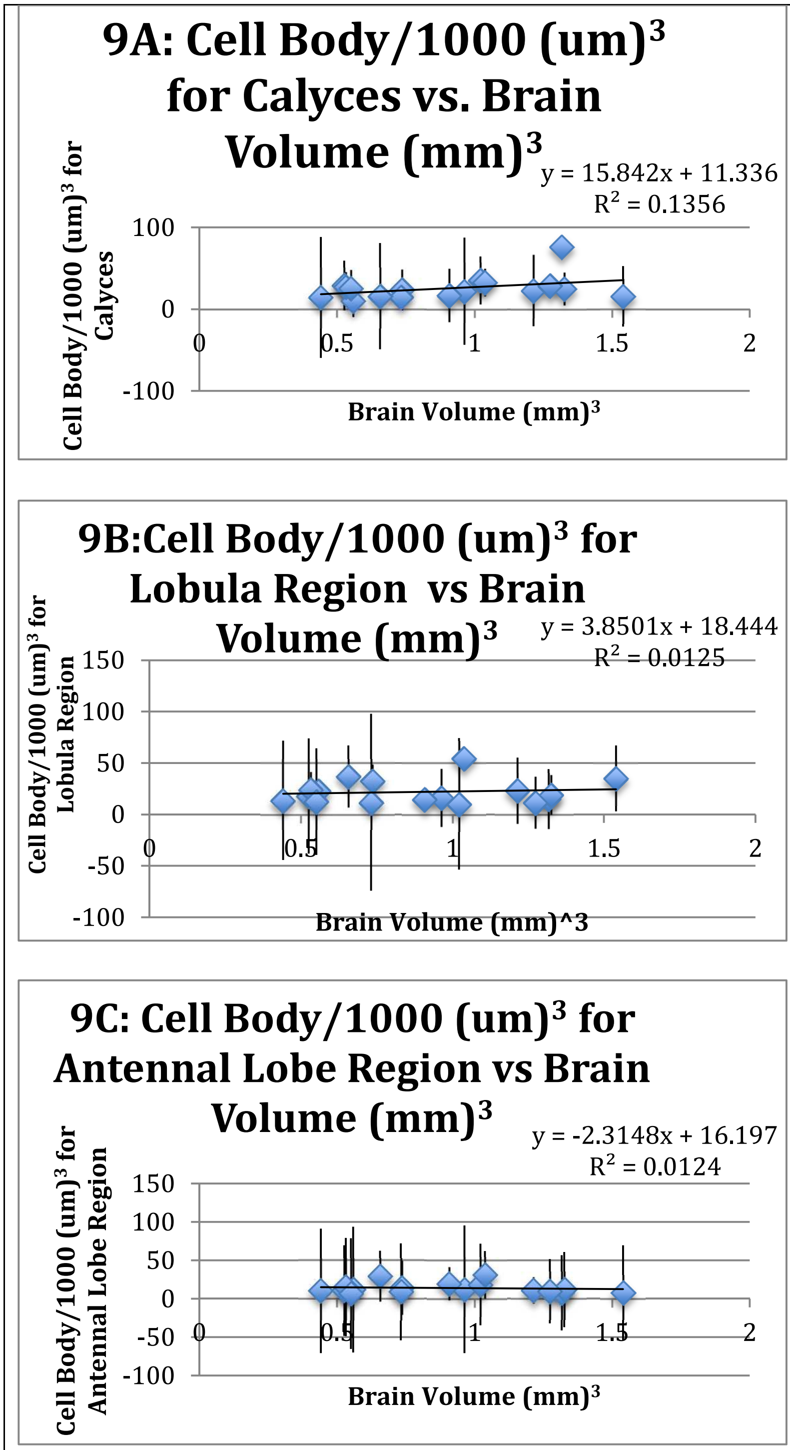

Figure 9 (A,B,C): Correlation between brain volume and cell body/unit of volume. For the cell body/unit of volume for calyces region **(A)** $y = 15.842x + 11.336$; $R^2 = 0.1356$, ANOVA: P-value: 0.21 For the cell body/unit of volume for the lobula region **(B)** $y= 3.8501x + 18.444$; $R^2 = 0.01247$, ANOVA: P-value: 0.20 and for the cell body/unit of volume for antennal lobe region **(C)** $y= -2.3148x + 16.197$; $R^2 = 0.01244$, ANOVA: P-value: 0.21.

Assuming that each cell body takes up the same space, the cell body density had no correlation with brain volume sizes (stayed the same) in all three regions of the brain (calyces, lobula region, and antennal lobe region). Thus, there would be a higher total number of neurons in those regions and in larger brains in general as larger brains have more total volume. The reasons as to why there might have been slight variations in the data might have been due to human error of counting the neurons and calculation error of the area. It is also possible that a larger sample of animals or sections would statistically resolve any variation that complicates interpretation.

Cell Size (Diameter)

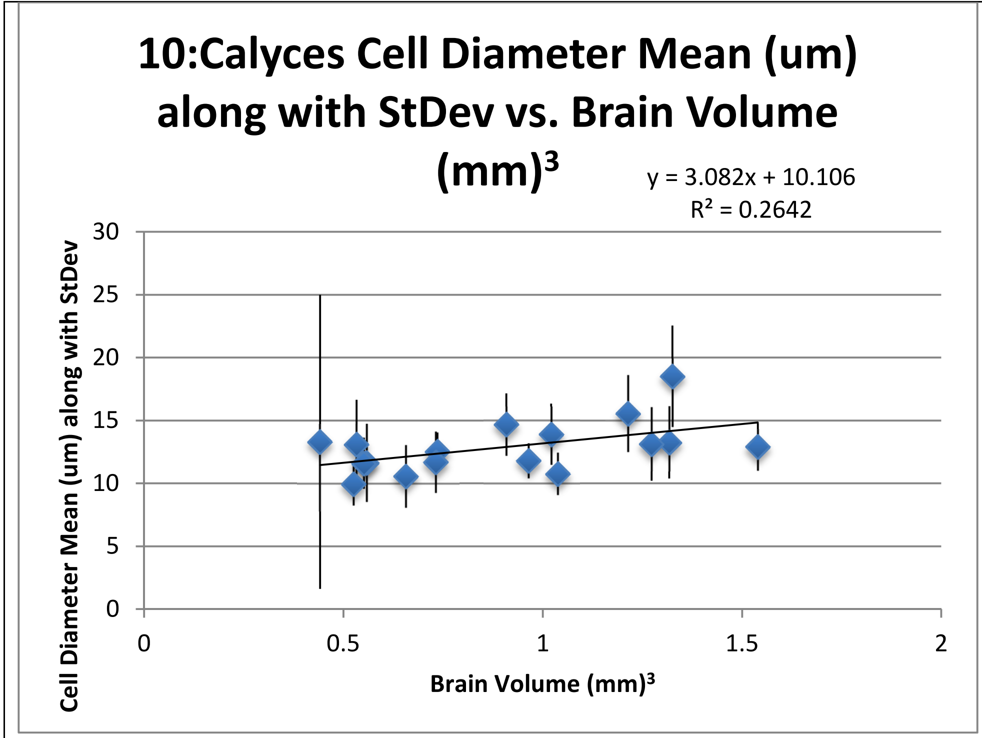

Figure 10: Correlation between brain volume and cell diameter mean for calyces region: $y = 3.082x + 10.106$; $R^2 = 0.26416$; ANOVA: P-value: 0.1828 = no significance.

The last goal was to examine neuron size in the brain. In order to examine the cell sizes of neurons in the calyx, the neuron diameter (size) was plotted along with brain volume. Across the bees, the cell diameter increased in the calyx region with brain volume (Figure 10), showing that larger brains correspond to larger neurons. The P-value (0.1828) for the cell diameter was greater than .05 (although smaller than the P-values for the density correlations with brain volume) and this suggests there was no significance.

Discussion

My work demonstrates for the first time the physical cause of brain size variations between individuals of a single insect species. Body size, head width, total brain volume, neuron size, and neuron number were examined and correlated. The usage of bumblebees allowed me to examine the variation in these factors in a single species (*Bombus impatiens*). The fact that size variation can be studied in bumblebees within a single species results in fewer errors compared to studies spanning different species, which are confounded by species-specific differences.

Brain Volume Versus Head Width and Weight

For the first part of the data, head width and weight without abdomen were both found to be positively correlated with brain volume (Fig 8A and Fig 8B). All the bees chosen were females to reduce any gender specific confounding errors. These results are in good agreement with previous studies (Jerison, 1973; Mares *et al*., 2005; Chittka *et al*., 2009; Cole, 1985; Jerison, 1985) and it was expected because, as a general rule, brain size increases with body size (Gould 1966, Healy and Harvey, 1988). Since (Jerison (1985)) concluded that brain size is a rough estimator of brain capacity to perform tasks, it can be inferred that insects with larger brains have more complex behavior (Jerison, 1985, Reader *et al*., 2002). More importantly, this finding suggests that larger brains permit larger insects (bigger head width and more body weight) to have more advanced behaviors, superior cognitive abilities, and to fulfill complex tasks (Howse, 1974, Mares *et al*., 2005, Rensch, 1956, Morse 1978, Foster *et al*., 2004). However, this might not be applicable to all scenarios. For example, an elephant brain size is larger than a human brain size although, in the ranking of intelligence, a human is considered more intelligent.

Neuron Size

In *Bombus impatiens*, larger brains tended to have larger neurons in this study. This is in agreement with previous studies (Coelho and Leevers, 2000; Kozlowski, 2010; Chittka, *et al*., 2009). This is also in agreement with the hypothesis that larger brains have larger sized neurons than smaller brains (Figure 4D). Additionally, larger neurons can lead to better learning ability and better capacity (Chittka *et al*., 2009). However, the positive correlation between brain and neuron size found in this study was not a strong one. In the calyces region of the brain, R^2 value was 0.26416 and P-value was 0.1828 showing no significance. Since the correlation was not strong enough (the "control" correlation between neuronal cells and brain size was .969 in Kozlowski, 2010 leading to factor difference of 3.67), this might not apply to all scenarios and all species. The reasons as to why the correlation was not strong enough or was not significant could have been due to smaller sample size or potential neuronal damage based on the health of bumblebees. Future studies examining a larger sample of brains, categorizing smaller and larger brains into separate groups, and using more brain regions, and/or more brain sections may reveal that this correlation is insignificant. Alternatively, a larger sample size may confirm this slight but significant correlation. In either case, it is likely that neuron size is not wholly responsible for variations in brain size.

Neuron Number

Another question I addressed is: Do larger brains correlate with greater numbers of neurons? In the experiment, an assumption was made that each cell body takes up the same space in a particular region of the brain. Based on that assumption, the average density of the neurons was calculated. In this study, density refers to the number of neurons per volume in a given region of the brain across the brain volume. For all three regions (calyces, lobula, and antennal lobe region), there was almost no correlation between density of neurons for a given brain region and overall brain volume with R^2 values being 0.13, 0.01 and 0.01, respectively, and p-values were 0.21, 0.20, and 0.21, respectively. This suggests that the neuronal density for these three regions is basically the same for large and small brains, and hence there would be an overall higher number of neurons in larger brains. This is because larger brains have more volume and they would have more neurons if the cell body/unit of volume stays the same across different brain sizes. These values (0.13, 0.01, and 0.01) are in agreement with the hypothesis that larger brains have the same density of neuron bodies as smaller brains (Figure 4B). All the correlations were close to zero (Figure 9). This agrees with data from Braitenberg, 2001 showing no apparent correlation between neuronal density and brain size. The slope of the lines in Figure 9 is not significantly different from zero, supporting Braitenberg's (2001) findings. Slight deviations from zero correlation could potentially be due to smaller sample size or potential neuronal damage based on the health of bumblebees. The slightly positive correlation in neuron density in the calyx and optical lobe, and the slightly negative correlation in antennal lobe region may be explained by observer error. The neurons were counted physically using camera lucida (microscope) and this data might not have been accurate enough. Because brain region size was not directly measured, it may be that neuron number per unit volume may be more closely correlated to variations in brain region size rather than whole brain size. It is also possible that with a greater number of section samples (30-50 sections instead of 5-6), any correlation would be found to be negligible with greater sample size. Thus, our model for brain size is best depicted by Figure 4B also along with 4D. However, these hypotheses would not be applicable to all scenarios and all species. Additionally, an increase in neuron number in larger brains could potentially relate to better brain capacity such as mentioned in Roth, (2005).

There appears to be some discrepancy in the findings because of increase in cell diameter in larger neurons while density remains the same across different brain sizes. This could be because of a potential error in cell density analysis. The correlation between neuron density and brain volume in all three regions (calyces, lobula region, and antennal lobe region) was positive but it was not a very strong correlation. This was because of very low R^2 values (0.13, 0.01, and 0.01) and less significant P values (0.21-calyces, 0.20 –lobula region and 0.21-antennal lobe region) greater than 0.05. These both show no significance of the correlation. Thus, all these findings indicate that the cell density correlation with brain volume size might not be a significant one.

Conclusion and Future Directions

It has been shown that intelligence is positively correlated with brain size (Rensch, 1956; Howse, 1974; Jerison, 1985; Reader *et al*., 2002; Mares, 2005). Furthermore, behavioral complexity has been linked to both neuron size (Amaya-Guerra, 2006 (positive correlation between neuron size and maze performance in rats); Chittka *et al*., 2009) and total neuron number (Roth, 2005; Chittka *et al*., 2009; Kelley, 2014 (positive correlation between medial septum basal forebrain cholinergic neuron number and spatial memory proficiency in Down syndrome mouse models). In bumblebees, I show that brain size is positively correlated with neuron size and total neuron number, but neuron density (number of neurons per specific brain volume) remains consistent across brains of different sizes. Thus, in larger brains (with a greater total volume), there are a larger number of neurons. This was based on the assumption that all neurons were the same size when neuron density was calculated. The idea of comparable neuron count per unit volume (cell density) might be compatible with increased neuron size in larger brains (as illustrated in Figure 4D) but it is not applicable in all scenarios and all species. In Figure 4B, the small and large brains have similar densities of cell bodies even though the larger brains have the larger neurons. When the density of neurons was calculated, neurons were counted manually first and then the value was divided by the volume occupied by the neurons. In that process, the cell size did not matter in the calculation. However, given the errors in cell density calculations (low R^2 and less significant P-values) more data are needed to confirm the relationship between number of neurons counted per unit volume, neuron size, and brain volume.

Supposedly, if all the strategies are revisited, experiments are performed again and significant correlations are found, this study could contribute to the correlation between animals' brain size and neuronal number that has intrigued people for decades. To further hone in on the link between intelligence and brain size, future work in bumblebees will need to combine behavioral studies with neuron size and neuron number quantification. Are larger bumblebee workers capable of more complex behaviors, and/or better memory? This question could be addressed by performing behavioral tests, then sacrificing tested individuals to examine their brains for total volume, neuron cell body diameter, and neuron number as described above. With a greater number of animals, healthy specimens, categorization of smaller brains and larger brains, and a greater number of brain sections examined, there may be sufficient statistical power to resolve whether neuron density, total neuron count, and/or average neuron size is associated with behavioral complexity and/or enhanced memory.

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