Effects of Pesticide Exposure on the Morphological Development of the Bee Brain and the Consequences for Learning Performance

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Declaration of Originality

All work presented in this thesis is my own with the following acknowledgements:

Behavioural assays in chapter 2 were carried out by both me (70%) and undergraduate project student Philipp H. Bischoff (30%). Staining and scanning of specimens used in chapter 3 was carried out by Galina Bernhardt and Inti Pedroso and performed prior to my PhD. The segmentation of some of the optic lobes (~30%) analysed in chapter 5 was carried out by undergraduate project student Daisy Burris.

Work presented in Chapter 3 in developing a method to explore soft brain tissue of insects has been published in a peer reviewed journal: Dylan B. Smith, Galina Bernhardt, Nigel E. Raine, Richard L. Abel, Dan Sykes, Farah Ahmed, Inti Pedroso & Richard J. Gill (2016). Exploring miniature insect brains using micro-CT scanning techniques. *Scientific Reports*, 6, 21768.

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Summary

How pesticides contribute towards insect pollinator declines is a question of global ecological and economic importance. In social bees, a critical but understudied step in evaluating pesticide risk is to understand how contaminated food brought back to the colony can affect developing individuals. Within social bee colonies individuals are likely to be exposed to pesticides during two principal developmental stages - brood (larval & pupal) and early adulthood. Understanding how the developmental timing of pesticide exposure affects adult phenotypes is important in revealing the stages at which workers are most vulnerable to exposure and thus uncovers windows of susceptibility in colony development.

This thesis investigates how exposure to a neurotoxic pesticide in a major insect pollinator – the bumblebee (Bombus *terrestris audax*), at different key developmental stages affects learning behaviour at two adult ages, and links variation in learning performance with volumetric changes in brain development. In Chapter 2 I investigate the olfactory learning behaviour of individuals, finding that older bees have reduced learning performance, whether exposed during brood or early-adult development. In chapter 3 I present the development of a new method for exploring morphological variation in tiny soft tissue structures using micro-Computed tomography and image analysis. In Chapters 4 and 5 I apply this new protocol to investigate the volume of the major brain neuropils of individual workers that were experimentally treated and assessed for learning performance in Chapter 2. I find that exposure during either developmental phase can impede growth of the structures associated with olfactory learning and that these reductions in volume correlate with impaired learning performance. My findings show that in response to pesticde exposure during brood development or early adulthood, bumblebee colonies can produce workers with developmentally and functionally impaired brains. This is worrying as it suggests that social bee colonies foraging on pesticide contaminated food may be producing a behaviourally impaired workforce.

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Chapter 1

General Introduction

Insects are both numerous and diverse and play a variety of functional roles important for ecosystem stability (Losey and Vaughan 2006, Yang and Gratton 2014, Schowalter, Noriega et al. 2018). It is therefore of great concern that recent reports have shown significant insect declines over the last few decades (Hallmann, Sorg et al. 2017). Insect pollinators are one such functional group under decline (Biesmeijer, Roberts et al. 2006, Potts, Biesmeijer et al. 2010, Vanbergen and Initiative 2013), which has implications for flowering plant species given that close to 90% of plant species depend, at least in part, on insect pollination (Ollerton, Winfree et al. 2011). Insect pollinator declines can affect wild flowering plant species that are a vital part of almost all ecosystems, as well as impact food security with around 75% of agricultural crop species depending to some degree on insect pollination; comprising 35% of the global food supply (Klein, Vaissiere et al. 2007) with an estimated global economic value of over €153 billion (Gallai, Salles et al. 2009). A huge increase (more than 300%) in the cultivation of insect pollinator dependent crops has occurred across the developed and developing world in the last 50 years (Aizen,

Garibaldi et al. 2008, Aizen, Garibaldi et al. 2009), and with the human population set to reach 9 billion by 2050 (Godfray, Beddington et al. 2010, Crist, Mora et al. 2017) the demand on the free service provided insect pollinators is predicted only to rise (Aizen and Harder 2009, Bommarco, Kleijn et al. 2013). The current trends in insect pollinator declines is thus worrying as demand is quickly outstripping and diverging from pollination supply, with the potential to for this to lead to a pollination deficit (Aizen and Harder 2009, Garibaldi, Aizen et al. 2011, Breeze, Vaissière et al. 2014, Schulp, Lautenbach et al. 2014). Whilst management of insect pollinators, such as honeybee domestication, has attempted to combat this deficit (for example, the import of honeybee hives to the almond crop blooms in California (Lee, Sumner et al. 2018)), a pollination deficit still remains. Furthermore, it has been argued that domestic honeybees cannot perform the same pollination service as many wild insect pollinators. Honeybee pollination efficiency has been shown to be comparatively lower than that of their wild bee cousins for a number of important world crops, and as such cannot replace many of the specialist mutualistic relationships of wild bees with wild plants (Garibaldi, Steffan-Dewenter et al. 2013, Woodcock, Edwards et al. 2013, Mallinger and Gratton 2015, Gill, Baldock et al. 2016).

Halting or even reversing current insect pollinator declines has thus been recognised as vital for ecological, economic and societal reasons (Potts, Imperatriz-Fonseca et al. 2016), as recently highlighted by the IPBES report on pollinators, pollination and food production (Potts, Imperatriz-Fonseca et al. 2016). The IPBES report recognised declines in species occurrence, diversity and abundance at both local and regional scales in North West

Europe and North America, along with local declines recorded in Latin America, Africa, Asia and Oceania (with their regional status unknown due to lack of data). A principal approach to safeguarding insect pollinators is to develop mitigative steps in reducing population declines by better understanding the factors that threaten individuals and aspects of fitness. To achieve this requires knowledge of the mechanistic basis of harmful effects, allowing the formulation of targeted action to protect against these susceptibilities and vulnerabilities. It is for this reason that controlled studies investigating the effects and responses of insect pollinator species to potential causative stressors are so valuable (Goulson, Nicholls et al. 2015, Gill, Baldock et al. 2016).

This thesis focuses on a key wild insect pollinator – the bumblebee – and seeks to establish how exposure to a sublethal environmental stressor during early stage individual development can lead to effects on adult behaviour known to be important for survival and the pollination service they provide. It then investigates how these effects on behaviour are reflected in changes to morphological development of the brain: first through developing and validating a novel methodology for imaging and analysis of the bumblebee brain, followed by application of this protocol to investigate brain structural volume in experimentally exposed individuals.

1.1 IMPORTANCE OF EUSOCIAL BEES AS INSECT POLLINATORS, AND CONCERNS OVER DECLINES.

Eusocial species share three key characteristics: multigenerational group living, reproductive division of labour and cooperative care of young (Wilson 1971). The order Hymenoptera represents an animal group with the largest number (and proportion) of eusocial species, where this social strategy has arisen independently multiple times (Hughes, Oldroyd et al. 2008). Though only 10% of the 4000 species of bee are described as eusocial, the size of colonies and success of this strategy make them abundant and dominant across a wide variety of landscapes. In North America, the pollination services of the non-native honeybees is worth an estimated $15 billion alone through pollination of apples, avocado, cantaloupes, cucumbers, alfalfa, blueberries and almonds (Calderone 2012). Additionally, pollination by honeybees and wild bees has been shown to increase crop yield and fruit set (Garibaldi, Steffan-Dewenter et al. 2013, Bartomeus, Potts et al. 2014, Classen, Peters et al. 2014, Stein, Coulibaly et al. 2017), including parameters of quality (including size, shape, seed weight, oil content) and commercial value (Bommarco, Marini et al. 2012, Garratt, Breeze et al. 2014, Klatt, Holzschuh et al. 2014). Eusociality is therefore the key to this pollination service, and this can be further supported by the dominance seen in another taxonomic group of eusocial Hymenoptera. Indeed, if just considering the cousins of the bees, the ants, species are known to make up just 5% of all tropical insect species, yet despite their comparatively small individual size ant colonies constitute over 1/3 of this total insect biomass. In turn eusocial versus solitary bees contribute a disproportionate level of pollination relative to species number – providing around 60% of pollination services to pollinator-dependent crops (Klein, Vaissiere et al.

2007, Rader, Bartomeus et al. 2016). So whilst eusociality appears to bring such benefits in dominating environments, are eusocial bees doing well?

Figures for domestic honeybees suggest severe regional declines, with reported colony (hive) losses of 59% between 1947 and 2005 in the USA (Council 2007). This trend has persisted in the USA, with losses of 35.8% of colonies recorded from 2007-2008 - an 11.4% increase in losses compared to the previous year (vanEngelsdorp, Hayes et al. 2008), and further losses reported more recently (2013-2015) (Lee, Steinhauer et al. 2015, Seitz, Traynor et al. 2015). Moreover, similar trends have been reported across Europe, with 25% loss of domestic honeybee colonies between 1985 and 2005 (Potts, Roberts et al. 2010). These patterns of decline are also being seen in bumblebees, which are reported to be in decline across Europe where species have suffered major range contractions and localized extinctions (Goulson, Lye et al. 2008, Williams and Osborne 2009, Marshall, Biesmeijer et al. 2018). In support of this view, the European Red List of bees classified 23.6% of *Bombus* species as threatened with extinction, 4.4% near threatened and 45.6% of bumblebee species to have declining populations (Nieto, Biesmeijer et al. 2014). In the UK specifically, over half of the 16 (non-parasitic) bumblebee species have been considered rare or in decline (Williams 1986, Goulson, Hanley et al. 2005, Benton 2006). Similar trends have also been seen in North America (Cameron, Lozier et al. 2011), South America (Martins and Melo 2010), China (Xie, Williams et al. 2008) and Japan (Inoue, Yokoyama et al. 2008), highlighting the global scale of bumblebee declines. Furthermore, it is unclear as to whether we could be underestimating this problem, as the majority of bumblebee species (56.7%) are considered data deficient and hence the true extent of

their 'threatened status' is unknown. As a side note, improved monitoring of species abundance and distribution in many regions, is therefore required to improve our understanding of the current plight of pollinator declines and to inform conservation going forward.

1.2 Are eusocial bees more or less vulnerable to environmental stressors than their solitary cousins?

1.2.1 Advantages of eusociality in buffering stress

Eusocial bee species are subject to various benefits associated with group living, such as a shared nest that can provide better defence against predators (Hamilton 1971). It has also been shown in certain species that larger group size can confer advantages against competitors. For example, improved territory defence in the ant *Azteca trigona* (Adams 1994), increased productivity in the hover wasp *Liostenogaster flavolineata* (Shreeves and Field 2002), and the ability to exploit temporary resources quickly as shown in discovery and foraging recruitment in eusocial ants, bees and wasps (Beekman, Sumpter et al. 2001, Mailleux, Deneubourg et al. 2003). Eusociality has also been suggested to buffer against parasite infection, a term known as social immunity (group-level, behavioural immune responses), through behaviours such as allogrooming of infected individuals (Cremer and Sixt 2009, Walker and Hughes 2009). This ability to buffer against stress is likely to be applicable to eusocial bees as well, having a large cohesive workforce allows plasticity and task partitioning to increase efficiency as exhibited in bumblebees (O'Donnell, Reichardt et al. 2000, Goulson, Peat et al. 2002). In eusocial bees, queens are in most cases the

principal source of reproduction for the colony (Michener and Press 1974, Röseler and van Honk 1990, Barron, Oldroyd et al. 2001) and therefore the loss of the queen will have major consequences to colony fitness. In contrast, individual workers do not (typically) reproduce (Barron, Oldroyd et al. 2001), so the loss of an individual does not impede colony reproduction and has minimum effects on colony productivity. The importance of these roles are reflected in their characteristic reproductive division of labour and cooperative brood care (Winston 1991, Goulson 2010), while the workers undertake tasks with high risk of exposing them to stressors such as foraging, queens are relatively sheltered, residing in the nest for most of their reproductive life with the exception of mating flights and nest initiation (Wilson 1971).

1.2.2 Potential costs of eusociality in bees

There can be general costs to living in social groups, including increased transmittance of parasites and disease within groups (Waddington and Rothenbuhler 1976, Krause, Ruxton et al. 2002, Otterstatter and Thomson 2007) and easy detection of the group by predators and parasites (Krause, Ruxton et al. 2002). Group-living requires a variety of tasks to be conducted (e.g. nest maintenance, colony defence, foraging); hence this task partitioning means that individuals are dependent on others to allow the colony to function. If any one of these 'cogs in the machine' are impaired it can create potential vulnerabilities in which the impairment of a certain cohort of the workforce could impact the health of other colony members (Bryden, Gill et al. 2013). For example, being central place foragers, bumblebee workers require good navigation, learning and memory to locate food resources and return to the nest (Von Frisch 1967, Michener 2000). If these

capabilities were to be compromised it could lead to the loss of foragers or inefficient foraging individuals, reducing the resources provided to the colony or resulting in the need to recruit more foragers, potentially exposing further individuals to the dangers of foraging or reducing the number of individuals to undertake nest duties (Gill, Ramos-Rodriguez et al. 2012, Henry, Beguin et al. 2012). Furthermore, social colony living requires high cognitive and communicative capabilities to maintain order and functioning, and impairment to enough individuals could have major disruptive effects and potentially lead to colony collapse (Bryden, Gill et al. 2013, Tison, Hahn et al. 2016). Overall, this means that whilst colony efficiency can be high when acting as multi-task performing 'superorganism' (Wilson 1971), each cohort of the workforce relies on the other, and if sublethal impairment were to reduce the efficiency of one or more of these cohorts below a certain threshold, then the superorganism is at increased risk of death.

The threats to bee health are considered multi-factorial (Vanbergen and Initiative 2013), with a number of different factors known to challenge and/or sub-lethally harm individual members of the colony in eusocial bees. Habitat loss and fragmentation can increase the foraging demands on foraging workers and dispersal in sexuals (Carvell, Roy et al. 2006, Winfree, Aguilar et al. 2009); climate change can place thermal limits on individuals and de-couple plant-pollinator mutualisms (Burkle, Marlin et al. 2013, Kerr, Pindar et al. 2015, Miller-Struttmann, Geib et al. 2015); invasive species can affect competition and predation (Morales, Arbetman et al. 2013) and parasites and pathogens can cause individual health and performance to be lowered (Meeus, Brown et al. 2011). The sublethal effects that pesticide exposure has on bee behaviour and physical ability,

however, has received increasing recent attention (Godfray, Blacquière et al. 2015, Woodcock, Isaac et al. 2016, Baron, Jansen et al. 2017). With the increased demands on food security, insecticides, fungicides and herbicides are now heavily used to protect crops (Cooper and Dobson 2007, Lamichhane, Dachbrodt-Saaydeh et al. 2015). However, the potential for these compounds to negatively affect non-target and beneficial insects must be considered, especially given our increased reliance on pollinator dependant crops (Aizen, Garibaldi et al. 2008), and that the costs of losing beneficial species has the potential to outweigh the gains in pest reduction (Budge, Garthwaite et al. 2015). Following reported dramatic declines in managed honeybees in the mid-2000's in the US, including symptoms of colony collapse disorder (CCD) (vanEngelsdorp, Evans et al. 2009, Ellis, Evans et al. 2010) and concerns raised about wild bee population declines (Goulson, Lye et al. 2008) attention was turned towards undertaking pesticide exposure studies, with the heaviest focus on eusocial bees (Table 1.1). Indeed, there is an increasing number of studies showing that pesticide residues can be found in pollen and nectar collected by foragers (Chauzat, Faucon et al. 2006, Botías, David et al. 2015, Rundlof, Andersson et al. 2015, David, Botías et al. 2016) and on individual foraging bees (David, Botías et al. 2016, Calatayud-Vernich, Calatayud et al. 2018). Consequently, pesticide residues have been reported to build-up inside colonies, with studies showing that up to 10 different active ingredients can be found inside bee colonies (Mullin, Frazier et al. 2010, David, Botías et al. 2016, Mitchell, Mulhauser et al. 2017, Calatayud-Vernich, Calatayud et al. 2018). Understanding how such pesticide residues affect individual bees, both inside and outside of the colony, is therefore of paramount importance.

Table 1.1 Summary of pesticide exposure studies on bees

Paper	*Desneux et al. 2007*	*Dai et al. 2010*
Title	The Sublethal Effects of Pesticides on Beneficial Arthropods	Effects of sublethal concentrations of bifenthrin and deltamethrin on fecundity, growth, and development of the honeybee *Apis mellifera ligustica*
Study Species	Multiple with focus *on Apis mellifera*	*Apis mellifera ligustica*
Pesticides	Multiple	Bifenthrin and Deltamethrin
Impact and Insight	Review of a range of behavioural and physiological sublethal effects reported in published literature	Experimental exposure to honeybees shows that both bifenthrin and deltamethrin reduce bee fecundity and decrease the rate of development to adulthood. Also a toxicity assay of bifenthrin and deltamethrin on workers show the median lethal effects were 16.7 and 62.8 mg/L, respectively.

Whitehorn et al. 2012	***Wu et al. 2011***	***Decourtye and Devillers 2010***
Neonicotinoid pesticide reduces bumble bee colony growth and queen production	Sub-lethal effects of pesticide residues in brood comb on worker honey bee (Apis mellifera) development and longevity	Ecotoxicity of Neonicotinoid Insecticides to Bees
Bombus terrestris	*Apis mellifera*	Multiple bee species
Imidacloprid	Multiple including fungicides, herbicides and insecticides.	Multiple neonicotinoids
Bumblebee colonies exposed to field-realistic levels of a neonicotinoid in laboratory conditions had a significantly reduced growth rate and suffered an 85% reduction in production of new queens.	First study to show sub-lethal effects of pesticide residue exposure from contaminated brood comb in honeybee workers. Sub-lethal effects found include delayed larval development and adult emergence along with shortened adult longevity.	Review of the toxicity of neonicotinoid insecticides to bees

Gill et al. 2012	***Laycock et al. 2012***	***Schneider et al. 2012***
Combined pesticide exposure severely affects individual- and colony-level traits in bees	Effects of imidacloprid, a neonicotinoid pesticide, on reproduction in worker bumble bees (Bombus terrestris)	RFID tracking of sublethal effects of two neonicotinoid insecticides on the foraging behavior of Apis mellifera
Bombus terrestris	*Bombus terrestris*	*Apis mellifera*
Cyhalothrin and Imidacloprid	Imidacloprid	Imidacloprid and Clothianidin
Showed that chronic combinatorial exposure of bumblebee colonies to a neonicotinoid and a pyrethroid pesticide impaired foraging performance and increased worker mortality resulting in reductions in brood development and colony success.	Found that queenless microcolonies of worker bumble bees exposed to dietary imidacloprid had a dose-dependent decline in fecundity.	Used radiofrequency identification (RFID) method to monitor the effects of sublethal doses of neonicotinoids on individual honeybee foragers - finding both imidacloprid and clothianidin exposure led to reduced foraging activity and longer foraging flights.

Williamson et al. 2014	***Feltham et al. 2014***	***Palmer et al. 2013***
Exposure to neonicotinoids influences the motor function of adult worker honeybees	Field realistic doses of pesticide imidacloprid reduce bumblebee pollen foraging efficiency	Cholinergic pesticides cause mushroom body neuronal inactivation in honeybees
Apis mellifera	*Bombus terrestris*	*Apis mellifera*
Imidacloprid, thiamethoxam, clothianidin and dinotefuran.	Imidacloprid	Neonicotinoids imidacloprid and clothianidin, and organophosphate miticide, coumaphos oxon
Showed that following a 24hr oral exposure period to imidacloprid, thiamethoxam or clothianidin, honeybees had impaired motor control, they were more likely to lose postural control and fail to right themselves. Additionally bees exposed to thiamethoxam spent more time grooming.	Showed that field-realistic doses of imidacloprid reduced the foraging ability of bumblebee workers collecting pollen, with treated bees bringing back pollen less often and in smaller quantities.	Showed that field-realistic levels of the neonicotinoids imidacloprid and clothianidin, and the organophosphate miticide coumaphos oxon, caused a depolarization-block of neuronal firing and inhibited nicotinic responses in mushroom body Kenyon cells of honeybee brain tissue.

Tsvetkov et al. 2017	***Woodcock et al. 2017***	***Rundlof et al. 2015***
Chronic exposure to neonicotinoids reduces honey bee health near corn crops	Country-specific effects of neonicotinoid pesticides on honey bees and wild bees	Seed coating with a neonicotinoid insecticide negatively affects wild bees
Apis mellifera	*Apis mellifera, Bombus terrestris* and *Osmia bicornis*	Multiple wild bee species and *Osmia bicornis* L, *Apis mellifera* and *Bombus terrestris.*
Multiple included miticides, fungicides, herbicides, and insecticides.	Clothianidin and thiamethoxam	Neonicotinoid clothianidin and the pyrethroid β-cyfluthrin
Colonies near neonicotinoid treated corn had increased worker mortality and were associated with declines in social immunity and increased queenlessness. Also showed that the presence of a commonly encountered fungicide can double the acute toxicity of neonicotinoids to honey bees.	Showed oilseed rape treated with neonicotinoids negatively affected honeybees during crop flowering and that effects persisted over winter, leading to smaller colonies the following year associated with clothianidin. Also, in *Bombus terrestris* and *Osmia bicornis*, neonicotinoid residues were negatively correlated with reproduction.	Showed that the oilseed rape seeds treated with the insecticide seed coating Elado, containing a combination of the neonicotinoid and a non-systemic pyrethroid, reduced wild bee density, solitary bee nesting, and bumblebee colony growth and reproduction in field conditions.

Paper
Title
Study Species
Pesticides
Impact and Insight

1.3 Effects of pesticides on honeybee and bumblebee behaviour: case study of the neonicotinoids

Of the various classes of insecticide implicated with bee declines it is the neonicotinoids that have featured most prominently in recent years (Feltham, Park et al. 2014, Rundlof, Andersson et al. 2015, Tsvetkov, Samson-Robert et al. 2017, Woodcock, Bullock et al. 2017). The first compound in the neonicotinoid class to be introduced to the market was Imidacloprid in 1991 (Nauen, Jeschke et al. 2008), and by the mid-90s became one of the most widely used pesticide across the world. A number of new neonicotinoids (thiamethoxam, clothianidin, acetamiprid, nitenpyram, thiacloprid and dinotefuran) came to market soon after, and the high uptake of neonicotinoids has been driven by their perceived safety compared to the pyrethroids, chlorinated hydro-carbons, organophosphates, and carbamate insecticides (Jeschke and Nauen 2008), with more neonicotinoids in development or nearing commercial release (Shao, Liu et al. 2013). Neonicotinoids are also versatile regarding application method due to their systemic nature in being dissolvable in water, as they can be applied in a range of forms including seed treatment, soil drench, foliar and stem application, all of which can reduce spray drift into the surrounding environment (Pflüger. W 1991). With the most adopted strategy for treating arable crops being seed dressings, the active ingredient is absorbed by the plant providing long-term protection for all tissues throughout growth, maximising exposure to their target pests and facilitating single application (Elbert, Haas et al. 2008). However, this systemic nature does mean that residues can get into the pollen and nectar

of treated or contaminated flowering plant species (Botías, David et al. 2015, Rundlof, Andersson et al. 2015) typically at 2 and 6 ng/g (Godfray, Blacquière et al. 2015) but can be present at up to 16 ng/g and 36.88 ng/g in pollen and nectar respectively (EFSA 2013, EFSA 2013, EFSA 2013). Consequently, foraging bees visiting treated flowers can be exposed to doses of neonicotinoids (Botías, David et al. 2015, Rundlof, Andersson et al. 2015, David, Botías et al. 2016, Mitchell, Mulhauser et al. 2017), see table 1.2 (Wood and Goulson 2017).

Neonicotinoids have a reduced toxicity to vertebrates yet are highly toxic to a wide range of invertebrate species – another property that has made them an attractive pesticide to adopt (Goulson and Kleijn 2013). Neonicotinoids are agonists of insect post-synaptic nicotinic acetylcholine receptors (nAChR), receptors found exclusively in the central nervous system (Breer and Sattelle 1987) where they mediate fast excitatory synaptic transmission. They have stronger affinity to insect nAChRs which is why they are selectively more toxic to insects than vertebrates (Tomizawa and Casida 2005). For instance, the neonicotinoids imidacloprid and clothianidin exhibit extremely low binding affinity to mammalian nAChRs (the α4β2-nAChRs) due to the prominent difference in structural architecture of insect and mammalian nAChRs (Tomizawa, Lee et al. 2000, Tomizawa and Casida 2003).

When neonicotinoid nAChR agonists bind insect nAChRs they elicit a transient inward current, leading to the generation of action potentials and thus the agonistic activation of the receptors, this being the same effect as the natural neurotransmitter acetylcholine (Ach). Whereas normal, modest levels of activation cause nervous stimulation, high levels

can overstimulate and block the receptors causing continuous excitation of the neuronal membranes, eventually leading to paralysis and death (Sheets 2010, Tomizawa 2013). This prolonged activation can occur because whereas acetylcholine is then broken down by acetylcholinesterase terminating the signal from the receptor, it is unable to break down neonicotinoids (Thany 2010, Simon-Delso, Amaral-Rogers et al. 2015). So, in sufficiently high concentrations they cause paralysis and death but even at low concentrations cause nervous stimulation (Suchail, Guez et al. 2001, Goulson and Kleijn 2013). Therefore, even at the sub lethal concentrations used in agriculture they are likely to be affecting the nervous system of any species for which they have affinity, with the potential to alter their behaviour.

Table 1.2 Summary of studies published since 2013 that document neonicotinoid residues in pollen and nectar collected by free flying bees at sites adjacent to treated and untreated flowering crops. SS spring-sown, WS winter-sown, US unclear sowing date. Note. Reprinted from "The environmental risks of neonicotinoid pesticides: a review of the evidence post 2013" by Wood, T.J and Goulson, D., 2017, *Environmental science and pollution research international*, 25(21), p. 17285-17325.

Species	Sample type	Samples collected	Nest location	Mean total neonicotinoid concentration (ng/ml or ng/g)	Reference
Apis mellifera	Nectar	2005–2009 (dates unknown)	Adjacent to untreated US OSR fields	<1 (limit of quantification)	Pilling et al. (2013)
Apis mellifera	Nectar	2005–2009 (dates unknown)	Adjacent to treated US OSR fields	0.7–2.4 (range of reported median values)	Pilling et al. (2013)
Apis mellifera	Nectar	6th May 2014	Adjacent to untreated WS OSR fields	<0.3 (limit of detection)	Rolke et al. (2016)
Apis mellifera	Nectar	6th May 2014	Adjacent to treated WS OSR fields	0.68	Rolke et al. (2016)
Apis mellifera	Nectar	10th–14th May 2014	Adjacent to untreated WS OSR fields	<0.3 (limit of detection)	Rolke et al. (2016)
Apis mellifera	Nectar	10th–14th May 2014	Adjacent to treated WS OSR fields	0.77	Rolke et al. (2016)

Apis mellifera	Nectar	June 2013 (peak OSR flowering)	Adjacent to untreated SS OSR fields	0.1	Rundlöf et al. (2015)
Apis mellifera	Nectar	June 2013 (peak OSR flowering)	Adjacent to treated SS OSR fields	10.3	Rundlöf et al. (2015)
Bombus terrestris	Nectar	June 2013 (peak OSR flowering)	Adjacent to untreated SS OSR fields	0	Rundlöf et al. (2015)
Bombus terrestris	Nectar	June 2013 (peak OSR flowering)	Adjacent to treated SS OSR fields	5.4	Rundlöf et al. (2015)
Apis mellifera	Pollen	2005–2009 (dates unknown)	Adjacent to untreated maize fields	<1 (limit of quantification)	Pilling et al. (2013)
Apis mellifera	Pollen	2005–2009 (dates unknown)	Adjacent to treated maize fields	1–7 (range of reported median values)	Pilling et al. (2013)
Apis mellifera	Pollen	2005–2009 (dates unknown)	Adjacent to untreated US OSR fields	<1 (limit of quantification)	Pilling et al. (2013)
Apis mellifera	Pollen	2005–2009 (dates unknown)	Adjacent to treated US OSR fields	<1–3.5 (range of reported median values)	Pilling et al. (2013)
Apis mellifera	Pollen	First 2 weeks of July 2012	Located in untreated SS OSR fields	0.24	Cutler et al. (2014)

Apis mellifera	Pollen	First 2 weeks of July 2012	Located in treated SS OSR fields	0.84	Cutler et al. (2014)
Apis mellifera	Pollen	June 2013 (peak OSR flowering)	Adjacent to untreated WS OSR fields	<0.5 (limit of detection)	Rundlöf et al. (2015)
Apis mellifera	Pollen	June 2013 (peak OSR flowering)	Adjacent to treated WS OSR fields	13.9	Rundlöf et al. (2015)
Apis mellifera	Pollen	May to September 2011	Non-agricultural area	0.047	Long and Krupke (2016)
Apis mellifera	Pollen	May to September 2011	Adjacent to untreated maize fields	0.078	Long and Krupke (2016)
Apis mellifera	Pollen	May to September 2011	Adjacent to treated maize fields	0.176	Long and Krupke (2016)
Apis mellifera	Pollen	6th May 2014	Adjacent to untreated WS OSR fields	<0.3 (limit of detection)	Rolke et al. (2016)
Apis mellifera	Pollen	6th May 2014	Adjacent to treated WS OSR fields	0.5	Rolke et al. (2016)
Apis mellifera	Pollen	10th–14th May 2014	Adjacent to untreated WS OSR fields	<0.3 (limit of detection)	Rolke et al. (2016)

Apis mellifera	Pollen	10th–14th May 2014	Adjacent to treated WS OSR fields	0.97	Rolke et al. (2016)
Bombus terrestris	Pollen	10th May 2014	Adjacent to untreated WS OSR fields	<0.3 (limit of detection)	Rolke et al. (2016)
Bombus terrestris	Pollen	10th May 2014	Adjacent to treated WS OSR fields	0.88	Rolke et al. (2016)
Bombus impatiens	Pollen	July to August 2013	Adjacent to untreated maize fields	<0.1 (limit of detection)	Cutler and Scott-Dupreee (2014)
Bombus impatiens	Pollen	July to August 2013	Adjacent to treated maize fields	0.4	Cutler and Scott-Dupreee (2014)
Osmia bicornis	Pollen	14th May 2014	Adjacent to untreated WS OSR fields	<0.3 (limit of detection)	Rolke et al. (2016)
Osmia bicornis	Pollen	14th May 2014	Adjacent to treated WS OSR fields	0.88	Rolke et al. (2016)

1.3.1 Colony level effects

Whilst the public consensus appears to be that neonicotinoid exposure is unquestionably harmful to colonies when exposed to field realistic concentrations, the empirical evidence actually provides mixed results. Starting with controlled exposure experiments, these

have been carried out in the lab or as semi-field studies to distinguish pesticide exposure effect on colony level traits from other variables. Colonies of *B. terrestris* exposed to 10 and 2.4 parts per billion (ppb) of thiamethoxam showed no significant difference in growth in either weight or number of individuals (Stanley and Raine 2017). *Bombus impatiens* colonies exposed to 6 and 36 ppb of the neonicotinoid clothianidin, exhibited no effect on pollen consumption, weight of newly emerged workers, or the number of brood, workers, males, and queens (Franklin, Winston et al. 2004). However, other studies have showed contrasting results. For example, Bumblebee *(Bombus terrestris*) colonies exposed to the neonicotinoid imidacloprid at field realistic concentrations (6 ppb in pollen and 0.7 ppb in nectar) suffered a significantly reduced growth rate and 85% reduction in queen production (Whitehorn, O'Connor et al. 2012). Gill *and colleagues* (Gill, Ramos-Rodriguez et al. 2012) reported reduced colony growth and higher mortality in bumblebee colonies exposed to 10 ppb of the neonicotinoid imidacloprid. In a controlled, semi-field experiment, bumblebee colonies exposed to the neonicotinoid clothianidin, at a concentration of five ppb for five weeks had fewer adult workers and sexuals when colonies at the end of the experiment were censused (Arce, David et al. 2017). Additionally, bumblebee colonies exposed to 10 ppb imidacloprid via sucrose solution showed reduced colony growth compared to control colonies, beginning to shrink after 33 days of a 42 day study (Bryden, Gill et al. 2013).

The mixed findings in controlled experiments could be a product of the variable dosages, exposure routes and neonicotinoids used across studies or the different stages of the colony life cycle at which they were exposed (Table 1.1). Indeed, to understand field

realistic dosages requires carrying out field experiment in which colonies are placed next to treated and untreated fields and flowers. However, such investigations have yielded similarly varied findings. Honey bee colonies placed in clothianidin seed-treated canola fields during bloom presented no effects on colony weight gain, honey production, pest incidence, bee mortality, number of adults, and amount of sealed brood (Cutler, Scott-Dupree et al. 2014). Pilling *et al*. (Pilling, Campbell et al. 2013) found honeybee colonies to be at low risk to long term exposure to thiamethoxam in the field following a four year study that presented no effects on colony traits including; colony size, colony weight, brood development and mortality. With similar results found by Pohorecka *et al*. (Pohorecka, Skubida et al. 2012) in honeybee hives placed near neonicotinoid treated oilseed rape fields which suffered no effect on bee mortality, colony size or brood development. Through surveying wild bees in agricultural landscapes of oilseed rape treated with a seed coating of a combined neonicotinoid (clothianidin) and pyrethroid (β-cyfluthrin) insecticide, it was found that there was reduced colony growth and reproduction in bumblebees along with reduced wild bee density (Rundlof, Andersson et al. 2015). Honey bee colonies around corn fields exposed to field realistic concentrations (2.0 – 4.9 ppb) of neonicotinoids had increased worker mortality and increased likelihood of queen loss (Tsvetkov, Samson-Robert et al. 2017). Woodcock and colleagues (Woodcock, Bullock et al. 2017), found that honey bee and bumblebee colonies foraging on oilseed rape grown commercially with seed coatings containing clothianidin or thiamethoxam exhibited negative effects; with reduced colony size (-24%) compared to control colonies in honey bees, and lower queen production in wild bumblebees (*Bombus*

terrestris) which was associated with neonicotinoid residues found in the nests. Conversely, Pilling *et al*. (Pilling, Campbell et al. 2013) found no effects on mortality, foraging behaviour, colony weight or brood development in honeybee colonies foraging on flowering maize and oilseed rape grown from thiamethoxam treated seeds. These contrasting colony level effects found in both lab and field experiments highlight the need to gain a better understanding of neonicotinoids exposure on the individual level to elucidate under what circumstances they translate to colony level impact (Crall, Switzer et al. 2018). Yet our mechanistic understanding as to how colony level effects are directly linked to pesticide induced impacts on individual traits, such as physiology and behaviour, is still limited (Bryden, Gill et al. 2013) (Farooqui 2013, Klein, Cabirol et al. 2017, Crall, Switzer et al. 2018).

1.3.2 Understanding how impaired individual behaviour translates to colony level effects: using effects on foragers as a case study

One of the most cognitively demanding tasks for individual bees to carry out is foraging, it requires the use of numerous sensory cues (colour, pattern, shape and scent) for the recognition and learning of the best flowers to utilize as sources of pollen and nectar (Chittka and Raine 2006, Kulahci, Dornhaus et al. 2008, Leonard, Dornhaus et al. 2011), in combination with navigating a large complex environment between these resources and the nest (Goulson and Stout 2001, Osborne, Martin et al. 2008, Lihoreau, Raine et al. 2012). Foraging is vital for colony growth, as the supply of food is closely associated with colony reproductive output in bumblebees (Pelletier and McNeil 2003), and therefore studying the impacts on foraging performance can provide insights into how colony

success can be affected. The effects on foraging can be assessed across these aspects of behaviour that comprise it:

Foraging efficiency – considered as the quantity of food brought back to colony. Foraging efficiency of *Bombus terrestris* workers foraging in a natural environment has shown to be reduced in colonies exposed to field realistic levels of imidacloprid (Gill, Ramos-Rodriguez et al. 2012, Feltham, Park et al. 2014, Gill and Raine 2014). When Bumblebee colonies were exposed to the neonicotinoid imidacloprid at concentrations of 10 ppb individuals suffered impaired natural foraging performance, specifically the efficiency of pollen foraging resulting in further effects on forager recruitment, productivity of workers and ultimately worker mortality (Gill, Ramos-Rodriguez et al. 2012). Additionally, it has been found that pesticide-exposed (2·4 ppb (Gill and Raine 2014) thiamethoxam) bumblebees brought back pollen less frequently compared to control (Stanley, Russell et al. 2016). This form of measurable reduction in food resources provided by workers has clear knock-on effects to colony health (Pelletier and McNeil 2003, Brodschneider and Crailsheim 2010), and studies have tried to test this in bumblebees showing that whereas control workers would increase the amount of food they brought back with increased experience, imidacloprid exposed workers exhibited no experience related increase (Gill, Ramos-Rodriguez et al. 2012, Gill and Raine 2014). However, it is still not known what aspects of behaviour were impaired to cause such reduced foraging performance as workers aged.

Activity levels – the duration, distance and speed of flights and general physical performance are key components of foraging and have been shown to be affected by

exposure to neonicotinoids. In a study by Tosi et al (Tosi, Burgio et al. 2017), honeybees given an acute sub lethal dose (1.34 ng/bee) of thiamethoxam showed significantly increased flight duration and distance on flight mills. In contrast, chronic exposure (1-2 days, 1.96–2.90 ng/bee/day) significantly decreased the flight duration, distance, and average velocity. Additionally, sub-lethal doses of imidacloprid (0.15–6 ng/bee) and clothianidin (0.05–2 ng/bee) have also been shown to increase the duration of free flight foraging bouts but reduce overall foraging activity of honey bees (Schneider, Tautz et al. 2012). Furthermore, Williamson *et al*. (Williamson, Willis et al. 2014) found that although honeybees exposed to neonicotinoids (imidacloprid, thiamethoxam, clothianidin, dinotefuran) did not exhibit significant changes in duration of flying or walking behaviour, they did show signs of impaired motor function in the form of more frequently losing postural control failing to right themselves. The impact of pesticides on these behavioural parameters of foraging is relatively underreported in bumblebees, a study by Lämsä *et al*. (Lämsä, Kuusela et al. 2018) found that bumblebees exposed to low levels (1 ppb) of imidacloprid showed no significant effects on physical performance (flight distance and speed). Although individuals did exhibit reduced motivation to forage, visiting fewer (artificial) flowers and being slower to initiate foraging. Therefore, reduced foraging efficiency, although potentially influenced by impairments of physical performance due to pesticide exposure, it would seem is likely underpinned by further effects on the cognitive capabilities of foraging bees.

Navigation and homing – the ability of individuals to located food resources in large complex environments then return from the foraging bout to the nest. Henry *et al*.

(Henry, Beguin et al. 2012) showed that sub-lethal doses of the neonicotinoid thiamethoxam decreased the chance of honey bee (*Apis mellifera*) foragers locating their colony leading to increased mortality. Similar results for honeybees were found by Fischer *et al*. (Fischer, Müller et al. 2014) showing individuals exposed to imidacloprid, clothianidin or thiacloprid all exhibited significantly lower successful return flights in catch and release experiments, along with reduced probability of responding to a salient landscape structure and less directed flights during homing. However, studies in bumblebees conversely found pesticide-exposed foragers found their way back to their nest more frequently during homing trials than individuals from control colonies (Stanley, Russell et al. 2016). Although, whether these impairments in navigation and homing are due to effects on the physical performance, cognitive capabilities or a combination of the two is unknown.

1.4 EVIDENCE GAPS

1.4.1 Is impairment to individual foraging performance from neonicotinoid exposure explained by effects on brain development?

The array of behaviours that underpin the foraging ability of social bees rely on the precise detection, integration and processing of information across the major brain structures and their functional and structural plasticity (Giurfa 2013). This neuroplasticity in relation to foraging has been demonstrated to occur in the mushroom bodies (MB); the brain neuropil associated with higher cognition, learning and memory in bees, for example a recent study reported structural changes at the MB input regions of honeybees, a

decrease in the number of synaptic boutons (the end of an axons and sites of synapses with other neurons) at the onset of foraging followed by an increase in conjunction with increased forging intensity (Cabirol, Cope et al. 2018). Therefore, it could be that disruption to the normal development and plasticity of these central brain structures, where the target (nAChR) receptors of neonicotinoids are found in high density (Breer and Sattelle 1987), is responsible for the impairment of these vital cognitive capabilities. However, our understanding of how pesticide contaminated food influences individual physiological development is still in its infancy (Gregorc and Ellis 2011, Wu, Anelli et al. 2011, Gill and Raine 2014), and research on whether this could translate to the forms of impaired behavioural performance (reported above) is urgently needed (Tomé, Martins et al. 2012, Yang, Chang et al. 2012, Tan, Chen et al. 2015, Siviter, Koricheva et al.).

1.4.2 What are the consequences of individuals exposed during development inside the colony?

Neonicotinoid residues have been reported in the nectar and pollen of crops and wild flowers occurring at concentrations of up to 16 ng/g and 36.88 ng/g in pollen and nectar respectively (EFSA 2013, EFSA 2013, EFSA 2013). The persistence of these residues leads to direct contact and ingestion of neonicotinoids through feeding by individual foragers, and this has been the primary justification for investigating the effects of direct adult worker exposure. However, these contaminants will also be transferred to the natal colony leading to exposure of members inside the colony, including developing brood. For example, pollen is the major food source for growing bee larvae and nurse workers, and therefore it is the contaminated products provided by the colony foragers on which they

feed and so acting as a route of exposure to neonicotinoids (Sanchez-Bayo and Goka 2014). Indeed, neonicotinoids have been found inside social bee colonies across the globe, in brood, wax and food stores (Chauzat, Martel et al. 2011, Pohorecka, Skubida et al. 2012, Botías, David et al. 2015, Mitchell, Mulhauser et al. 2017). This raises concerns as to how the omnipresence of these residues in the colony environment can affect colony development. Is the development of brood that are reared with this exposure affected, and could this lead to future cohorts of workers, that the colony depends on, being impaired? Yet to date we have overlooked this as being a cause for colony level problems.

1.5 Study Species

Here I have investigated the effects of neonicotinoid pesticide exposure during development and adulthood specifically on bumblebees for three primary reasons: first, social bumblebees can be reared within controlled laboratory conditions whereby individuals, including developing workers could be exposed in the social colony environment (as opposed to being in isolation) (Maleszka, Barron et al. 2009, Gill, Ramos-Rodriguez et al. 2012, Whitehorn, O'Connor et al. 2012), followed by learning assays of select individuals being readily undertaken (Riveros and Gronenberg 2009, Stanley 2015). Secondly despite the increased understanding of the importance of non-Apis bees, (Brittain, Williams et al. 2013, Garibaldi, Steffan-Dewenter et al. 2013, Gill, Baldock et al. 2016) and their differential sensitivity to pesticides (Garibaldi, Steffan-Dewenter et al. 2013, Rundlof, Andersson et al. 2015, Woodcock, Isaac et al. 2016, Heard, Baas et al.

2017) there are limited empirical tests of their physiological response to stress and postembryonic neuronal development compared to honeybees. Thirdly, bumblebees exhibit prominent worker body size variation compared to honeybees, with up to 10-fold mass difference between workers within the same colony (Alford 1975, Jandt and Dornhaus 2009) . This size variation has been linked to the observed division of labour seen in bumblebees (Brian 1952, Free 1955, Goulson, Peat et al. 2002, Jandt and Dornhaus 2009), whereby larger bees more likely to be foragers and smaller bees more likely to perform within-nest tasks and these behavioural differences likely make them differentially susceptible to pesticide exposure. Additionally, this size variation enables the assessment of how brain volume variation correlates with learning performance, and for the first time how a pesticide affects this relationship. As yet empirical testing for the 'bigger-is-better' hypothesis, with regards to bumblebee learning, has been restricted to measures of body, and not brain, size and has revealed mixed support (Worden, Skemp et al. 2005, Spaethe, Brockmann et al. 2007, Riveros and Gronenberg 2009, Smith and Raine 2014, Sommerlandt, Rössler et al. 2014).

1.6 THESIS OBJECTIVES

To test the effects of pesticide exposure on bumblebee development it is important to first consider the amount of inherent developmental plasticity in behaviour and brain growth. In social bees, worker maturation has been shown to correlate with stereotyped behavioural changes (Johnson 2010; Goulson 2010), as well as increased volumes of functional structures (neuropils) of the brain (Winnington et al. 1996; Withers et al. 1993;

Durst et al. 1994; Galizia et al. 2012)(Li, MaBouDi et al. 2017). It is therefore difficult to disentangle the inherent effect of age and the co-varying cumulative increase in sensory input (Jones et al. 2013; Maleszka et al. 2009; Riveros & Gronenberg 2010; Fahrbach et al. 1998). Distinguishing the effects of pesticide exposure from variation caused by other interacting factors, therefore: i) is facilitated by attempting to standardise experience and sensory input across tested workers so as to focus on experience-independent change; ii) requires testing workers of controlled age; iii) requires comparative analysis between young and old age cohorts. Furthermore, understanding how the timing of pesticide exposure affects adult phenotypes is important, as revealing developmental stages vulnerable to pesticide exposure reveals susceptibility windows in colony development (Gill et al. 2016). Individuals developing inside a colony are likely to be exposed during two main developmental stages - brood (larval & pupal) and early adulthood. These considerations lead us to ask, at what stage does pesticide exposure have the greatest impact on learning behaviour and/or brain growth, and critically can brain plasticity allow recovery during an unexposed later stage?

The aim of this thesis is to investigate how exposure to a neurotoxic pesticide in the bumblebee (Bombus *terrestris audax*) at different key developmental stages can affect learning behaviour as later adults, and whether I can link variation in learning performance with changes to an aspect of brain development.

1. In Chapter 2 I explore how individual worker bees of different ages; 3 and 12 days old, perform in an olfactory learning assay following exposure to field realistic

levels of a major neonicotinoid during larval development and the first few days of early adulthood.

2. In chapter 3, with the aim of investigating how variation in learning behaviour between age and pesticide exposure treatments correlates with growth of specific brain structures; I detail the development and validation of a new method of micro-CT and image analyses to explore variation in soft tissue structure allowing me to investigate changes to bumblebee brain morphology.

3. In Chapter 4, taking workers from the experiment described in chapter 2, I apply the new brain scanning protocol to estimate differences in volume of brain structures associated with olfactory learning and memory of individual bees and assess how this correlates with their learning performance.

4. In Chapter 5 I explore further brain structures of the bumblebee brain and assess whether brain structures associated with processing visual information and motor control respond similarly to neonicotinoid exposure as those in investigated in chapter 4 or if these functionally distinct neuropils respond differently.

Chapter 2

EFFECTS OF PESTICIDE EXPOSURE AT DIFFERENT LIFE STAGES ON OLFACTORY RESPONSIVENESS AND LEARNING IN BUMBLEBEES

2.1 INTRODUCTION

Neonicotinoid residues have been found inside social bee colonies across the globe (Mullin, Frazier et al. 2010, David, Botías et al. 2016, Mitchell, Mulhauser et al. 2017, Calatayud-Vernich, Calatayud et al. 2018), raising concerns as to whether the omnipresence of these residues in the environment can affect colony development (Cressey 2017). Indeed, field studies (Rundlof, Andersson et al. 2015, Tsvetkov, Samson-Robert et al. 2017) and controlled exposure experiments (Gill, Ramos-Rodriguez et al. 2012, Whitehorn, O'Connor et al. 2012) have associated neonicotinoid exposure with reduced colony growth. However, our understanding of how reduced colony functioning is explicitly linked to pesticide induced changes to the behaviour of colony members is relatively limited (Bryden, Gill et al. 2013, Gill and Raine 2014, Baron, Jansen et al. 2017).

Individual workers within bumblebee colonies can have an array of tasks to undertake during their lifetime such as foraging, feeding brood and other in nest tasks, with each reliant on their ability to recognize, learn and respond to sensory cues (Weidenmüller

2004, Jandt and Dornhaus 2009, Jandt, Huang et al. 2009). Bumblebees have also been shown to be capable of completing complex novel learning tasks (Loukola, Perry et al. 2017), and in laboratory tests it has been demonstrated that bumblebees can learn to associate rewards with olfactory cues (Riveros and Gronenberg 2009, Smith and Raine 2014), numerous visual cues; colour (Raine, Ings et al. 2006, Riveros and Gronenberg 2012), patterns (Fauria, Dale et al. 2002) and shapes (Muller and Chittka 2012), as well as electrical fields (Clarke, Whitney et al. 2013). Through a combination of these multisensory cues bees have been shown to improve the accuracy of their decisions during foraging (Kulahci, Dornhaus et al. 2008, Leonard, Dornhaus et al. 2011). Colony success relies on workers being effective foragers, requiring individuals to demonstrate these high levels of cognitive ability (Chittka 2017) that are crucial for foraging in large complex environments and underline behaviours required for optimal homing, navigation and overall foraging efficiency (Reinhard, Srinivasan et al. 2004, Raine and Chittka 2007, Raine and Chittka 2008, Riveros and Gronenberg 2009, Menzel 2012). Foragers must assimilate the navigational and spatial memory requirements of foraging along with an ability to learn to associate an array of specific host plant cues, such as chemical volatiles, colour, odour and electric fields with a food reward in the form of nectar and pollen (Clarke, Whitney et al. 2013, Knauer and Schiestl 2015, Muth, Papaj et al. 2015) if they are to bring back the resources required to rear colony brood and maintain optimum colony function (Knauer and Schiestl 2015). Therefore, understanding how these associative learning capabilities in bumblebees are affected by neonicotinoids is important to understanding how they translate to the observed colony level effects.

The ability to physically detect and respond to stimuli is also vital to the capacity of bees to forage effectively. Bees make decisions on which food sources they exploit based, in part, on the concentration of the nectar (Loo and Bitterman 1992, Hill, Hollis et al. 2001, Cnaani, Thomson et al. 2006) as such, variation in sucrose responsiveness influences the foraging behaviour of bees (Pankiw, Waddington et al. 2001, Scheiner, Barnert et al. 2003, Scheiner, Page et al. 2004). It has been shown that individuals can differ in their response thresholds through morphological differences in sensory organs (Riveros and Gronenberg 2010, Russell, Morrison et al. 2017) and increased responsiveness has been linked to learning performance (Scheiner, Barnert et al. 2003, Perez, Rolland et al. 2013). Furthermore, sucrose concentration positively correlates with learning performance, with bees learning to associate higher concentration faster than low concentrations of sucrose (Loo and Bitterman 1992, Scheiner, Erber et al. 1999, Cnaani, Thomson et al. 2006). However pesticides have been shown to alter sucrose responsiveness, in honeybees, neonicotinoids affected responsiveness to sucrose concentrations (Aliouane, el Hassani et al. 2009, Démares, Crous et al. 2016, Démares, Pirk et al. 2018), with stronger effects on responsiveness to higher concentrations. However, as bumblebees differ in their sucrose response thresholds (de Brito Sanchez, Chen et al. 2008, Mommaerts, Wäckers et al. 2013) and their sugar preference (Waller 1972, Pouvreau 1974), their responsiveness to sucrose is likely to be differentially affected by brood or adult exposure to neonicotinoids, though whether this is the case is yet to be explored.

Multiple studies have indicated that the associative learning capabilities, along with memory formation in bees can be impeded by neonicotinoid exposure. For example,

visual associative learning performance, tested using T-tube maze assays was reduced in honeybees exposed to imidacloprid (Han, Niu et al. 2010). Additionally, Decourtye and Devillers (Decourtye and Devillers 2010) report reduced associative learning of visual cues and a food reward by honeybees in a complex maze setup following oral exposure to thiamethoxam. Olfactory learning performance and memory has also been explored in numerous studies using the proboscis extension reflex conditioning assay, where it has been shown to be impaired in honeybees exposed to neonicotinoids (including imidacloprid, thiamethoxam and acetamiprid) (Decourtye, Devillers et al. 2004, El Hassani, Dacher et al. 2008, Aliouane, el Hassani et al. 2009, Han, Niu et al. 2010, Williamson and Wright 2013, Tan, Chen et al. 2015, Wright, Softley et al. 2015). Chronic exposure to Thiamethoxam at field realistic levels (2.4ppb) showed slower learning and impaired memory in proboscis extension reflex (PER) conditioning in the less studied bumblebee (Stanley 2015).

Individual performance in such lab-based learning studies has been positively correlated with the natural foraging performance of colonies: the average visual learning speed of individuals within bumblebee colonies was shown to correlate with colony foraging performance (nectar collection rate) in the field (Raine and Chittka 2008). And foraging experience was associated with increased olfactory learning performance assessed using the proboscis extension reflex assay in bumblebees (Riveros and Gronenberg 2009). However, while increased experience seems to correspond with better learning performance, age alone has been suggested not to correlate with the ability to learn in bumblebees, with one day old workers able to perform as well in olfactory learning tests

as much older individuals (Riveros and Gronenberg 2009). And bumblebees as young as 2 days after emergence are known to be able to leave the nest to forage (Pouvreau 1989, Yerushalmi, Bodenhaimer et al. 2006). Although disentangling age and experience isn't easy, even within the nest older bees will have been exposed to a greater number of sensory signals, social interactions and potential learning experiences. Therefore to test the effects of pesticide exposure on bumblebee learning it is still important to consider the level of age and experience dependent plasticity in behaviour; indeed worker maturation has been shown to correlate with stereotyped behavioural changes, such as the onset of foraging (da Silva-Matos and Garófalo 2000, O'Donnell, Reichardt et al. 2000, Goulson 2010). Moreover, while bumblebees are considered not to exhibit strict age polytheism, as found in honeybees, their large variation in size has been linked to task specialization between individuals with larger workers more likely to be foragers and smaller workers likely to perform nest tasks (Free 1955, Garófalo 1978, Jandt and Dornhaus 2009). Further to this, it has been reported that larger bees exhibit higher foraging performance and collect nectar at a higher rate (Goulson, Peat et al. 2002, Spaethe and Weidenmüller 2002). In consideration of these factors, to distinguish the effects of pesticide exposure, from variation caused by other interacting factors, I would need to: i) endeavour to standardise experience and sensory input across tested workers to focus on experience-independent change; ii) test workers of controlled age; iii) compare between young and old age cohorts and iv) account for worker size.

That young, newly emerged bumblebees should be as capable of learning and performing colony tasks as older individuals may mean the colony is at greater risk to stress of

workers during development – with individuals potentially emerging impaired and less able to efficiently contribute to the colony with less scope for this ability to recover with age. Furthermore, understanding how the timing of pesticide exposure affects adult phenotypes is important, as revealing developmental stages vulnerable to pesticide exposure allows us to identify windows of susceptibility in colony development (Gill, Baldock et al. 2016). However, currently our knowledge on how developmental stress in the form of neurotoxic insecticides could influence behavioural capabilities as an adult is limited to just a couple of studies. For instance, Yang *et al*. (Yang, Chang et al. 2012) found impaired olfactory associative learning in adult honey bees that had been treated with 0.04 ng/larva imidacloprid in the larval stage and Tan *et al*. (Tan, Chen et al. 2015) reported that honey bees exposed as larvae to 0.24 ng/bee (total dose) of imidacloprid had significantly impaired olfactory short-term learning acquisition.

In this study, I implemented a complex factorial experimental design in which bumblebee workers, whilst reared inside their natal colonies, were exposed via food provision to the neonicotinoid imidacloprid at different developmental stages (during brood [*pre-eclosion*], early adult [*post-eclosion*] or both [*continual*]) to compare the effects on responsiveness and learning behaviour relative to unexposed [*control*] workers (Figure 2.1). Adult workers were then tested on their sucrose responsiveness followed by their olfactory associative learning performance at either 3 or 12-days post-emergence.

2.2 METHODS

2.2.1 Animal Husbandry

Bombus terrestris audax colonies were delivered by a commercial supplier (Agralan Ltd), each arriving with a queen and mean (±s.e.m.) of 13.3±0.77 workers (Table 2.1.) This colony size was deemed large enough to prevent colony failure but small enough to reduce the risk of a switch from worker to sexual production over the experimental period as the study design relied on the production of new workers. Upon arrival, each colony was housed in a plastic box and all were moved to a controlled environment (23°C; 60% humidity) red light room and remained so for the duration of the experimental period. Throughout the experiment, colonies were provisioned with ad-libitum pollen in a petri dish and 40/60% sucrose/water solution in a gravity feeder, with this food replenished every two days and the feeders thoroughly cleaned prior to refill (see Appendix 2.1 for colony consumption data). The sucrose solution concentrations provided remained accurate throughout the experiment by being checked before filling the gravity feeder using a refractometer (Bellingham + Stanley, E-Line 80, 0.5°Brix minimum). During Phase I (days 1-21; Figure 2.2), I conducted daily checks of all newly eclosed bees and marked each individual using a white paint pen (uni POSCA, Mitsubishi Pencil CO. LTD.), allowing us to distinguish between newly eclosed workers during Phase II (day 22 onwards) from eclosed workers before this (Table 2.1). Colonies were checked daily for any males, gynes or dead individuals which were removed and stored at -20°C.

2.2.2 Pesticide preparation

For the pesticide treated sucrose solution, 100mg of imidacloprid (powder; *grade*: PESTANAL®, analytical standard; *brand*: Fluka) was dissolved in 100ml of acetone to produce a primary stock solution (1mg/ml). An aliquot of the primary stock solution was then added to a 40/60% sucrose/water solution to produce a 5ppb imidacloprid solution. A control sucrose solution was made by repeating this process but adding the same volume of a pure acetone stock solution to a sucrose solution. Colonies were fed honeybee collected pollen obtained from a commercial supplier (Agralan Ltd).

Table 2.1 Colony census at start of experiment and at the end of each phase. All newly eclosed workers from the beginning of phase II were temporarily removed and tagged with a unique numbered Opalith tag for identification. Colony 22 (in bold) had ceased to produce new workers during the experiment and showed signs of colony failure and therefore was not considered in the tests.

Colony	Treatment	Starting Number of Ws	Number of workers eclosed	
			end of Phase I	end of Phase II (i.e. tagged)
1	Control	25	27	45
2	Control	17	48	58
3	Control	20	26	35
4	Pre.	9	17	16
5	Pre.	24	23	47
6	Pre.	18	36	13
7	Post.	12	41	6
8	Post.	20	54	42
9	Post.	20	34	22
10	Cont.	12	51	54
11	Cont.	14	42	29
12	Cont.	17	20	10
13	Control	10	28	40
14	Control	17	12	2
15	Pre.	16	36	41
16	Pre.	11	19	18
17	Pre.	8	24	8
18	Post.	15	24	47
19	Post.	12	41	28
20	Post.	7	33	13
21	Cont.	13	38	40
22	**Cont.**	**12**	**5**	**0**
23	Cont.	6	24	24

2.2.3 Experimental setup

On arrival colonies were randomly assigned to the four experimental treatments, with no significant difference in the number of workers between treatments (ANOVA: df=3, F=1.04, p=0.40; Table 2.1; Figure 2.1). Monitoring overall development of workers in colonies, I implemented a fully factorial design with our colony treatments comprising a

combination of two exposure phases: *Phase I* encompassing the majority of the brood (larval & pupal) development period (21 days) and *Phase II* comprising the early adult development period (up to 12 days; Figure 2.2). Phase I exposure period started two days after colonies arrived, and lasted for 21 days approximating development time from an egg or very small larva to adult eclosion (Alford 1975, Duchateau and Velthuis 1988, Cnaani, Schmid-Hempel et al. 2002). This ensured that all sampled adults (for PER testing and brain scanning) will have been exposed / unexposed during the vast majority of their brood development in a standardised manner (Figure 2.2). On the 22nd day Phase II started, and during which I checked daily for newly eclosed workers (adults recently emerged from their pupal case) with each worker temporarily removed and tagged with a unique numbered Opalith tag using superglue and placed back into the colony (lasted 15-20 minutes). On tagging, I randomly assigned half of the workers per colony per day to be removed 3 and 12-days later, which could be achieved without error by using the tag ID. These ages were chosen to represent newly emerged "naive" workers just after the initial burst of post eclosion brain growth (3 day) and an older experienced worker of at around maximum brain volume (12 day) (Jones, Leonard et al. 2013). This tagging period lasted for 11 days to provide us with a high number of workers to test (n=413 tested bees; Figure 2.3; Table 2.3). This window of opportunity approximates the time of pupal development, in which pupae evacuate their gut and stop feeding (Cnaani, Schmid-Hempel et al. 2002) hence giving us this window and relatively standardised pesticide exposure across all tested workers (Figure 2.2).

I applied four treatments: *control* – both phases unexposed (n = 5 colonies); *pre-eclosion exposure* – Phase I exposed to Imidacloprid, Phase II unexposed (n = 6); *post-eclosion exposure* – Phase I unexposed, Phase II exposed to Imidacloprid (n = 6); *continual* - both Phases exposed to Imidacloprid (n = 5; Figure. 2.1). Originally six colonies constituted the *continual* treatment, however by the end of Phase I, one colony had ceased to produce new workers and showed signs of colony failure; therefore, only the remaining five were used (Table 2.1).

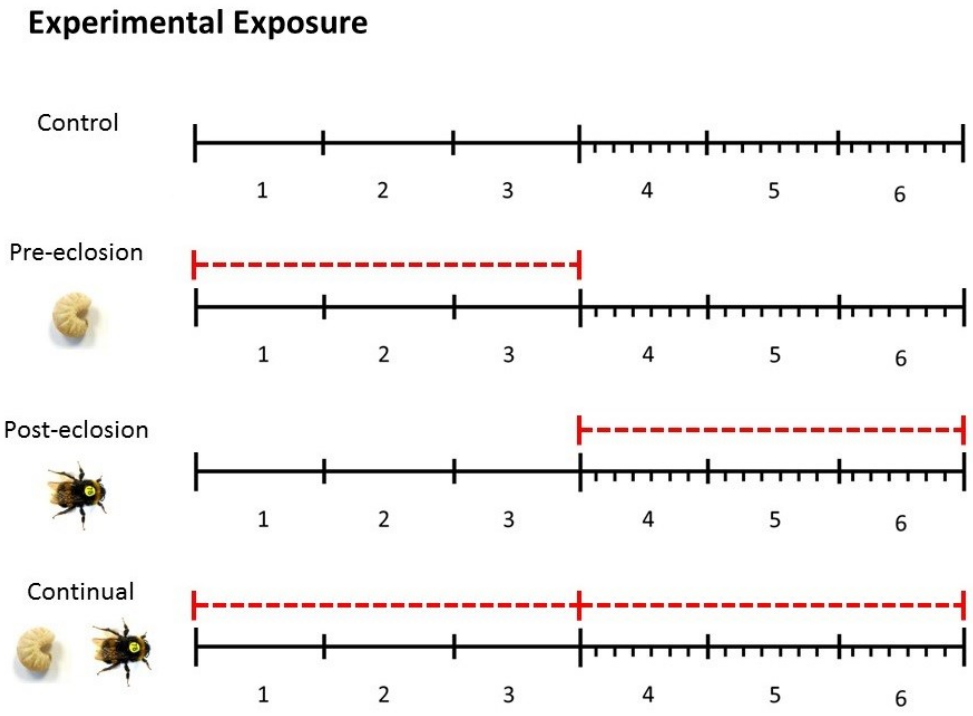

Figure 2.1 Colony experimental exposure. Colonies were exposed over 6 weeks (weeks 1-3 Phase I, 4-6 Phase II) for each treatment group: control, pre-eclosion (brood development, Phase I), post-eclosion (early adult development, Phase II) and continual (exposure during both developmental phases). The period of Imidacloprid exposure is indicated by red lines

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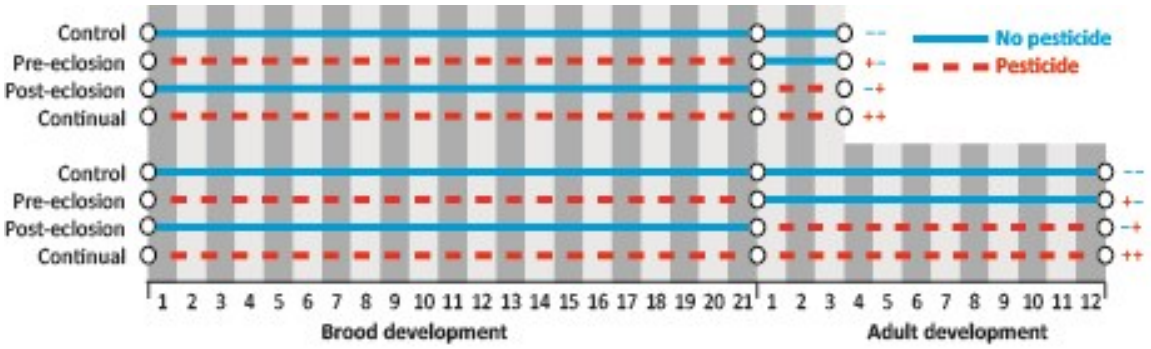


Figure 2.2 Graphic showing the eight treatment cohorts of individual bees derived from four colony treatments (control, pre-eclosion, post eclosion & continual). Blue solid lines represent non-treated food and red dashed line represents pesticide-treated food. Brood developmental represents the larval and pupal stages of individuals (phase I), with adult development representing the number of days after eclosion from the pupal case (phase II).

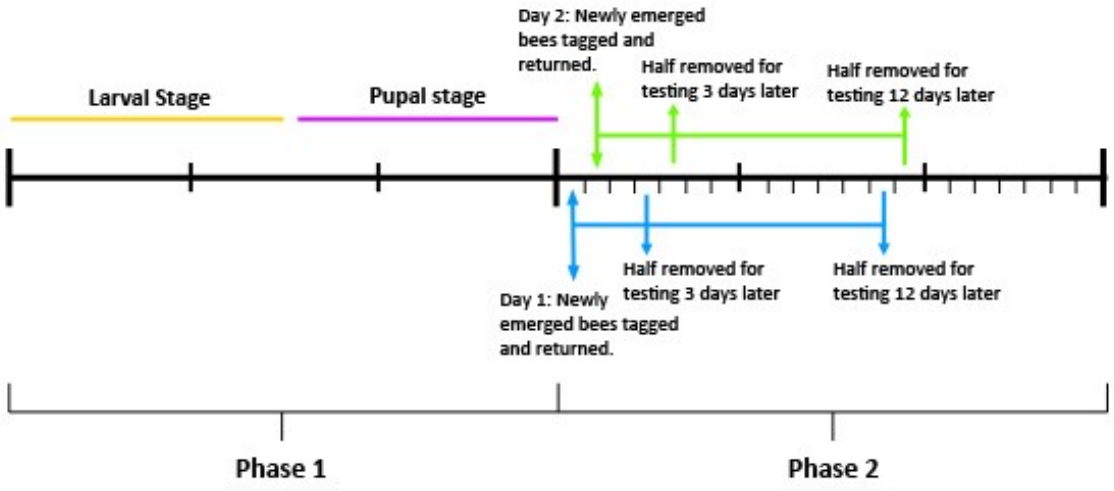


Figure 2.3 Sampling for 3 and 12-day cohorts. During Phase I: all newly eclosed bees were marked using a white paint pen, but this also represents the developmental time of workers with our sampled bees having been larvae (blue) and pupae (purple) during this 21 day development period. Phase II: for 11 days, colonies were checked daily with any newly eclosed workers being

tagged and returned to their natal colony with half randomly assigned to a 3-day cohort and the other half a 12-days cohort. The respective cohorts were then removed 3 days or 12 days later. This continual tagging and sampling over the 11 day period provided us with a large number of workers to test (here I provide an example for the first two days for demonstration purposes).

Table 2.2 Sample size across experimental stages. For each colony the number of tagged workers removed for PER testing: those that survived through the harnessing and overnight period, how many of those that showed a response and of those a learnt response.

Treatment	Colony	Tagged		Survived to Harnessing		Harnessed		Survived Overnight		Responsive		Learner	
		3	12	3	12	3	12	3	12	3	12	3	12
control	1	16	29	15	29	14	13	13	11	9	8	5	4
control	2	18	40	17	40	17	19	16	17	9	9	3	7
control	3	13	22	13	21	13	10	12	10	6	6	2	1
pre-eclosion	4	9	7	9	7	9	6	9	5	4	2	1	0
pre-eclosion	5	21	26	21	25	21	22	20	19	10	9	4	1
pre-eclosion	6	9	4	8	4	8	3	8	3	3	0	1	0
post-eclosion	7	4	2	4	2	4	2	4	2	1	0	0	0
post-eclosion	8	14	28	14	27	14	15	11	12	5	6	2	1
post-eclosion	9	11	11	11	11	11	7	11	7	4	4	2	1
continual	10	15	39	15	35	14	11	11	10	4	2	0	0
continual	11	13	16	13	15	12	10	12	8	5	2	1	1
continual	12	6	4	5	4	5	2	5	2	3	2	2	2
control	13	18	22	18	21	18	14	17	13	7	12	1	6
control	14	0	2	0	2	0	1	0	1	0	0	0	0
pre-eclosion	15	17	24	16	21	16	15	16	15	5	11	0	1
pre-eclosion	16	6	12	6	12	6	8	6	8	2	6	0	1
pre-eclosion	17	4	4	4	4	4	4	4	3	2	2	0	0
post-eclosion	18	19	28	19	26	19	17	18	16	2	8	0	1
post-eclosion	19	11	17	11	15	11	10	9	9	3	8	0	4
post-eclosion	20	4	9	4	6	4	5	4	5	2	2	0	0
continual	21	14	26	13	25	13	8	13	8	3	8	0	3
continual	22	0	0	0	0	0	0	0	0	0	0	0	0
continual	23	6	18	6	17	6	4	6	4	1	3	0	1
	Totals	248	390	242	369	239	206	225	188	90	110	24	35
			638		611		445		413		200		59

2.2.4 Assessing olfactory learning performance using proboscis extension reflex (PER) conditioning

Olfactory learning performance assessed using the established proboscis extension reflex (PER) conditioning paradigm (Figure 2.4) (Bitterman, Menzel et al. 1983, Laloi, Sandoz et al. 1999, Riveros and Gronenberg 2009, Sommerlandt, Rössler et al. 2014) as previously used to test pesticide effects on adult learning performance in honeybees exposed during the larval stage (Yang, Chang et al. 2012, Tan, Chen et al. 2015), and bumblebees exposed as adults (Stanley 2015).

On removal from the colony, workers were harnessed using a modified 2ml centrifuge tube and a split pin yoke, under natural light in the lab, (Stanley 2015) (Figure 2.4 for harness setup). This was done in the afternoon (between 13:00-14:00) and bees were left for 2hrs to settle. All bees were then fed to satiety using a Gilmont® syringe to present 40% sucrose solution droplets directly to the mouthparts, and harnessed bees were then left overnight for precisely 18 hours under red light in a separate CT room (same environmental conditions) to be used for testing the next day (between 08:00-09:00). Any individuals that did not survive overnight were removed from the test (n=32). Next, using 50% sucrose solution all remaining bees (n=413) were tested for responsiveness, touching each of the antennae of every individual with a droplet of sucrose three times, alternating between the left and right (6 touches in total). Unresponsive bees were removed from testing and responsive bees were fed a small droplet (0.8µl) of the sucrose solution for motivation, and 15 minutes later were ready to start the PER test (Figure 2.4; Table 2.3).

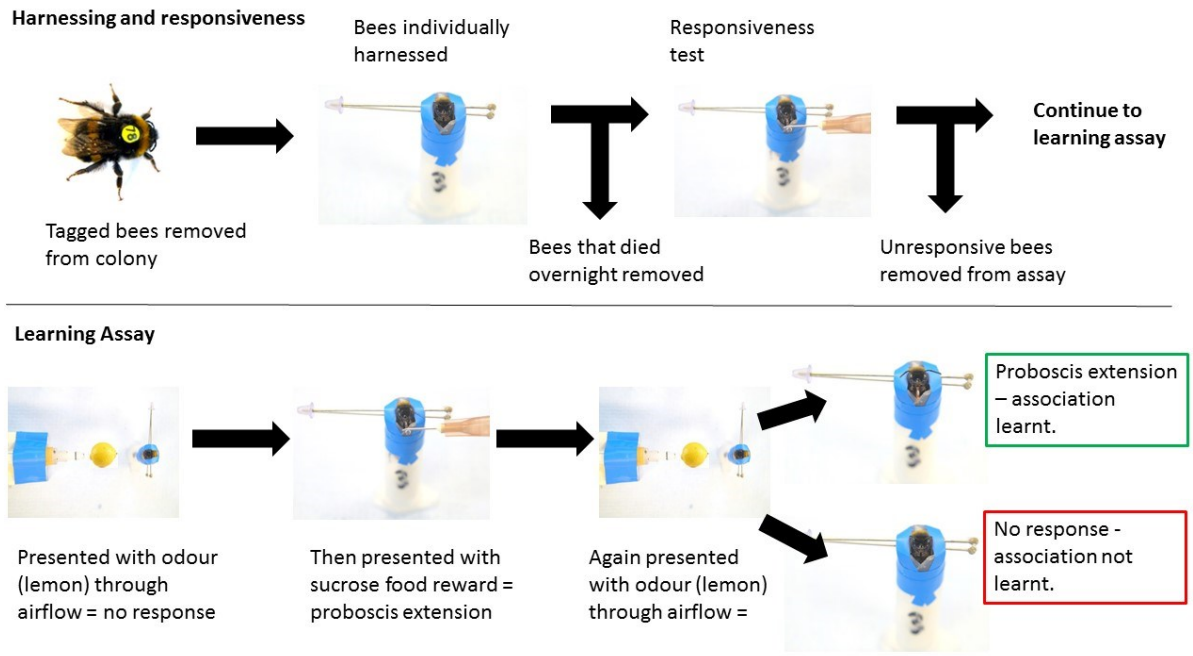


Figure 2.4 Proboscis extension reflex assay setup and step-by-step guide. Individuals were placed inside a plastic test tube and the tube was placed on ice for 10 minutes. Individuals were then harnessed in modified 2ml centrifuge tubes and a split pin yoke held them in place with electrical tape (blue). Harnessed bees were always placed the same distance from the air flow odour source and an extractor fan was mounted behind to remove excess odour.

The PER conditioning test involved each harnessed bee being positioned in front of a filtered ventilation system to stop any build-up of odour in the testing area preventing neighbouring harnessed bees or subsequent trials to be affected. First, each bee was conditioned by presenting a channelled flow of clean air for 5secs and then air mixed with a lemon odour for 10secs regulated at an air flow rate of 80 ml/sec (Figure 2.3). The air was channelled using an odour tube pointed at the bee and positioned 3cm away, with a Solenoid valve (Nass Magnet 108-030-0257 24vAC/12vDC) allowing us to switch between a tube with clean air flow or a tube containing a piece of filter paper with 1µl of lemon odour applied (lemon essential oil, Naturally Thinking Ltd.). For the bee to associate the lemon odour with a reward I presented 0.8µl of 50% sucrose solution 6secs into the 10sec odour delivery phase, by touching their

antenna with the sucrose droplet to elicit a proboscis extension and then allowing them to feed. For this initial conditioning trial, if a bee responded to the odour before the reward had been presented they were removed from the experiment.

Following a trial, each individual was allowed to rest in the conditioning arena for 15–25 seconds to allow for initial memory formation before being removed and the next individual put in place. The odour and reward presentation phase was repeated to each bee as described above over another nine consecutive trials with an inter-trial interval (ITI) per individual of 10 minutes, allowing for multiple bees to be trained in the same session. Ten conditioning trials were used to optimally balance the number of individuals that could tested within each session and thus overall, with the number of trials required for bumblebees to learn based on previous studies which indicated most bumblebees will learn the association within 10 conditioning trials (Riveros and Gronenberg 2009). A Raspberry Pi 2, Model B computer was connected to the air source allowing us to ensure precise stimulus delivery (in terms of air volume and time period). I recorded whether the bees showed a PER to the odour stimulus prior to or after the reward, which were defined as a learnt or non-learnt response respectively. A PER response before receiving the reward meant the workers was exhibiting an olfactory conditioned learnt response, and this allowed us to assess the proportion of leaners (workers showing at least one learnt response), identify how many trials were required before the first learnt response was exhibited, and provide a learning score which was the total number of learnt responses out of the nine trials. If bees showed no response after the reward was provided and did this three times concurrently, the individual was removed form testing.

2.2.5 Data Analysis

Statistical analyses were conducted in R version 3.0.1 (R Development Core Team 2014) using RStudio version 1.0.143, with mixed effects model using the lme4 package (Bates, Mächler et al. 2015). For responsiveness, learning and learning level the data was analysed using binomial generalized linear mixed models (GLMMs). In addition to treatment as a fixed factor, I included worker size (inter-tegula width) to consider any disproportionate effect on small or large workers (Samuelson, Chen-Wishart et al. 2016). Colony was incorporated into the model as a random factor. When considering repeated measures for learning level, worker ID was nested within colony unless a stepwise reduction increased the best fit of the model based on AICc criteria. Models (GLMMs) for responsiveness and learning were also performed for comparisons between treatments for 12-day workers separately. Mean values provided in the text for responsiveness, learners and learning level are back transformed from model outputs (additional averages values from raw data are provided in Data Tables and Appendicies). For Figure 2.5, all panels represent model means, with bayseian credible intervals (BCI) (see Appendix 2.4 for learning curves with BCI).

2.3 RESULTS

2.3.1 Initial Analyses – sucrose consumption, thorax width and sample sizes

Of the total provisioned sucrose (1,110ml per colony over the duration of the experimental period), *control, pre-eclosion, post-eclosion* and *continual* treatments consumed a median (IQR) of 54 (50-56), 47 (37-57), 61 (48-69) and 51 (35-63) % respectively (Appendix 2.1 for daily consumptions rates). The mean (range) thorax width of workers was similar between treatments (LMM, $p>0.07$) with control being 4.23mm (3.29-5.17), developmental 4.16mm

(3.09-5.12), adult 4.28mm (3.14-5.63) and continual 4.33mm (3.36-5.34). In total, 445 bees were harnessed in prepartion for the PER assay, with 413 bees (*control* = 110; *pre-eclosion* = 116; *post-eclosion* = 108; *continual* = 79) surviving overnight to be tested (Table 2.3).

2.3.2 Responsiveness of workers

I first tested the PER response of workers to the stimulation of their antennae by a 50% sucrose solution droplet (Figure 2.4). For 3-day adult workers (n = 225 see Table 2.3), relative to the proportion of responsive c*ontrol* exposure workers (mean = 0.51), I found a lower proportion from *pre-eclosion* exposure (0.40)*,* and a signficantly lower proportion from *post-eclosion* (0.29; z-2.53, p=0.039) and *continual* (0.31; z-2.03, p=0.014). For 12-day workers (n = 188), *control* exposure workers again showed the highest proportion (0.63), with a lower proportion in *pre-eclosion* (0.55), *post-eclosion* (0.51) and *continual* (0.49), although this was not signficantly lower than the *control*. Indeed, I found no signficant effect of age or any age × treatment interaction on responsivness (Figure 2.5a; Table 2.3; Appendix 2.2 and 2.3).

2.3.3 Learners

Responsive bees were then tested over 10 consecutive trials on their ability to learn to associate a lemon odour with a sucrose reward by demonstrating a PER response (N.B. by definition a worker could not learn on the first trial; see Methods; Figure 2.4), classifying any worker forming an association between the two stimuli (exhibiting at least one learnt response) as a 'learner'. For 3-day workers (n = 80), *control* exposure showed the highest proportion of learners (mean model estimate = 0.45), compared to *pre*-eclosion (0.23)*, post-eclosion* (0.25) and *continual* (0.19), although treatments were not signficantly lower relative to *control.* Focusing on *control* exposure workers I found a significant positive effect of age on the proportion of learners (z=2.13, p=0.033), demonstrating age-enhanced learning

capability. However this did not hold true for p*re-eclosion* exposure workers, where I detected a significant treatment*age effect on age-enhancement (z=-2.19, p=0.029) as evidenced by the proportion of 12-day 'learners' actually being lower than 3-day 'learners' from this treatment, demonstrating an impeded age-enhancement. Addtionally, I found consistent negative model estimates for the age*treatment interaction for p*ost-eclosion* and *continual* exposure workers (Figure 2.5b; Table 2.3; Appendix 2.2), showing a lower degree of age enchancement across all pesticide exposure treatments relative to *control*.

Next looking solely at 12-day workers (n = 97), the proportion of learners from all three exposure treatments was significantly lower (0.13, 0.24 & 0.41) relative to *control* exposure (0.75; z≥2.005, p≤0.045; Figure 2.5b; Appendix 2.3, indicating a more pronounced effect of the treatments in older workers.

Table 2.3 Proportion of responsive individuals and individuals showing a learnt response. Across all individuals: mean proportion across all tested workers regardless of colony. Per colony: mean proportion per colony over all colonies per treatment.

Responsive				
Treatment	Model Mean Estimate	Across all individuals	Mean per colony	Median per Colony (IQR)
3-day				
Control	51	53	54	53 (43-66)
Pre-eclosion	40	41	41	41 (33-50)
Post-eclosion	29	30	34	35 (22-47)
Continual	31	34	36	36 (20-51)
12-day				
Control	63	67	69	66 (55-87)
Pre-eclosion	55	57	50	57 (30-74)
Post-eclosion	51	55	48	50 (30-67)
Continual	49	53	64	75 (23-100)
Learners				
Control	45	48	45	41 (29-64)
Pre-eclosion	23	23	16	13 (0-35)
Post-eclosion	25	24	15	0 (0-43)

Continual	19	21	18	0 (0-46)
12-day				
Control	75	75	70	78 (38-95)
Pre-eclosion	13	10	7	9 (0-14)
Post-eclosion	24	26	22	17 (6-41)
Continual	41	44	44	38 (17-75)

2.3.4 Learning Level

For learners, I then looked at the proportion of learnt responses across the 10 trials for each indivdual per treatment. This comparative analysis, however, should be taken with some degree of caution as many individuals had been excluded from the experiment by this stage (See Table 2.4). Overall, I found that for 3-day workers (n=23), the proportion of learnt responses for all three pesticide exposure treatments (mean model estimates: *pre-eclosion* = *0.38*, *post-eclosion* = 0.35, *continual* = 0.30) showed no signficant difference relative to the *control* (0.28; Figure 2.5c; Appendix 2.2). However, for 12-day workers (n=35), and relative to to the *control* (0.48), I found a non-significant trend of a lower proportion of learnt responses for *pre-eclosion* (0.26; z=-1.84, p=0.066) and a significantly lower proportion of learnt responses for *post-eclosion* (0.23; z=-2.06, p=0.039), whilst this was also lower but a non-significant trend, for *continual* (0.31; Figure 2.5d; Appendix 2.2 & 2.3).

As found for the proportion of learners, by first focusing on *control* exposure workers I found a positive significant effect of age on the proportion of learnt reposnses (z=2.15, p=0.032) demonstrating an age-enhancement. Moreover, I again found a negative interactive effect of pesticide exposure on this enhancement, with *post-eclosion* exposure workers showing a signficant age*treatment interaction (z=-2.06, p=0.039), and *pre-eclosion* and *continual* showing consistent negative model estimates (Figure 2.5d; Appendix 2.2).

Table 2.4 Data summarised from the learning level analysis. Number of workers at each stage: Harnessed for PER, total=437; Responsive bees that performed learning assay, total=204; Learners whose learning score was assessed, total=59.

	PER	**Responsive**	**Learners**
3-control	61	31	11
12-control	56	35	18
3-developmental	64	26	6
12-developmental	57	33	3
3-adult	60	18	4
12-adult	54	29	7
3-continued	50	16	3
12-continued	35	16	7

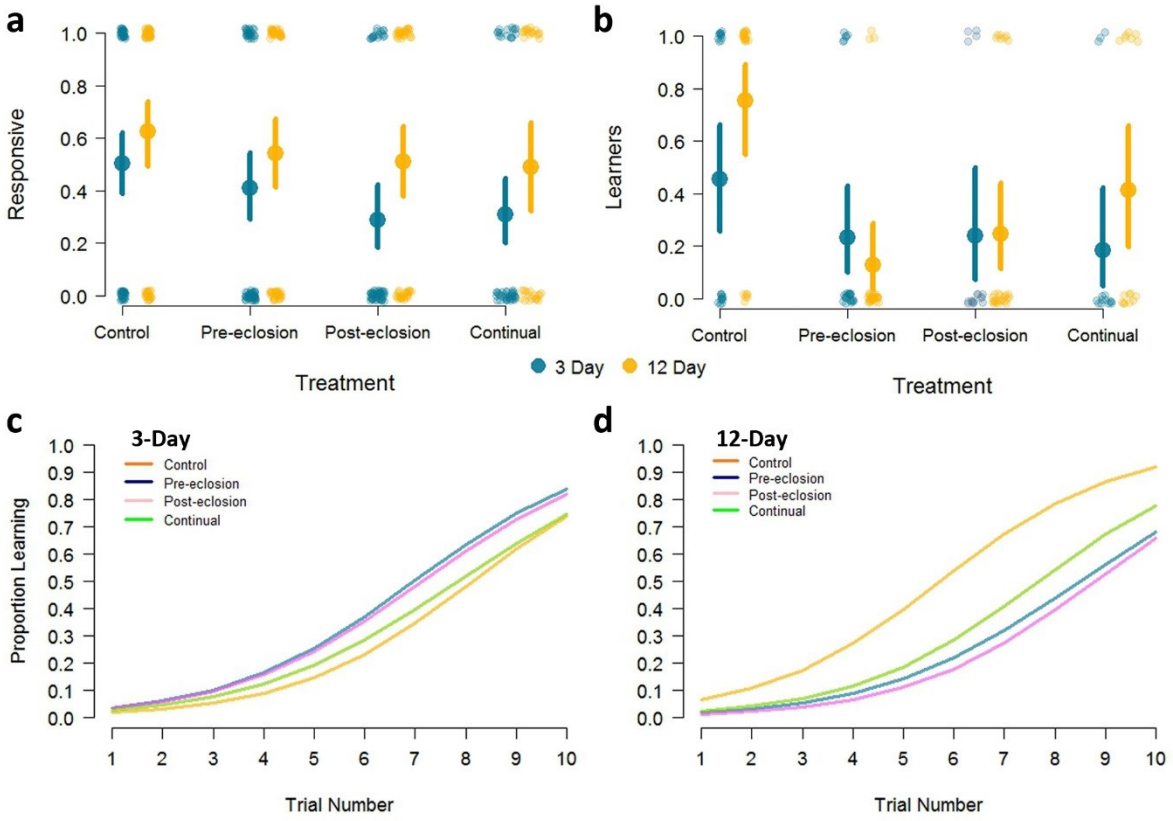


Figure 2.5 Comparisons between treatments of responsiveness and olfactory learning performance using proboscis extension reflex (PER) conditioning. a, the proportion of bees that responded to a sucrose solution droplet before PER testing; b, proportion of responsive workers that then exhibited a learnt response during the PER assay. The bars depict ±95% Bayesian credible intervals (BCI) with the intersecting circular point representing the estimated model mean taken from back-transformation of the Binomial GLMM. The semi-transparent, jittered circular points at the top and bottom of the graph, correspond to the raw binary data. c and d, the estimated probability of a worker showing a learnt response per trial, with trials 2-10 considered in the model. c, 3-day and d, 12-day adults are shown, with lines representing the mean model fit by treatment from a Binomial GLMM.

2.4 DISCUSSION

In this chapter I investigated how exposure to field-realistic levels (5ppb) of a neonicotinoid pesticide during key developmental stages (brood and early adult development) affects the olfactory learning performance of worker bumblebees at different ages (3- and 12-day). The evidence shown in this chapter indicates that worker bumblebees reared inside neonicotinoid contaminated colonies can suffer from impaired task performance later in life. Revealing that adult olfactory learning performance is affected by exposure during either brood or early-adult development and that the age-enhancement observed in learning performance can be impeded.

2.4.1 Pesticide exposure during early adulthood affects responsiveness to the provisioned sucrose solution droplet

First, I assessed the responsiveness of workers to sucrose, with non-responsive individuals omitted from the learning assay. I found that older (12-day) bees were no more likely to respond to 50% sucrose than younger (3-day) bees and that the overall proportion of responsive bees (when considering *control* treatment, Table 2.3) was similar to that observed previously for harnessed *Bombus terrestris* (Mommaerts, Wäckers et al. 2013). Considering the effects of pesticide exposure, I found that 3-day workers from both *post-eclosion* and *continual* colonies had reduced responsiveness, suggesting that exposure to imidacloprid during early adulthood post-emergence can affect the ability of younger bees to respond to sucrose. However, this level of reduced responsiveness was not seen in workers exposed only during brood development (*pre-eclosion*) which were not strongly impaired. Interestingly, 12-day workers in each pesticide treatment group showed no effects on responsiveness

compared to *control*, suggesting bees may be able to overcome these initial impairments in responsiveness as they mature. This study allowed me to demonstrate an effect of neonicotinoid exposure on sucrose responsiveness in bumblebees. It has been reported that the neonicotinoid Thiamethoxam affected responsiveness to high sucrose concentrations (3%, 10% and 30%) in honeybees (Aliouane, el Hassani et al. 2009, Démares, Crous et al. 2016) although as bumblebees differ in their sensitivity to sugar concentration (de Brito Sanchez, Chen et al. 2008, Mommaerts, Wäckers et al. 2013) and sugar preference (Waller 1972, Pouvreau 1974) it could be expected that they are differentially affected by neonicotinoid exposure.

Similar to the studies on honeybees, bumblebees in this study assessed on their ability to respond to a relatively high concentration (50%) of sucrose did exhibit reduced responsiveness when exposed to a neonicotinoid. However, whereas honeybees showed equally impaired responsiveness with age (7-day and 14-day bees) older bumblebees showed lesser effects compared to younger (3-day) individuals. These differences are perhaps driven by the comparatively higher sucrose concentration used in my study, and a more rigorous investigation on bumblebees considering different sucrose concentrations would be interesting to assess whether the ability to detect lower concentrations is affected, or whether this difference disappears under different concentrations. If bumblebees exposed to neonicotinoid pesticides suffer reduced sucrose responsiveness they may require even higher concentrations of sugars than usual to be stimulated to feed or given the importance of sucrose concentration in flower choice (Cnaani, Thomson et al. 2006) it could alter their foraging preferences (Pankiw, Waddington et al. 2001, Scheiner, Barnert et al. 2003, Scheiner, Page et al. 2004). Furthermore Impaired sucrose responsiveness could have significant knock

on effects for foraging performance due to its influence on learning ability (Scheiner, Page et al. 2001, Scheiner, Barnert et al. 2003, Perez, Rolland et al. 2013)

2.4.2 Pesticide exposure during early development impedes age-enhanced learning capability

The proportion of workers that exhibited an associative olfactory learning response was consistently lower for all three pesticide exposure treatments compared to *control* across both age cohorts. When considering 3-day workers, *pre-eclosion* workers (exposed during brood development) were as similarly impaired as *post-eclosion* and *continual* exposed workers. This similarity in adult learning behaviour between the exposure treatments suggests that exposure during brood development can translate to effects comparable to adult exposure in young bees. It also indicates that despite *pre-eclosion* workers experiencing up to 3-weeks of exposure as brood, throughout their larval and pupal development, their capability to learn was similar to that of an adult exposed for just the first three days after eclosion - signifying the first three days following emergence as a developmental window particularly susceptible to environmental stress.

The cohort of 12-day workers were found to be significantly more likely to exhibit a learnt response than 3-day workers, with this age enhancement also evident in the proportion of learnt responses across all trials. This is in contrast to what has previously been reported for bumblebees (Riveros and Gronenberg 2009, Riveros and Gronenberg 2009, Smith and Raine 2014) where age had no effect on the ability to learn, although it must be noted that individuals in these studies had access to foraging arenas. Given that foraging experience is also associated with learning performance (Riveros and Gronenberg 2009), perhaps an innate age enhancement in learning ability can be masked by the initial sensory experience or first

foraging very early after eclosion. Considering that workers were reared under a constant environment, restricted to the colony nest box, with no foraging experience and levels of sensory input were limited, would suggest that my finding is likely to be largely explained by an experience-independent age-enhancement in learning. If not experience dependent, the enhancement in learning capability between 3 and 12 days reported here may be related to the age dependent changes in brain volume shown to occur between these ages (Jones, Leonard et al. 2013). It is therefore concerning that I found a consistent negative interactive effect of pesticide exposure on age, with 12-day workers from all pesticide treatments showing impeded enhancement as evidenced by a decreased likelihood of learning. Furthermore, even for the pesticide exposed 12-day workers that did learn, individuals showed a lower proportion of learnt responses (relative to *control* exposure workers).

Impaired olfactory associative learning behaviour in bumblebees from chronic exposure to a neonicotinoid pesticide during adulthood has been reported before, although using foraging workers of unknown age (Stanley 2015). However, the effect of *pre-eclosion* (brood) exposure on the learning ability of 12-day workers shown in the study presented here provides important insights regarding the hazard posed by pesticide residues entering social bee colonies, and as a route leading to long term colony effects. Furthermore, the effects of *pre-eclosion* exposure reported here conforms to previous studies on other eusocial bee species, honeybees (*Apis mellifera*) and stingless bees (*Melipona quadrifasciata anthidioides*) in which larvae reared in under topical and oral neonicotinoid exposure exhibited negative effects on adult learning and motor function behaviour, respectively (Tomé, Martins et al. 2012, Yang, Chang et al. 2012, Tan, Chen et al. 2015). But perhaps more worryingly, is that comparing 3 and 12-day workers reveals that the effects of impaired learning performance from brood

exposure reported in my study, may be irreversible, as despite these individuals no longer being exposed to pesticides during their adult life in this treatment (or at least substantially less, as nectar stores can sometime last a few days (Heinrich 1979)), this study observed no learning enhancement in 12-day compared to 3-day *pre-eclosion* exposure workers. This indicates a high level of vulnerability of developing brood in bumblebee colonies and that even limited exposure for part of the colony life cycle may result in a behaviourally deficient workforce.

Chapter 3

A METHODOLOGY FOR NON-DESTRUCTIVE EXPLORATION AND ANALYSIS OF BUMBLEBEE BRAINS USING MICRO-CT SCANNING TECHNIQUES

3.1 INTRODUCTION

Despite their small size, insect brains are capable of rapidly detecting and responding to a plethora of diverse stimuli in a wide range of sensory modalities, facilitating their global ecological success and establishing them as an essential model system for cognitive biology and neuroscience (Wehner 2003, Collett, Graham et al. 2006, Srinivasan 2010, Chittka and Skorupski 2011, Warrant and Dacke 2011, Menzel 2012). And although much smaller and simpler than their vertebrate counterparts, the brains of insects such as social bees bestow an impressive display of cognitive performance (Chittka and Geiger 1995, Giurfa, Eichmann et al. 1996, Giurfa, Zhang et al. 2001, Dyer, Neumeyer et al. 2005, Boisvert and Sherry 2006, Collett, Graham et al. 2006, Leadbeater and Chittka 2007, Dacke and Srinivasan 2008, Chittka and Niven 2009, Avarguès-Weber, Deisig et al. 2011, Sheehan and Tibbetts 2011, Lihoreau, Raine et al. 2012, Collett, Chittka et al. 2013, Giurfa 2013, Lihoreau, Raine et al. 2013). Therefore, knowledge of insect brain structure allows us to understand how comparatively small (and simple) brains can generate complex patterns of behaviour and act as a gateway to understanding more complex brains and their evolutionary development (Menzel and

Giurfa 2001, Wehner 2003, Greenspan and van Swinderen 2004, Chittka and Niven 2009, Chittka and Skorupski 2011). Importantly and in the context of this thesis, the capacity to explore small soft tissue structures in detail is vital to understanding the link between animal physiology and behavioural features like learning, and how exposure to stressors in the environment influence this relationship.

Variation in the volume of brain regions in insects - as traditionally examined using histological techniques - has been reported to be linked to differences in innate responses to stimuli (Julian 2002), age/experience related behavioural transitions (Durst, Eichmüller et al. 1994, Withers, Fahrbach et al. 1995, Riveros and Gronenberg 2010), behavioural syndromes (Molina and O'Donnell 2007, Jandt, Bengston et al. 2014) and rates of learning and performance in cognitive tasks (Riveros and Gronenberg 2009, Gronenberg and Couvillon 2010). Yet, there remains much to discover about how insect brain structure and functional complexity determines the diversity of behaviours observed in individuals (Chittka and Niven 2009, Abbott 2013, Jandt, Bengston et al. 2014). Closing such a fundamental knowledge gap requires the development of new imaging protocols and the application of novel strategies to measure, record and robustly quantify aspects of brain morphology across multiple individuals.

Past investigations of brain structure, and its role in determining specific behavioural traits, have propelled critical developments in imaging methodology, such as magnetic resonance imaging (MRI) and X-ray computed tomography (CT). For example, CT scanning has highlighted that variation in volume, shape and density of particular human brain regions can be correlated with phenotypic syndromes and diseases (Shear, Sullivan et al. 1995, Honea 2005). The implementation of such technologies to the study of smaller organisms with tiny brains (such as insects), however, is notably lacking (Holdsworth and Thornton 2002,

Metscher 2009, Schambach, Bag et al. 2010). A major barrier to progress in this area is that imaging at this minute scale is a more challenging task due to problems with low image resolution and the practicalities of manipulating, preparing and observing miniature composite structures (Miklos 1993, Schambach, Bag et al. 2010).

The majority of investigations that have explored insect brain morphology have used traditional histological techniques (See Tables 3.1 and 3.2 for a list of representative studies). Producing 2D images with these techniques requires invasive dissection for image preparation followed by relatively time-consuming fixing and physical tissue slicing using a microtome (Friedrich and Beutel 2008). Typically brain samples prepared using this method can suffer tissue distortion, desiccation and permanent damage, leading to biased measurements that impede accurate quantification of morphology (Andersen and Gundersen 1999, Dorph-Petersen, Nyengaard et al. 2001, Ju, Warren et al. 2006, Simmons and Swanson 2009). Magnetic resonance and confocal microscopy imaging have been used to study insect brains, eliminating the need for tissue slicing and/or staining, but these approaches suffer from comparatively low resolution and semi-destructive treatment of the samples (Galizia, McIlwrath et al. 1999, Haddad, Schaupp et al. 2004, Jenett, Schindelin et al. 2006, Rybak, Kuss et al. 2010). The development and application of micro-CT to investigate the small brains of insects is a significant milestone towards collecting unprecedented data and insight to brain structure (Brandt, Rohlfing et al. 2005, Ribi, Senden et al. 2008, Zhang, Li et al. 2010, Greco, Tong et al. 2012) and offers the potential for studies linking variation in brain structure with behavioural differences. The study by Ribi *et al.* (Ribi, Senden et al. 2008) showcased the use of micro-CT in exploring insect tissues, specifically in the honeybee (*Apis mellifera*) brain. After removing the head musculature and salivary glands, the authors used an osmium-based staining method to enhance contrast and showed that the main brain structures could be

distinguished from other tissues. Further studies have shown that the integration of brain reconstructions can provide a powerful method for producing structural atlases of standardised insect brain maps that facilitate fast, semi-automatic analysis (region annotation, segmentation and volume extraction) of multiple individuals (Miklos 1993, Rohlfing, Brandt et al. 2001, Rohlfing, Brandt et al. 2004, Brandt, Rohlfing et al. 2005, Jenett, Schindelin et al. 2006, Kvello, Løfaldli et al. 2009, Rybak, Kuss et al. 2010, Schambach, Bag et al. 2010). While Ribi *et al.* (Ribi, Senden et al. 2008) provided a proof-of-concept that high resolution imaging of insect brains is possible, brain atlases to date have typically been constructed using lower resolution and invasive techniques. Therefore, developing protocols to apply high-resolution imaging (such as micro-CT) coupled with segmentation, for quantitative volumetric and 3D morphological analysis, would improve our ability to understand the intricate details of brain morphology, and link this structural variation with organismal function through comparative analyses both within and among species, across a range of ontologies and life histories.

(Mares, Ash et al. 2005)	Study
Bombus impatiens and *Apis mellifera*	**Species**
Decapitated, head capsule cut open frontally, brain dissected out under fixative (4% formaldehyde and 0.1% picric acid in phosphate buffer, pH 6.8) and fixed for 3 hours. Rinsed in four repeated changes of buffer, stained in 1% aqueous osmium tetroxide solution for 2 hours at 4°C and for one additional hour at room temperature. Next, brains rinsed in water for 4 hours, dehydrated in acidified 2,2-dimethoxypropane [Thorpe and Harvey, 1979], plastic-embedded (Fluka, Durcupan) and polymerized at 65°C. Sectioned on a sliding microtome.	**Sample Preparation**
1% aqueous osmium tetroxide	**Stain**
10-20µm	**Slice Thickness**
Area measurements multiplied by the section thickness and number of sections. Every second section (20-um sections) or every third section (10-um sections) was traced and measured	**Volume Calculation**

(Gronenberg and Couvillon 2010)	**(Riveros and Gronenberg 2010)**
Apis mellifera	*Bombus occidentalis*
Heads embedded in wax, opened frontally and brains dissected under 50% ethanol, then transferred to water and block-stained in toluidine blue (1% toluidine blue in 1% aqueous borax solution) overnight at room temperature on a rotator. The staining then differentiated in water for 3–6 hours, transferred to 50% ethanol and then dehydrated in acidified 2,2-dimethoxypropane (Thorpe & Harvey, 1979), embedded in Spurr's low viscosity embedding medium (Electron Microscopy Science; Hatfield, PA) and polymerized at 70°C. Brains sectioned on a sliding microtome.	Dissected from head capsule, fixed in 4% phosphate-buffered formaldehyde for 2 hours, then rinsed in phosphate buffer. Brains then stained in the dark using 1% osmium-tetroxide for 2 hours at 4°C and an additional 30 minutes at room temperature. After repeated rinses with distilled water, dehydrated brains with 50% ethanol (10 min), acidified 2,2-dimethoxypropane [10 min; Thorpe and Harvey, 1979] and acetone (10 minutes). Next, brains plastic-embedded (Spurr's low viscosity medium, RT 14300 Electron Microscopy Sciences) and sectioned on a sliding microtome.
1% toluidine blue in 1% aqueous borax solution	1% osmium-tetroxide
20µm	10-20µm
Area measurements multiplied by section thickness. Every second section was measured, probing the brains at 40µm intervals.	Multiplying the area of each region by the section thickness. Outlined and measured every second (20µm) or every third (10µm) section.

(Tomé, Rosi-Denadai et al. 2014)	(Jones, Leonard et al. 2013)
Melipona quadrifasciata anthidioides	*Bombus impatiens*
Brains were dissected in insect physiological solution; the tissues then fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 hours. Fixed samples then rinsed in 0.1 M phosphate-buffered saline (PBS), dehydrated in an increasing series of ethanol concentrations (70–100 %), and embedded in JB4 historesin (EMS) according to the manufacturer's instructions. Brains were subjected to serial sectioning using a glass knife on an automatic microtome.	Decapitated and removed mandibles & part of each eye to allow fixative to penetrate. Brains fixed in head capsule in 4% formaldehyde in cacodylate buffer (pH 6.8) overnight on a rotator. After fixation, brains rinsed with and stored in cacodylate buffer at 4°C. Brains dissected from head capsule, stained in the dark with 1% aqueous osmium tetroxide for 2 hours on ice, then additional 30 minutes at room temp. Rinsed with distilled water, then dehydrated using 50% ethanol, acidified 2,2-dimethoxypropane, and acetone for 10 minutes each. Next, brains were plastic-embedded in Spurr's low-viscosity medium. Blocks polymerized at 65°C for 12 hours, sectioned on a sliding microtome.
Hematoxylin and eosin	1% aqueous osmium tetroxide
7µm	10 or 15µm
Measurements performed at each of six-section intervals, following Cavalieri's method.	Multiplying area by the section thickness. Every other section (15-µm section thickness) or every third section (10-µm section thickness) was measured.

Study	Species	Sample Preparation	Stain	Slice Thickness	Volume Calculation

Table 3.1 Studies of bee brain volumes using a variety of traditional histological techniques for sample preparation and staining.

Table 3.2 Studies investigating correlations between insect brain morphology, behaviour and performance.

Study	Species	Brain Structure(s)	Findings
(Withers, Fahrbach et al. 1993)	*Apis mellifera*	Mushroom bodies	Mushroom body volume increased with foraging experience
(Durst, Eichmüller et al. 1994)	*Apis mellifera*	Mushroom body calyces	Experience dependent volume changes of calyces sub-compartments (lip and collar) in foragers compared to nurses and newly emerged bees
(Winnington, Napper et al. 1996)	*Apis mellifera*	Antennal lobes	Behaviour and task dependent volumetric changes of antennal lobes
(Sigg, Thompson et al. 1997)	*Apis mellifera*	Antennal lobes	Olfactory learning performance increased with increased antennal lobe volume and an activity dependent volume increase in antennal lobes
(Fahrbach, Moore et al. 1998)	*Apis mellifera*	Mushroom bodies	Mushroom body exhibited experience-expectant volume increase independent of light stimulus and social interaction
(Julian 2002)	*Messor pergandei* and *Pogonomyrmex rugosus*	Medulla, lobula and mushroom bodies	Decreasing phototaxis correlated with decreased medulla volume
(Withers, Day et al. 2008)	*Osmia lignaria*	Mushroom bodies	Mushroom body volume increased with foraging experience
(Gronenberg and Couvillon 2010)	*Apis mellifera* European and Africanized Honeybee	Mushroom bodies and lobula	Mushroom body volume increased with olfactory learning performance but lobula volume correlated negatively with olfactory learning performance
(Riveros and Gronenberg 2010)	*Bombus occidentalis*	Mushroom body, MB calyces and antennal lobes	Foraging experience did not correlate with mushroom body total volume or volume of calyces but did positively correlate with volume of the medial calyx. Found no experience-dependent volume increase of the antennal lobes
(Jones, Leonard et al. 2013)	*Bombus impatiens*	Mushroom bodies and antennal lobes	Found that bee deprived of visual stimuli for 7 days had increased mushroom body and antennal lobe volume than bees exposed to visual stimuli

In developing a new Micro-CT and image analysis protocol I considered two principle aims: First, to provide a relatively cost-effective and time-efficient toolkit to enable a wide-range of researchers to

explore intra- and inter-individual variability of soft tissues (in this case brain structure), and their links to organismal phenotypes (minimizing sample preparation artefacts), which can be applied to both insects and other invertebrate taxa. Secondly, to provide a methodology with which to investigate whether exposure to specific stressors, such as disease or agrochemicals, can significantly affect brain development, such as volumetric growth and shape, for experimentally sampled subjects.

With these stated aims I have developed a methodology to allow exploration of the composite structure of small-scale soft tissues without disturbing the insect brain within the head case, hence retaining its natural stereo-geometry and minimising any potential tissue destruction. Through exploring the brain of a common European bumblebee (*Bombus terrestris*) I outline a relatively easy-to-use protocol for high resolution micro-CT scanning, and couple this with the application of open source (freeware) visual analysis software SPIERS to generate non-destructive, 3D reconstructed brain images at relatively high resolution (achieving a standardised 4.6µm voxel size per scan – an improvement on 7µm reported by previous studies (Ribi, Senden et al. 2008)). Furthermore, the application of this method is demonstrated by segmenting and virtually extracting five of the primary brain composite structures: mushroom bodies (MBs), antennal lobes (ALs), medullas (Mes), lobulas (Los) and the Central Body (CB) (see Table 3.3 for brief description and associated studies of each structure), for 19 individual bees. Followed by quantitative volumetric measurements for each of these brain structures. This study provides an appreciably large number of samples, providing a deeper insight into the degree of variation in brain morphology and how to achieve the appropriate level of statistical power required for its subsequent application in comparative analyses.

Table 3.3 Functions assigned to the insect brain structures I focus on in this study.

Structure	Function	References
Mushroom Bodies	Associated with higher cognition and learning with the processing of multimodal sensory information. They possess distinct subcompartments of functional specialisation.	(Durst, Eichmüller et al. 1994) (Homberg 1979) (Erber, Homberg et al. 1987) (Mobbs 1982) (Fahrbach 2006)
Antennal Lobes	The principle olfactory centre associated with processing of chemical stimuli.	(Hansson and Anton 2000), (Homberg, Christensen et al. 1989)
Medullas	Processing of visual information	(Paulk, Dacks et al. 2009a) (Paulk, Dacks et al. 2009b)
Lobulas	Processing of visual information	(Paulk, Dacks et al. 2009a) (Paulk, Dacks et al. 2009b) (Paulk, Phillips-Portillo et al. 2008)
Central Body (Fan Shaped Body)	Considered to be involved in locomotion and orientation	(Strauss and Heisenberg 1993) (Strauss 2002) (Li, Pan et al. 2009)

3.2 METHODS

3.2.1 Animal husbandry, bumblebee sampling and **sample preparation**

Five bumblebee colonies (*Bombus terrestris*) were obtained from a commercial company (Koppert Biological Systems, the Netherlands). Upon arrival, colonies (each containing a queen and a mean of 95.4 workers (range = 71-127)) were transferred to wooden nest boxes within 24 hours. All colonies were then provided with *ad libitum* pollen and sucrose solution (40/60% sucrose/water). To understand the standing variation in brain morphology and to consider the accuracy of our measurements, I sacrificed individuals for brain scanning that were all of the same age – four days old. These early-adults had eclosed from their pupal case

after the colonies had spent at least 21 days (approx. time taken for a worker to be reared from egg to adult (Duchateau MJ 1998)) under the same laboratory setting. Age matched individuals were sacrificed because adult maturation, changes in social environment and increased experience can all have effects on brain morphology (Durst, Eichmüller et al. 1994, Jones, Leonard et al. 2013). I monitored colonies twice per day and marked any newly eclosed individuals using numbered Opalith tags (Christian Graze KG, Germany), which consequently allowed us to track the age of each individual throughout the sampling period.

Young bees were used for brain scanning to limit any potential changes in brain structure/size during adult development associated with allocation to different tasks in the colony. Bees were sacrificed by removing an individual from the colony using forceps, and then swiftly decapitating the live individual using a disposable surgery scalpel (Figure 3.1a). Once cut, the head was fully submerged in a 70/30% ethanol/de-ionised water solution in a 1.5ml centrifuge tube and stored at 5°C until the staining process was undertaken.

The head case was prepared for staining by removing the front part of the head case (just below the antennae; see Fig. 3.1a) with a scalpel under 10x magnification (using a stereomicroscope) ensuring that I did not cut into the brain. The head was then fully submerged in the staining solution in one well as part of a multi-well cell plate. I measured thorax width for each decapitated individual, just above the tegula (Fig. 3.1b), using a set of digital callipers (accuracy = 10µm) as this has been shown to be strong estimator of body mass in bumblebees (Hagen and Dupont 2013). Thorax width measurements were taken twice per individual and the mean average taken. I sacrificed 34 bees for staining (n = 6 from one colony for the staining optimization test; n = 28 from five colonies for brain 3D reconstruction).

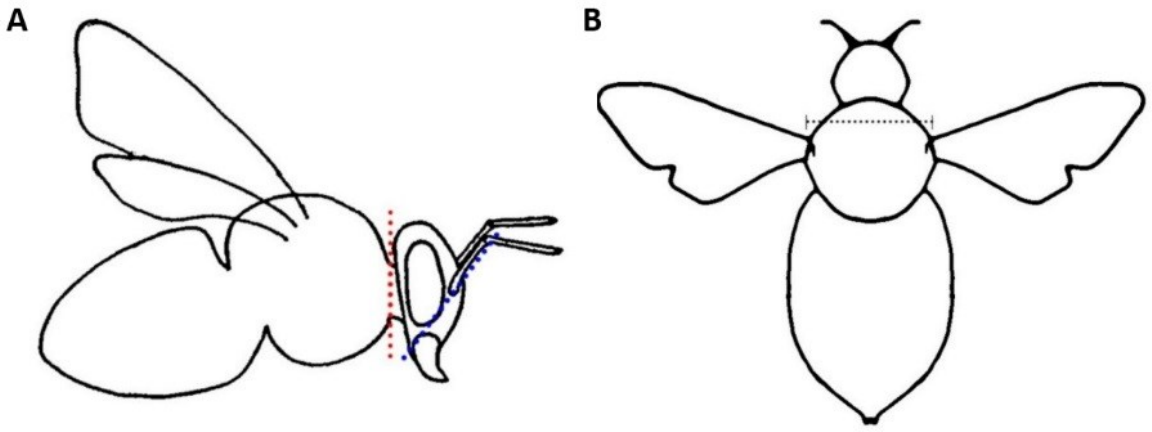


Figure 3.1 Cutting and Thorax measurements. A) Lateral view showing the cutting planes to remove the bee's head from the body (decapitation: red dotted line) and to open the head case to expose the brain for staining (blue dotted line). B) Dorsal view showing the standardised thorax width measurement (black dotted line) across the widest parts of the tegulas (insertion points for the wings on each side of the thorax). The mean thorax width across the 19 workers was 4.63 mm (range = 3.61 – 5.61).

3.2.2 Selecting staining conditions

Measures of contrast enhancement to view staining success have been used for imaging animal soft tissue in previous studies (Metscher 2009a) although each have employed differing stains, techniques and preparation. In order to develop an accurate and repeatable protocol for visualising bumblebee brain structures, I first focused on establishing a staining procedure. There are a number of widely used tissue stains including uranyl acetate (UA) (Hyafil, Cornily et al. 2007, Seo, Lim et al. 2014), iodine (I) (Hyafil, Cornily et al. 2007, Metscher 2009a, Jeffery, Stephenson et al. 2011), phosphotungstic acid (PTA) (Metscher 2009, Metscher 2009a, Faulwetter, Vasileiadou et al. 2013) and osmium tetroxide (Ribi, Senden et al. 2008, Seo, Lim et al. 2014). For our investigation I examined the effectiveness of: i) 1% I solution (1mg/ml concentration in 70/30% ethanol/water solution); ii) saturated (0.9-1%) aqueous solution of UA; iii) 0.5% PTA solution (0.5mg/ml concentration in 70/30%

ethanol/water solution). Osmium tetroxide was excluded due to its high toxicity and cost (Bentley, Jorgensen et al. 2007, Metscher 2009a).

For each optimization test I prepared two heads per stain. Each of the six heads were individually scanned on days 1, 3, 6, 7, 8 and 9 to observe how well the stain penetrated the brain (see Appendix 3.1). *Uranyl acetate* – the brain scans indicated little staining across all nine days. As differentiation of brain regions could not be seen or was very difficult to detect I concluded this to be a poor staining method for soft brain tissue CT scanning. *Iodine* – this permeated the brain more extensively than UA and was quicker to stain tissue than PTA as it only required around 1-3 days. However, the contrast threshold for iodine was not as good, tissues were not as readily identifiable, and edges of brain structure tissues were less defined compared to PTA stained samples. Moreover, after just six days iodine began to bleed causing very blurry edges and then poor tissue differentiation. PTA showed the highest level of contrast enhancement after seven days of staining of all stains, and in comparison to Iodine, I found no evidence of stain bleeding, even after nine days. Additionally, when comparing identical phases of perfusion for each stain, PTA produced superior definition of brain regions compared to Iodine and UA. Therefore, I decided to use seven-day exposure to PTA stain based on the achievable image quality (see Appendix 3.2 and 3.3). Scans for all staining assays were performed using the following settings: 80-90kV at 100-110µA, gain 6dB, with no noise reduction and no beam hardening corrections, and was balanced by increased projections to 6284 and exposure time of 354ms to provide a suitable noise to signal ratio. The relative performance of the stains I assessed could vary under different scanner settings, therefore, additional testing of these settings could be further optimized for each stain. For example, small decreases to the voltage to align with the excitation edges for tungsten, and to increase the current to increase flux.

3.2.3 Scanning of specimens

Micro-CT scans were carried out at the *Imaging and Analysis Centre*, London Natural History Museum, using a Nikon Metrology HMX ST 225 system (Nikon Metrology, Tring, UK), with cone beam projection system, four megapixel detector panel, maximum voltage output of 225kV for the reflection target and a maximum current output of 2000µA (Appendix 3.4). The focal spot size is 5µm and the exposure ranges from 0.25-5.6 frames per second. Reconstructed data were visualised in VG Studio Max 2.1 in which samples were rotated and re-sliced along the orientation plane that gave the optimum view for segmentation (Fig. 3.2a; mean slices per brain = 377, range = 294-580).

From the 28 brains that were prepared and PTA stained for micro-CT scanning, and of the possible 308 separate structures of interest (left and right MB lobes, MB calyces, ALs, Mes and Los, and the CB), I obtained images to allow full and detailed 3D reconstruction of 297 (96.4%) of these structures. In this study I decided to use the 19 brains (17 workers, and 2 males due to an ID error) that provided us with reconstructions of all the brain structures of interest per individual (see Appendix 3.5). I scanned the seven-day stained brains by scanning two heads per scan run. This was achieved by inserting two heads inside a plastic straw, held firmly in place by rigid foam at each end and separated by tissue, before being placed in the scanner.

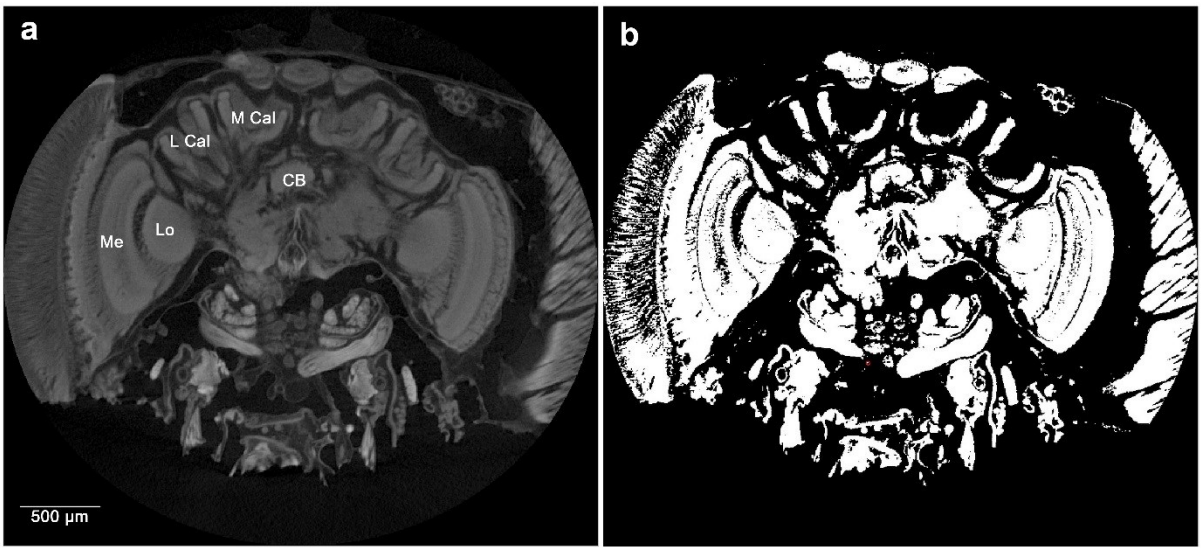


Figure 3.2 Bumblebee brain Micro-CT scan slice. Raw image a) showing optimum orientation and slice plane for viewing brain structures for segmentation, visible structures of interest: lateral calyx (L Cal), medial calyx (M Cal), central body (CB), medulla (Me), lobula (Lo), and b) the optimum threshold for segmentation of the mushroom body calyces of the same slice.

3.2.4 Image segmentation and geometric morphometric analyses

SPIERS 2.20 was run on a standard laptop computer (Samsung Series 3 Notebook 1TB HDD NP3530EC, Intel® Core™ i5-3210M CPU @ 2.50GHz, RAM 6.00 GB, Intel® HD Graphics 3000, 64-bit Operating System). SPIERS is a custom software suite made up of three independent programs: SPIERS edit, SPIERS view and SPIERS align. SPIERS was chosen ahead of other potential software based on two key and advantageous features: i) financial cost – SPIERS is freely available; ii) system requirements – SPIERS has relatively low system requirements and will run on most standard desktop and laptop operating systems. Segmentation was carried out using the SPIERS edit program (see supplementary Smith *et al*. 2016 Step-by-Step Guide (Smith, Bernhardt et al. 2016)).

For each sample, segmentation of five of the key composite structures of the brain (MBs; ALs; Mes, Los and the CB) was performed, with each MB being segmented as calyces and lobes

separately. Each individual scan was opened in SPIERS edit in the form of an 8-bit greyscale bitmap (BMP) image stack. The sequence of slices could be viewed in either greyscale or threshold form (a black and white, binary visualization of the image) with a simple interchange between the two representations. For a single scan slice showing the brain region of interest, thresholding was performed by adjusting the *Base* and *Top* values representing the range of shades to be assigned on the grey-value scale covered where 0 = black and 255 = white. The optimum threshold being that which achieved the best ratio of white, active 'on' pixels comprising the structure of interest and black, inactive 'off' pixels for the surrounding tissues. To implement our 'tracing method' I outlined the following criteria for our threshold: i) it would separate all 'background' material from the bee head tissue, ii) it would retain the identifying features of each brain structure of interest (MBs, ALs, Mes, Los and CB), iii) and it would separate each structure of interest from the tissue directly surrounding it. This was achieved by overlaying the greyscale and threshold views, adjusting the base level until the binary image fulfilled our criteria - to effectively trace the structures of interest. For the 'pixel intensity histogram' method, I based the threshold point on the base value corresponding with the top of the second peak on the working image histogram. This second peak value was applied as the constant determinant of the threshold for the second method. The optimum threshold (for each structure) was independently determined for 15 slices, at 10 slice intervals, across one brain scan to account for slight changes in second peak values across slices. This interval spacing was selected to provide a robust subsample, as each structure is typically visible in 150 scan slices (this slice interval was adjusted for those smaller structures visible across fewer slices). The average of the ranges established for each of our 15 slices was taken to obtain a single range to apply and achieve an optimum threshold, across all scan slices for each individual scan (Fig. 3.2b).

Working in this 'threshold view', a central slice - of those in which the structure of interest was visible - was selected as a starting point. To filter down to the specific brain structure of interest I drew a looped spline around the object(s) to be followed across multiple slices and used a series of landmarks (nodes) to refine the loop to match the shape of the object as accurately as possible. I copied the fitted spline to the next slice (either five slices up or down from the starting slice) and adjusted it to fit the structure outline in the new slice (using the same number of nodes). Spline adjustment was performed on every fifth slice for the region in which the structure of interest was visible. I then interpolated the splines between each start and end slice over the five slice intervals, this process estimated and positioned nodes to define a curve on each intervening slice to create an accurate transition between curve shapes for every first to fifth slice in sequence. This produced a framework around the outline of the structure of interest across all relevant slices, which was then used as the defining foundation to create a 'mask', or in other words isolating the complete structure across all slices to be used to form a complete 3D image. The mask could then be exported as an independent object for reconstruction in SPIERS view rendering software, allowing 3D viewing and manipulation of the structure.

Following image reconstruction, I applied an automated processing feature in SPIERS view (called 'island removal' combined with 'smoothing') to reduce the amount of artefactual extraneous tissue and smooth the mask to depict a more accurate representation of the structure (Figs 3.3 & 3.4; see Smith *et al*. 2016, Step-by-Step Guide for details (Smith, Bernhardt et al. 2016)). In SPIERS edit I then calculated the volume of the brain structure using a tool to calculate the number of voxels making-up each independent object (each segmented structure), which I could then multiply by the known voxel volume.

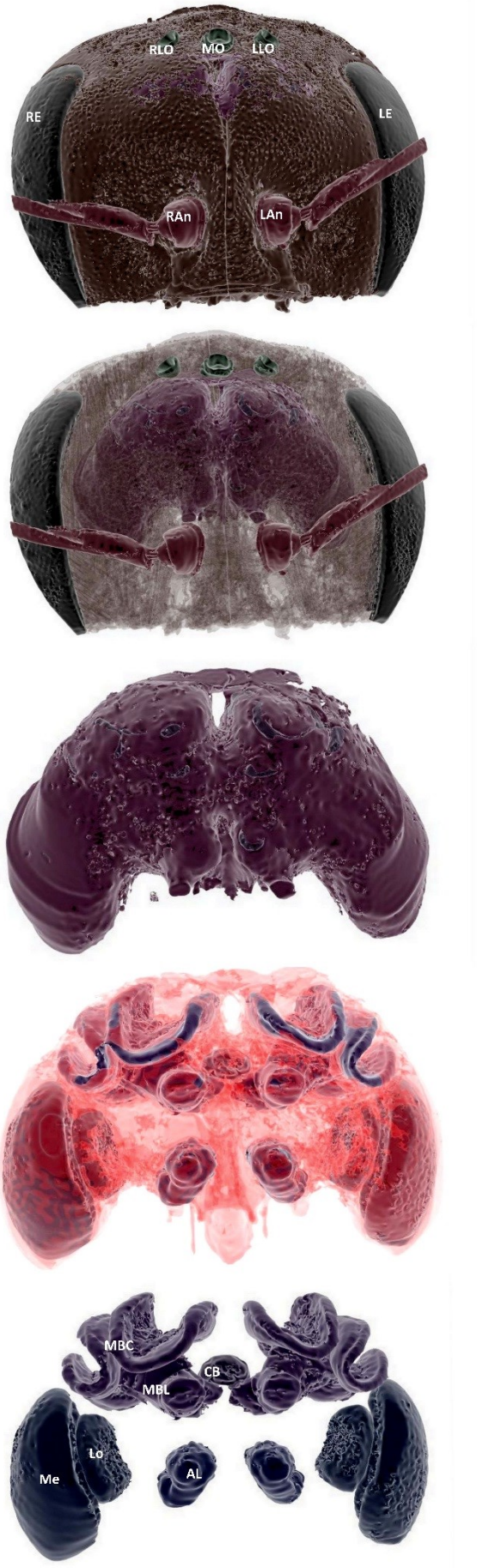


Figure 3.3 3D rendered views from micro-CT imaging of bee head case, brain and brain structures. a) External head case with independently segmented structures: right eye (RE), right ocellus (RO), right antenna (RAn). b) transparent head case showing brain in situ; c) brain tissue, virtually segmented from head case; d) transparent brain tissue showing brain structures in situ; e) individually segmented brain structures independent of additional brain tissue showing: Central Body (CB), and one of the pair of Lobulas (LO), Medullas (Me), Antennal Lobes (AL), Mushroom Body Calyces (MBC) and Mushroom body Lobes (MBL). The images show false colour application to the head and brain structures, and images **c**, **d** and **e** have been magnified 1.5x in size compared to **a** and **b**.

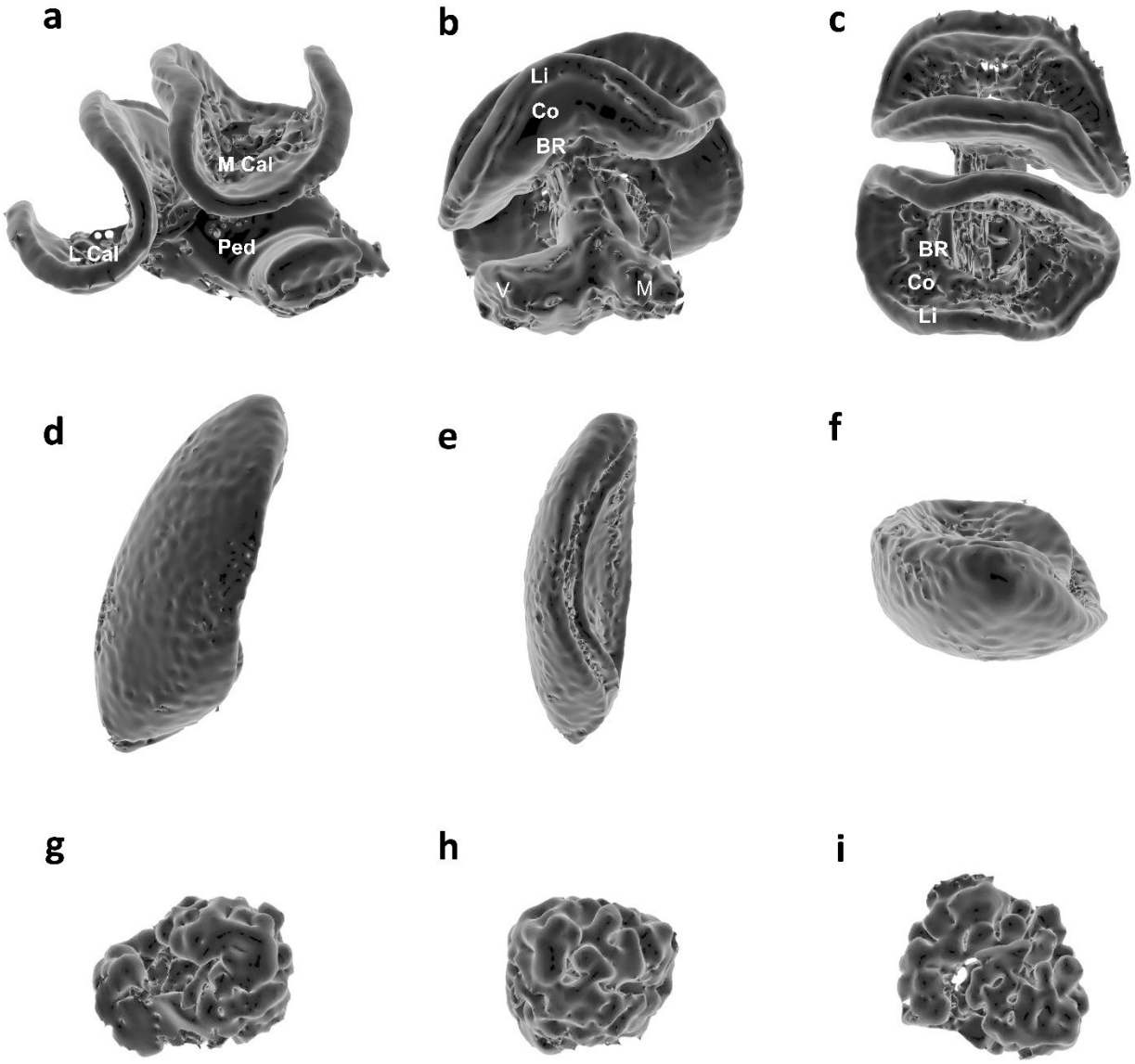


Figure 3.4 3D SPIERS View renderings from micro-CT imaging of a mushroom body (a-c), medulla (d-f) and antennal lobe (g-i). a) Right mushroom body frontal view highlighting the: lateral calyx (L Cal), medial calyx (M Cal), and Pedunculus (Ped); b) medial side view highlighting the: lip (Li), collar (Co), basal ring (BR), vertical lobe (V) and medial lobe (M); c) dorsal view; d) right medulla frontal view; e) medial side view; f) dorsal view; g) right antennal lobe frontal view; h) medial side view; i) dorsal view.

3.3 RESULTS

Working towards the objective of attaining high resolution images and precise 3D morphological measurements of insect brain morphology I addressed several of the key challenges faced by researchers when attempting to use X-rays to image small brain structures: a) sample preparation: I optimised a protocol for specimen preparation, using a non-hazardous dye and simple staining procedure, that produces images with appropriate contrast for distinguishing soft tissues of interest for downstream image analyses; b) scanning of specimens: our protocol uses settings to scan multiple individuals at once to reduce cost and machine running time while still producing high quality images; c) image segmentation and validating the biological veracity of the data: I have developed a useful, freeware-based, protocol for image analysis to segment brain regions with high confidence, capable of systematically providing precise and repeatable measures of morphological features.

Generating accurate visualisations of independent brain structures critically requires the ability to differentiate multiple tissues based on density, shown by the degree of staining that provides effective contrast enhancement. To facilitate stain perfusion throughout the brain tissues I removed the front cuticle of the bee head-case, without disturbing the brain (see Fig. 3.1a). Standardizing the staining process involved studying the dynamics of tissue staining over time (i.e. the rate at which specimen's take-up the stain, and contrast provided when repeatedly imaged). I tested the efficacy of three staining solutions previously used on insect tissues: iodine (I), uranyl acetate (UA) and phosphotungstic acid (PTA). I excluded osmium-tetroxide, used by Ribi *et al*. (2008), from the comparison as its toxicity profile precludes widespread general use. To examine the effectiveness of staining, each head was micro-CT scanned once per day for specified days over a nine-day period (see Methods). I found that seven days of PTA staining provided the best contrast enhancement and resolution of brain

structures and was thus the number of days chosen as the staining method for bee brains before scanning in this study (Appendix 3.1-3.3).

Whilst 19 of the 28 worker brains that were scanned had all structures of interest appropriately stained, the remaining nine worker brains had some structures that did not fully stain (n = 21 separate structures in total over all nine brains; see Appendix 3.5). I found that 15 of the 21 incompletely stained structures were components of the mushroom bodies (either the MB lobes or calyces). Given that mushroom bodies are the structures furthest away from the anterior sliced opening in the head-case this could suggest that the reason for poor staining could have been because there wasn't sufficient time for the stain to penetrate and perfuse throughout the brain. I found that the nine bees that were incompletely stained were significantly larger than the workers that had fully stained brains (median (IQR): 5.09 (4.73-5.38) vs 4.57 (4.22-4.94) mm; Mann Whitney: $U = 18$, $n_1 = 9$, $n_2 = 19$, $p = 0.044$). It therefore may be advisable to stain the brain for an extra day (up to 8 days staining) if the individual in question is comparatively large.

Micro-CT scans were performed using a Nikon Metrology HMX ST 225 system (Abel, Laurini et al. 2012) with a molybdenum target (Appendix 3.4). The raw data for each brain scan were reconstructed using CTPro 2.1 software (Nikon Metrology, Tring, UK) and visualised using VG Studio Max 2.1 (Volume Graphics GmbH, Heidelberg, Germany) for alignment and re-slicing of all the samples along the same optimum orientation plane (see Fig. 3.2a). For each sample I exported scan images at a standardised voxel size representing the upper limit of the range which was 4.6µm (scan resolution ranged between 3.1–4.6µm). Segmentation and volume analysis of brain structures were carried out using the freeware SPIERS 2.20 (Serial Paleontological Image Editing and Rendering System). Using SPIERS required us to develop a novel protocol to specifically segment insect brains. Individual scans were opened in the form

of an 8-bit greyscale bitmap (BMP) image stack and could be viewed in greyscale or binary form (a black and white visualization of the image; Fig. 3.2b). For segmentation, I first compared two different methods of thresholding on two brain structures (Mes and MB Calyces): i) a manual 'tracing method' judging by eye; and ii) a second method based on 'pixel intensity histograms' (see 3.2 Methods). Volumes calculated from the each of the tracing - and pixel intensity histogram methods gave almost identical results for the simpler structure of the Medullas. Seventeen of the 19 samples had exactly the same calculated volume, with a mean difference between the two methods for the 19 brains of just 0.45% (median (IQR) = 0% (0 - 0)). For the more complex mushroom body calyces 10 of the 19 structures had exactly the same volume, with a mean difference in calculated volumes between the two methods for the 19 brains being 3.8% (median (IQR) = 0% (0 - 5.5); see Fig. 3.5 & Table 3.4). Each method had its pros and cons, with the tracing method potentially suffering from subjective decisions for node placement but benefitting from making common-sense decisions as to what should constitute the tissue of interest, whereas the pixel intensity histogram provides an objective method but may include neighbouring pixels that are not part of the tissue of interest. For this paper, I used the tracing method to calculate volumes of the remaining brain structures.

I further validated the method of brain segmentation by testing the precision of our estimations. I repeated full segmentation and volumetric analysis four times for both the left and right medullas from one brain, finding low test re-test repeatability scores of 0.0016 for the left medulla (mean = 0.0745mm^3; s.d. = 0.0008, c.v. = 1.08%) and a score of 0.0011 for the right medulla (mean = 0.0751mm^3; s.d. = 0.0006, c.v. = 0.74%; see Table 3.5), showing high precision of our volumetric estimates.

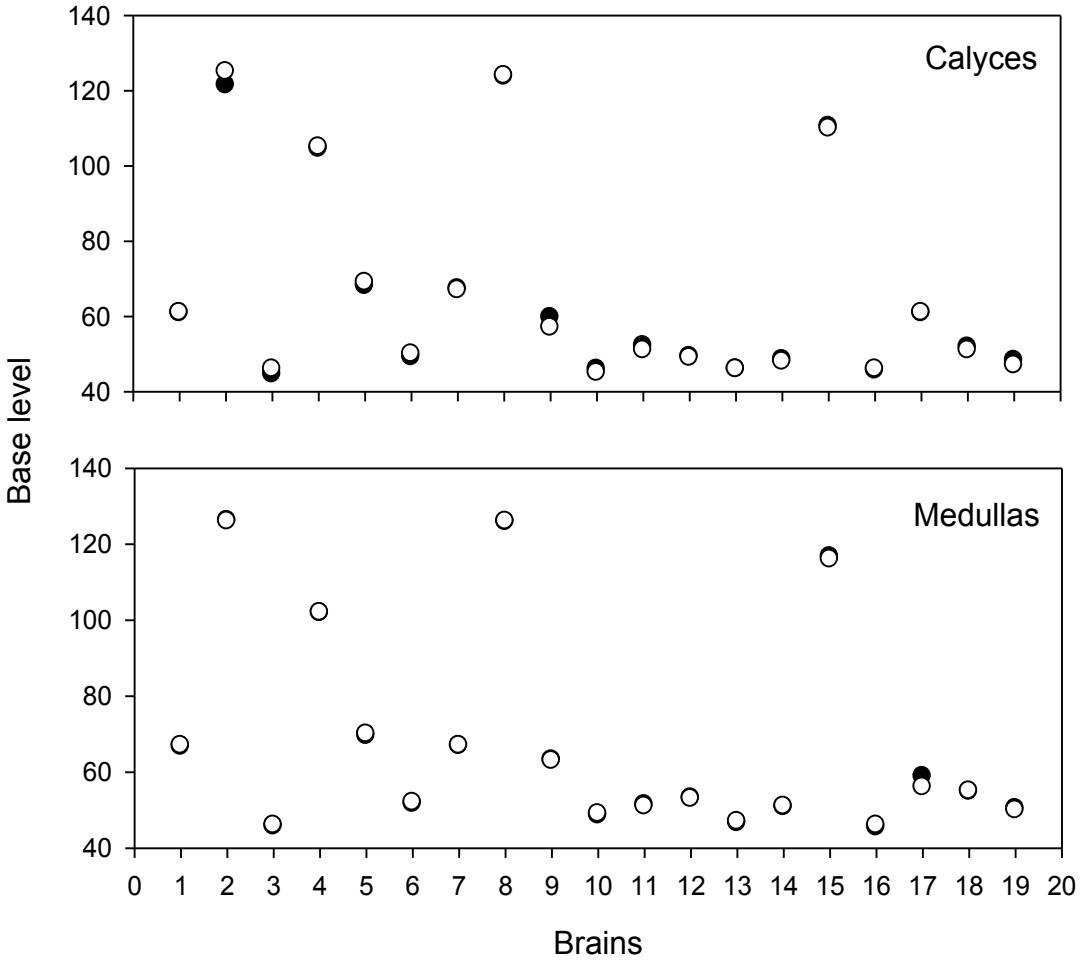


Figure 3.5 Comparison of the optimum base levels used with the manual tracing method (open circles) versus the histogram pixel intensity method (filled circles). As calyces are complex structures there is more likelihood of finding differences in base level using each method, whereas the simpler structure of medullas results in lower disparity.

Table 3.4 Comparison of the base level (bl) threshold value and resulting volumetric calculations based on the manual tracing and histogram pixel intensity methods, showing the percentage difference between the two for the MB calyces and medullas. The complex structure of the calyces shows that using alternative methods can result in different volume calculations for nine of the 19 brains because of *bl* differences. For the simpler structure of the medulla, however, only two of the 19 brains showed different calculated volumes. The brain number corresponds to the x axis number in Figure S6. Note that as previously mentioned the MB calyces of bee C3W7 was observed to be malformed which made tissue differentiation for segmentation difficult and likely explains why there is a big disparity when using the two methods.

Bee	Brain	MB Calyces					Medullas				
		manual		histogram		% diff.	manual		histogram		% diff.
		bl	*vol. / mm3*	*bl*	*vol. / mm3*		*bl*	*vol. / mm3*	*bl*	*vol. / mm3*	
C3W41	1	46	0.129	46	0.129	0	46	0.171	46	0.171	0
C2G3	2	45	0.085	46	0.096	11.8	46	0.131	46	0.131	0
C2G8	3	124	0.098	124	0.098	0	126	0.132	126	0.132	0
C2G27	4	52	0.105	51	0.100	6.0	51	0.129	51	0.129	0
C3W5	5	105	0.070	105	0.070	0	102	0.108	102	0.108	0
C3W25	6	67	0.104	67	0.104	0	67	0.149	67	0.149	0
C12G28	7	61	0.122	61	0.122	0	59	0.154	56	0.146	5.6
C12G13	8	61	0.072	61	0.072	0	67	0.075	67	0.075	0
C12G12	9	48	0.114	48	0.114	0	51	0.111	51	0.111	0
C11R21	10	60	0.082	57	0.077	5.3	63	0.124	63	0.124	0
C6W11	11	46	0.107	45	0.101	5.5	49	0.117	49	0.117	0
C6W13	12	49	0.091	50	0.095	4.5	52	0.113	52	0.113	0
C11R13	13	52	0.110	51	0.105	4.2	55	0.129	55	0.129	0
C3W1	14	46	0.108	46	0.108	0	47	0.181	47	0.181	0
C2G29	15	110	0.109	110	0.109	0	117	0.127	116	0.124	3.0
C3W7	16	121	0.081	125	0.100	18.3	126	0.119	126	0.119	0
C6W52	17	48	0.119	47	0.113	5.5	50	0.136	50	0.136	0
C12G1	18	49	0.108	49	0.108	0	53	0.144	53	0.144	0
C12G11	19	68	0.087	69	0.097	10.6	70	0.121	70	0.121	0

Table 3.5 Repeated segmentation and volumetric calculations of the right and left medullas from a single brain using the manual tracing method. The top table shows the basic statistics of the four repeated measures per structure, with the 'test re-test repeatability' calculated by multiplying the standard deviation by 1.96. The bottom table shows a pairwise comparison for each repeated volume estimate per structure and takes the mean and median percentage differences over all six comparisons to give an inaccuracy value.

Repeated measure	**Vol. Right Medulla**	**Vol. Left Medulla**
1	0.0749778	0.0744753
2	0.0750145	0.0757387
3	0.0733002	0.0749277
4	0.0746532	0.0754171
Mean	0.0745	0.0751
Standard deviation	0.0008	0.0006
Standard error of mean	0.0004	0.0003
Test re-test repeatability score	0.0016	0.0011
Co-efficient of variation (%)	1.0838	0.7378

Pair-wise comparison of % differences between each repeated measure				
Medulla 1				
	0.0749778	0.0750145	0.0733002	0.0746532
0.0749778	x			
0.0750145	0.048881778	x		
0.0733002	2.237461569	2.285226288	x	
0.0746532	0.433011087	0.481657423	1.845748393	x
Mean % diff	1.2220	(mean of all six pairwise comparisons)		
Median % diff	1.1637	(median of all six pairwise comparisons)		
Medulla 2				
	0.0744753	0.0757387	0.0749277	0.0754171
0.0744753	x	x	x	x
0.0757387	1.6964	x	x	x
0.0749277	0.6075	1.0707	x	x
0.0754171	1.2645	0.4246	0.6531	x
Mean % diff	0.9528	(mean of all six pairwise comparisons)		
Median % diff	0.8619	(median of all six pairwise comparisons)		

Secondly, I examined the self-consistency of our volumetric estimates by comparing left and right-paired structures for each brain across all individuals (the single structure of the CB was therefore excluded). Finding highly similar (or the same) paired volumetric calculations would support the precision of our method - when assuming that such paired structures typically develop symmetrically. I found strong linear relationships, and low mean percentage differences, between the left and right paired structures (whole MBs: $r^2 = 91.2$, %diff = 3.85%; MB lobes only: $r^2 = 74.8$, %diff. = 7.48%; MB calyces only: $r^2 = 93.4$, %diff. = 4.10%; ALs: $r^2 = 89.3$, %diff = 4.92%; Mes: $r^2 = 95.7$, %diff = 3.06%; Los: $r^2 = 72.1$, %diff = 6.58%; Figure 3.6A, 3.7 & Table 3.6).

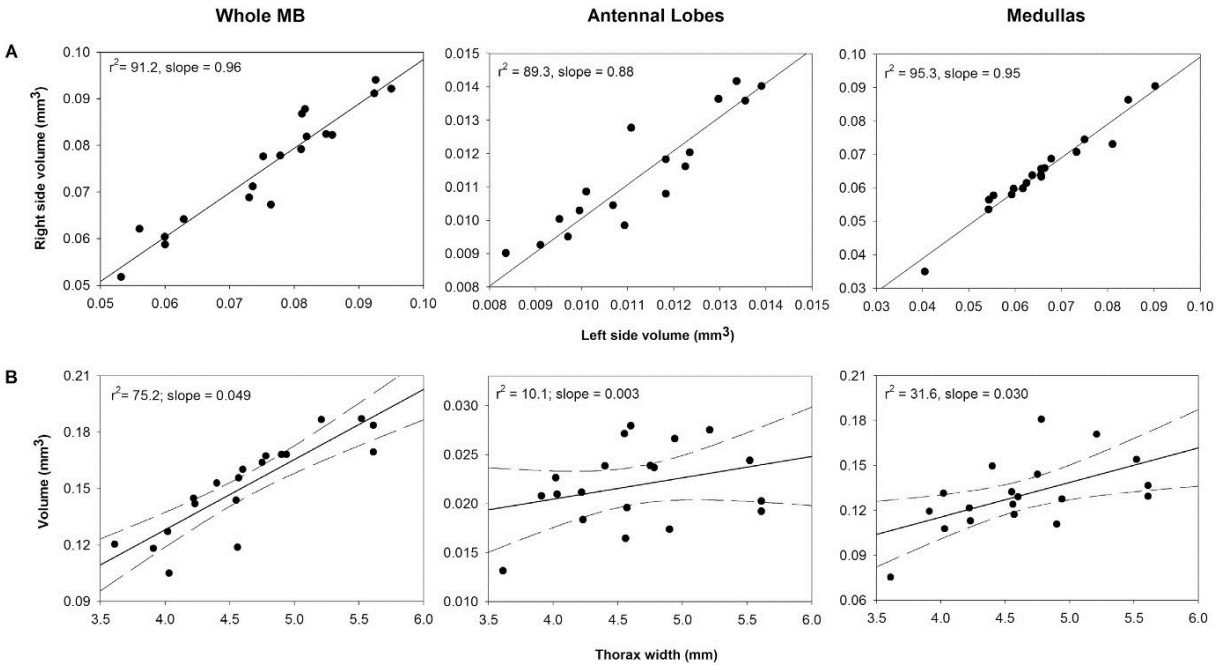


Figure 3.6 Isometric and allometric relationships. A) Isometric relationship between the calculated volumes (mm^3) of three paired structures found on the left (x-axis) and right (y-axis) sides of the brain (n = 19), and B) allometric relationship between body size (thorax width (mm)) and total volumes of each of the paired structures combined (n = 19). Using the manual tracing method; A) Fitted linear regression lines are plotted with r^2 values and slope gradients shown. The very high degree of congruence between the volume of left and right paired structures strongly supports that our method is effective at differentiating and extracting the structural tissue. B) Fitted linear regression lines are plotted with 95% confidence limits (dashed line), and r^2 values and slope gradients are shown. For the whole mushroom body (MB) I explored the relationship when the lobes and calyces were combined.

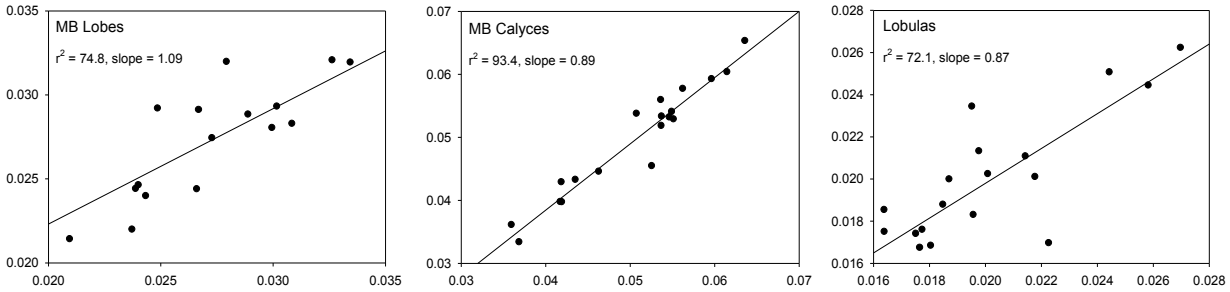


Figure 3.7 Correlations between calculated volumes (mm3) using the manual tracing method of paired structures found on the left (x-axis) and right (y-axis) sides of the brain (n = 19). Fitted linear regression lines are plotted with r^2 values and slope gradients shown.

Table 3.6 Statistical outputs from paired t-tests comparing the calculated volumes of each brain structure from the right and left side of the brain. The table shows the mean values (± s.e.m.) for each of the paired structures (excluding the central body as this is not a paired structure) as well as the total volumes when both of paired structures are combined. All volumes were calculated using the manual tracing method, except those values underlined calculated using the histogram intensity method for comparison.

Structure	Right side (mm3)		Left side (mm3)		n	t	p	Total	
	mean	s.e.	mean	s.e.				mean	s.e.
Whole MBs	0.07606	0.00288	0.07563	0.00287	38	0.48	0.637	0.15169	0.00567
MB Lobes	0.02554	0.00123	0.02612	0.00097	38	-0.84	0.413	0.05166	0.00211
MB Calyces	0.05052	0.00188	0.04951	0.00203	38	1.97	0.065	0.10002	0.00386
MB Calyces	0.05095	0.00168	0.05011	0.00186	38	1.42	0.173	0.10100	0.00347
Antennal Lobes	0.01088	0.00044	0.01095	0.00051	38	-0.27	0.790	0.02183	0.00092
Medullas	0.06558	0.00268	0.06460	0.00272	38	1.54	0.142	0.13019	0.00540
Medullas	0.06516	0.00261	0.06437	0.00270	38	1.53	0.144	0.12956	0.00532
Lobulas	0.01978	0.00078	0.01962	0.00074	38	0.62	0.543	0.03941	0.00150
Central Body	-	-	-	-	19	-	-	0.00333	0.00014

I then tested how my estimations compared to the allometric relationships observed in previous studies, I found a significant positive correlation between predictors of body size and each brain structure (MBs, ALs, Mes and Los), with the exception of the CB, which is consistent

with previous findings (Mares, Ash et al. 2005, Riveros and Gronenberg 2010) (Figure 3.6B, 3.8 & 3.9). I compare my dataset to two case-studies (Mares, Ash et al. 2005, Jones, Leonard et al. 2013) predicting that they would have overestimated the volume of structures more complicated in shape (such as the MBs, Mes, ALs and Los which are more morphologically complex than CB) compared to the volume estimations presented here. Indeed, in confirmation of this prediction I found all of the structures to have lower volumes, with my calculated volumes of the MBs, ALs, Mes and Los being, respectively 49/69%, 44/53%, 60/56% and 63/59% (Mares/Jones) of the volumes estimated in case-studies (Mares, Ash et al. 2005, Jones, Leonard et al. 2013), and the CB being 88/80% of the size (see Table 3.7 for details). I also compared the different brain structures against each other in a pair-wise manner to examine whether the ratios between pairs of each structure remained consistent with increasing body size (in this analysis I considered the calyces and lobes independently rather than mushroom body as a whole). I did not find any significant change after correcting for multiple testing for the 15 paired comparisons (p-value <0.033: see Figure 3.9).

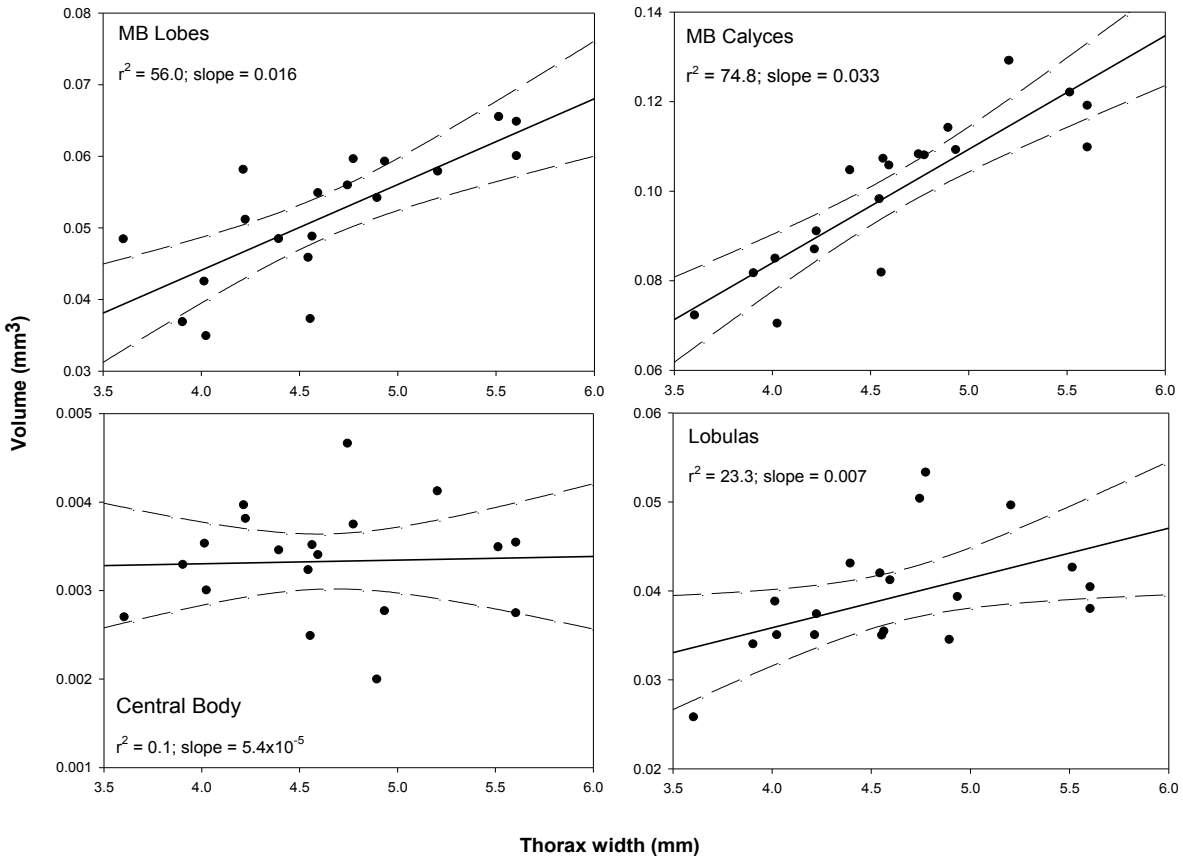


Figure 3.8 Body size and brain structure relationship. Comparison between body size (determined here by thorax width/ mm) and volume (mm^3) of brain structures of interest calculated using the manual tracing method: mushroom bodies (MBs), central body and lobulas (n = 19 workers; volumes of paired structures were combined to provide a total volume for this analysis). Fitted linear regression lines are plotted with 95% confidence limits (dashed line), and r^2 values and slope gradients are shown.

al tracing method for each structure from our s … igh quality
al. 2013).** Volumes for each paired structure w … vidual, and
e to the respective individual's whole brain vol … = 46, Jones
d Jones *et al.* (2013) were on *Bombus impatien* … study on *B.*
nsidering values at four decimal places I compa … re to those
e absolute different in value by subtracting th … resent this
erence) from the volumes calculated from Mar …

	Our Study		Mares 2005		Jones 2013*		Our study minus Mares		Our study minus Jones	
	mean	s.e.m	mean	s.e.m	mean	s.e.m	value difference	% difference	value difference	% difference
Whole MBs	0.118660	0.002962	0.24	0.02	0.1709	-	-0.1213	-50.6	-0.0522	-30.6
MB Lobes	0.040434	0.001244	0.05	0.01	0.0670	0.0048	-0.0096	-19.1	-0.0266	-39.7
MB Calyces	0.078226	0.002068	0.19	0.02	0.1039	0.0062	-0.1118	-58.8	-0.0257	-24.7
Antennal Lobes	0.017532	0.000838	0.04	0.01	0.033	0.0034	-0.0225	-56.2	-0.0155	-46.9
Medullas	0.102012	0.003386	0.17	0.02	0.1815	0.0088	-0.0680	-40.0	-0.0795	-43.8
Lobulas	0.031373	0.001177	0.05	0.01	0.0535	0.002	-0.0186	-37.3	-0.0221	-41.4
Central Body	0.002628	0.000118	0.003	0.001	0.0033	0.0003	-0.0004	-12.4	-0.0007	-20.4

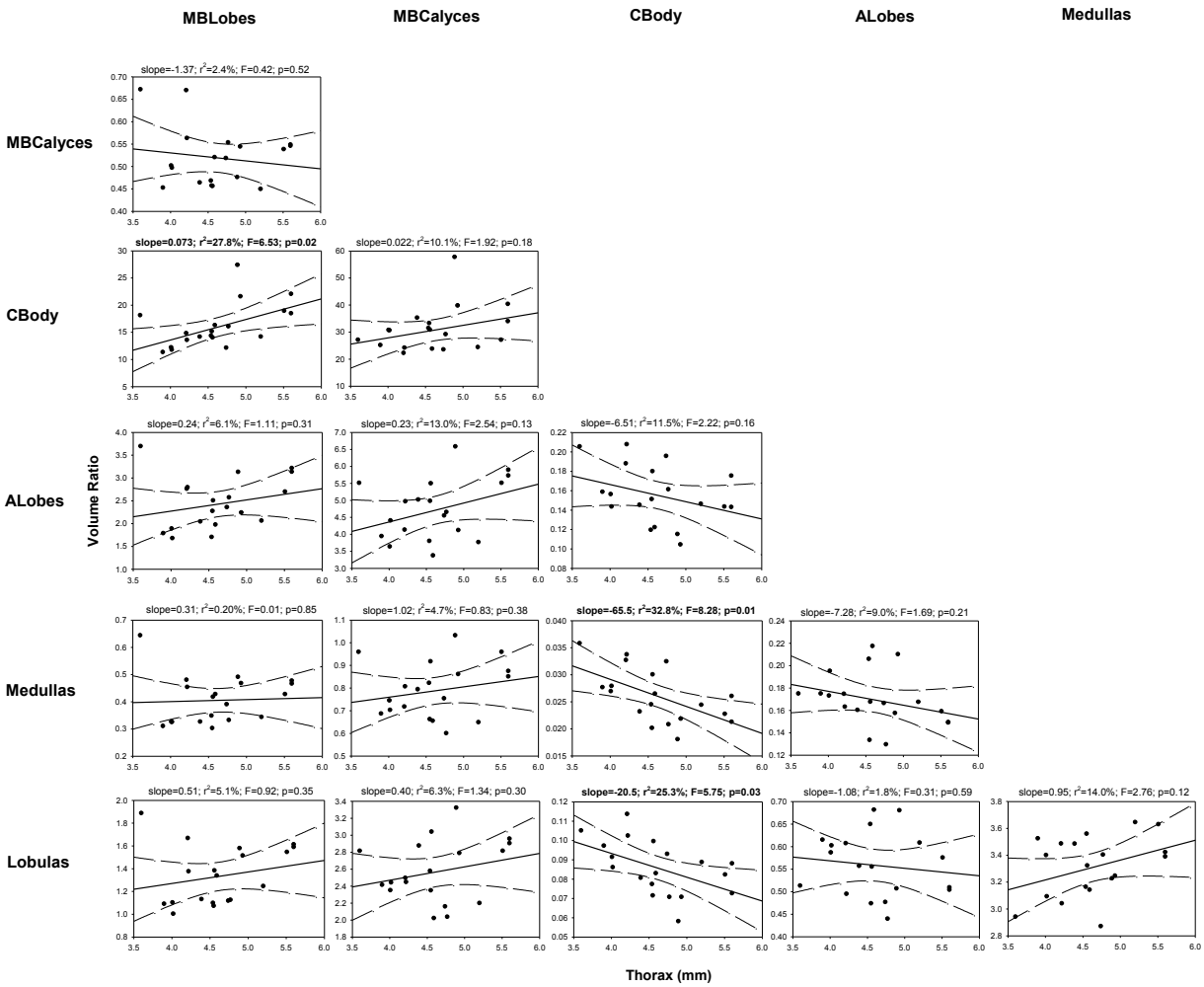


Figure 3.9 Pairwise comparisons of the associated volumes (mm3) of each brain structure to provide a comparative size ratio across all 19 individuals. These ratios were then plotted against the associated individual body size (thorax width /mm) to explore whether there were relative differences between structure sizes in different sized bees. Fitted linear regression lines are plotted with 95% confidence limits (dashed line) along with the statistical output from the regression analysis with p-values falling under the 0.05 significance value highlighted in bold. For the mushroom body (MB) I explored the relationship when the lobes and calyces were combined (Whole MB) as well as independently. Volumes calculated using the manual tracing method.

Additionally, for one of the 19 individuals I found an apparent pronounced deformation in the right mushroom body (MB) lobe: being both visually smaller in volume and unusual in shape. Given the typically high precision when scanning and reconstructing tissues, and the relative uniformity between right and left sides, I am confident that this was not caused by human

error when segmenting the brain but reflects true tissue deformation. Indeed, this was also the brain that showed the highest percentage difference in volume calculation using the two thresholding methods.

3.4 DISCUSSION

That the results presented here conformed to the general allometric relationships between brain structures and body size found in previous histological studies supports the view that my volume estimates do not suffer from significant artefacts or misrepresentations of brain tissue. Indeed, our methods should have generated structural volumes that more accurately reflect real volumes, whereas histological techniques are likely to suffer from multiple sources of error. For instance, histological techniques estimate structural volumes by using only a subset of microtome slices with each slice being typically thicker than ours (e.g. 10-20 vs 4.6µm), whereas our methodology incorporates structural tissue across all slices (i.e. mean = 377 slices) into an automated volume calculation. Furthermore, from our optimum thresholding procedure, I can differentiate tissues based on density and thus facilitate the exclusion of extraneous internal tissue from each slice facilitating our ability to examine real representations of these complicated structures, with surfaces textured with grooves, pits, crevices and hollows (Figure 3.4). Histological techniques, however, are unable to do this as volumes are calculated by visually tracing the structure of interest for each of the subset of slices, taking this calculated area and then interpolating between the subset of slices. Subsequently, the histological approach is forced to assume that the structure has a relatively uniformed and even surface between slices, yet this is unlikely to be (as shown by our images; Figure 3.4). These results therefore validate the predictions that previous studies will have

overestimated the volumes of these complex brain structures and that our methodology would produce consistently lower volume estimations in comparison.

To further validate the precision of this new protocol I assessed the left/right volumetric symmetry of the five paired brain structures (MBs, ALs, Mes and Los) assuming that such paired structures typically develop symmetrically. The differences between the paired volumes were very small and these findings support that the methods can objectively assess the volume of morphologically complex brain structures with relatively high precision. Indeed, the differences I found likely represent an upper limit of error considering that true asymmetry between brain hemispheres may naturally develop, although the extent of this has not been quantified.

Through developing and combining the advances in imaging technology (Ribi, Senden et al. 2008, Zhang, Li et al. 2010, Greco, Tong et al. 2012) with a detailed imaging analysis protocol I have been able to produce high quality 3D reconstructions of soft tissue samples with high resolution and precision that can be revisited for repeated new exploration, virtual dissection and comparative analyses of tissue structure without the need for additional sample preparation. The initial primary goal of the development of this protocol was to provide a reliable and precise way to study comparative changes in brain volume between experimentally stressed and non-stressed bees. The relatively high precision and reduced error (compared to existing methods) demonstrated within this protocol will allow me to confidently apply it to the investigation of how the exposure of bees to chemical stressors such as neurotoxic insecticides may alter brain development and growth, reflected in potentially very small but significant changes in volume, of key structures or even their functional components in the brain of an important insect pollinator species – the bumblebee *Bombus terrestris*.

Chapter 4

EFFECTS OF PESTICIDE EXPOSURE ON THE DEVELOPMENTAL GROWTH AND FUNCTION OF THE MUSHROOM BODY AND ANTENNAL LOBES OF THE BUMBLEBEE BRAIN

4.1 INTRODUCTION

A number of studies have shown social bee colonies to perform poorly under exposure to neonicotinoids, with reduced colony growth and sexual production (Gill, Ramos-Rodriguez et al. 2012, Whitehorn, O'Connor et al. 2012). It is generally considered that such colony level effects are caused by neonicotinoid induced impairment to the physiology and behaviour of the workers that perform the colony tasks, with the cumulative effect across workers proposed to cause colonies to become functionally weakened (Bryden, Gill et al. 2013, Crall, Switzer et al. 2018). One possibility is that neonicotinoids, being a neurotoxin, affect neuronal processes important for the cognitive and learning abilities that colony tasks require, such as foraging ability. Indeed, previous studies have shown reduced foraging performance in workers from exposed colonies (Gill, Ramos-Rodriguez et al. 2012, Feltham, Park et al. 2014, Gill and Raine 2014), and in Chapter 2 I show and discuss the negative impacts of neonicotinoid exposure on learning performance. Learning ability relies on the detection,

assimilation and processing of sensory input from the environment all of which is dependent on brain development and its functional and structural plasticity (Cabirol, Cope et al. 2018). Here I investigate how neonicotinoid exposure effects the development and growth of brain structures associated with olfactory learning and the subsequent relationship with learning performance.

It is known that the environmental conditions experienced throughout development can significantly affect the brain, with subsequent repercussions for sensory processing, learning and memory (Rice and Barone Jr 2000). In bees, brain development can be particularly sensitive to changes in their natural developmental environment as demonstrated in honeybees reared artificially compared to hive-reared bees which exhibited smaller mushroom body (MB) lateral calyces (Steijven, Spaethe et al. 2017). Additionally, minor deviancies from optimal rearing temperature during pupal development has been shown to result in severe defects in MB neuronal architecture (Groh, Tautz et al. 2004) along with effects on learning performance (Tautz, Maier et al. 2003) of honeybees. It is based on this observed level of behaviourally mediated plasticity and sensitivity to developmental environment that it could be suggested that certain environmental stressors – such as pesticides - can interfere with brain development

Box. 1. Olfactory associative learning tested using the proboscis extension reflex assay requires individuals to detect an odour stimulus followed by antennal stimulation with sucrose, and through this partnering of olfactory cue and reward establish a conditioned learnt response. In Chapter 2 I showed that neonicotinoid exposure during brood development or early adulthood impairs this ability in both young (3-day) and older (12-day) adults (pages 67-72). Therefore, I expect that this level of exposure to have correspondingly harmed the key neuropils responsible for the detection, processing and learning of olfactory information ***- the antennal lobes and mushroom bodies.***

and predispose bees to be poorer learners later in life, degrading their functional contribution to the colony, such as impairing foraging performance. Indeed, the neonicotinoid pesticide imidacloprid is known to: i) act as neuronal agonists by targeting nicotinic acetylcholine receptors found in high densities in Kenyon cells of social bee brain, particularly the mushroom bodies (Jeschke and Nauen 2008, Palmer, Moffat et al. 2013); ii) affect learning performance in social bees exposed during individual development (Yang, Chang et al. 2012, Stanley 2015, Tan, Chen et al. 2015).

The first stage in learning olfactory cues requires the detection of olfactory and gustatory stimulus with the antennae (Hansson and Anton 2000). Following which olfactory information is initially processed through the glomeruli of the antennal lobes (ALs) (Gascuel and Masson 1991, Sachse, Rappert et al. 1999) (functional subdivisions of the AL (Galizia, Sachse et al. 1999)). Projection neurons from the AL glomeruli then output to the mushroom bodies (MBs) (Menzel, Galizia et al. 2005). The ALs exhibit experience and age related plasticity (Withers, Fahrbach et al. 1993, Winnington, Napper et al. 1996, Sigg, Thompson et al. 1997, Riveros and Gronenberg 2010, Jones, Leonard et al. 2013) and volume increases of glomeruli are associated with improved learning performance (Sigg, Thompson et al. 1997). However nicotinic agonist have been shown to act on neurons of the ALs (Barbara, Grünewald et al. 2008) and neonicotinoid exposure is known to impair olfactory learning and memory in bees (Williamson and Wright 2013, Stanley 2015) suggesting impaired function. Based on which, along with my findings in chapter 2, I would predict the ALs of these individuals to also exhibit impaired growth and this to be most prominent in older (12-day) workers.

The mushroom bodies (MBs) of the brain play a major role in sensory integration, learning and memory in insects (Erber, Homberg et al. 1987, Heisenberg 1998, Zars 2000). Olfactory

learning is one of the functions most strongly linked to the MBs of insects (Heisenberg 2003, Perisse, Burke et al. 2013, Aso, Hattori et al. 2014) and this form of associative learning in social bees is considered to be physiologically underpinned by the MB development (Hammer and Menzel 1995, Heisenberg 1998). Indeed, learning performance is dependent on normal MB function and growth: honeybees whose MBs were treated with mitotic blocker hydroxyurea (HU) (that kills proliferating cells) as first-instar larvae exhibited reduced sucrose responsiveness and poorer learning performance (Scheiner, Weiß et al. 2001), olfactory memory formation is impaired by local cooling of sub-units of the MB (Erber, Masuhr et al. 1980). And MB volume has been suggested to be correlated with higher olfactory learning performance (number of learned responses in the proboscis extension reflex assay) (Gronenberg and Couvillon 2010).

The MB's also display a large amount of plasticity in relation to behaviours and experiences that require olfactory learning. For example, honeybee foragers have larger MB volume compared to nurse bees, perhaps to account for the increased cognitive requirements, and this growth is further increased through foraging experience (Farris, Robinson et al. 2001). Similarly honeybee foragers exhibit greater MB volume compared to 1 day old (non-foragers) and volumetric growth of the MB occurs in young, precocious bees that are induced to forage (Withers, Fahrbach et al. 1993, Durst, Eichmüller et al. 1994, Withers, Fahrbach et al. 1995). Foraging experience has also been shown to correlate positively with MB volume in bumblebees (Riveros and Gronenberg 2010). Further to this, structural changes of the honeybee MB intput regions occur at the onset of foraging, in the form of a decrease in synaptic boutons, followed by an increase in conjunction with increased forging intensity (Cabirol, Cope et al. 2018). Thus, I would expect workers in chapter 2 that showed the highest learning performance (*control*) to correspondingly possess greater MB volumes; with the

fewer learners and lower learning performance seen in pesticide treatment groups to be consistent with smaller MB volume due to an impaired experience-dependent plasticity.

In addition, I would further expect a differential effect of pesticide exposure on MB volume between age cohorts: first because of my findings in chapter 2 where 12-day (compared to 3-day) workers exhibited the largest effects on learning performance. Secondly due to the age-related growth of the MB components, that can occur independent of experience (Gronenberg, Heeren et al. 1996, Fahrbach, Moore et al. 1998, Kühn-Bühlmann and Wehner 2006, Riveros and Gronenberg 2010, Jones, Leonard et al. 2013). Pesticide exposure may interfere with learning performance through inhibiting this innate age-dependent growth of the MB.

It also is important to note that studies have highlighted that experience related plasticity in MB volume can be localized or differentially effect sub-components – such as the medial calyx in bumblebees which showed a significant volume increase with foraging experience where the lateral calyx did not (Riveros and Gronenberg 2010). Specific regions of the calyx themselves show differentially plasticity, volume of the lip and collar region of the calyces in honeybees was positively correlated with foraging intensity (Cabirol, Cope et al. 2018) and the collar volume of the calyces in Desert Ants, *Cataglyphis bicolor* increased with foraging experience (Kühn-Bühlmann and Wehner 2006). Calyx volume has been found to differ with division of labour in honeybees – whereby nurses had smaller visual and olfactory input regions (the collars and lips respectively) than foragers (Durst, Eichmüller et al. 1994). This highlights the importance of MB plasticity when considering the behavioural capacity of an individual bee, but it also suggests that the calyces exhibit the greatest levels of plasticity. Therefore, I might expect to find workers with better learning performance to have

disproportionately larger volumes in either or both calyces and that these structural components of the MB to be differentially affected by pesticide exposure compared to the rest of the MB or ALs.

This study took a subset of workers tested in Chapter 2 that were orally exposed to 5ppb Imidacloprid (see Chapter 2.2 for full details of experimental exposure), during *pre-eclosion* (brood development), *post-eclosion* (adult development) or *continual* (exposure during both developmental phases), to compare with unexposed *control* workers. I used workers from both age cohorts aged 3 and 12-days as brain development occurs during pupal-stage metamorphosis and the first few days after eclosion (Farris, Robinson et al. 2001, Jones, Leonard et al. 2013). This allows me to assess how learning enhancement correlates with both pre and post eclosion development of the MB and AL across treatments. I applied my high resolution Micro-CT scanning and 3D image analysis method (Smith, Bernhardt et al. 2016) (Chapter 3) to non-destructively perform volumetric measurements of the separate components of the MB (lateral calyces, medial calyces and lobes) and the ALs from the left and right side of the brain *in situ*, allowing me to detect the potentially minute and subtle, but important variations in volume caused by neonicotinoid induced stress. I investigated the differential response to Imidacloprid exposure of the functionally distinct multisensory input (LC, MC and Lo) and output regions (Lo) (Heisenberg 2003, Fahrbach 2006) of the MB and AL. I then looked at how the volume of these brain structures of these workers correlates with their responsiveness and learning performance (detailed in chapter 2).

4.2 METHODS

4.2.1 Animal Husbandry and experimental exposure

Bumblebee colonies were housed and experimentally exposed as described in Chapter 2.2.1.

4.2.2 Micro-CT scanning

From the bees tested in Chapter 2 (n=204 that passed the responsiveness test), I sampled 92 ensuring a balanced representation across treatments and age, but sampled blind of learning performance to avoid bias. Following the olfactory learning assay (see chapter 2), bees were humanely sacrificed by swiftly decapitating the live individual using a disposable surgery scalpel. Once cut, immediately the head was fully submerged in a 70/30% ethanol/de-ionised water solution in a 1.5ml centrifuge tube and stored at 5°C. Preparation of the heads followed precisely the protocol detailed in the methods of Chapter 3 (Smith, Bernhardt et al. 2016). Soft brain tissue was stained with phosphotungstic acid (PTA) before being CT scanned at a voxel size of 3.5-4µm using a Nikon Metrology HMX ST 225 system (Nikon Metrology, Tring, UK). The staining and scanning methodology employed has been shown to give confidence in the accuracy of our measurements of these complex morphological structures – the MB calyces and lobes. The raw µCT data for each brain scan was reconstructed using CTPro 2.1 software (Nikon Metrology, Tring, UK) and processed using VG Studio Max 2.1 (Volume Graphics GmbH, Heidelberg, Germany). Each 3D reconstructed scan was then re-oriented to the same optimum plane of view for visualizing all of the major brain neuropils including all components of the MBs then re-sliced into a new 2D series of images along this plane. For each sample, scan images were exported as 8-bit BMP image series at a standardized voxel size of 4µm.

4.2.3 Mushroom body volume measurements

Segmentation and volume analysis of brain structures was carried out using the software SPIERS 2.20 (Serial Paleontological Image Editing and Rendering System). For segmentation, scan slices were converted to binary threshold images (of white, active pixels and black, inactive pixels) adjusted to achieve the optimum ratio of active white pixels that comprise the structure of interest, and inactive black pixels, for the surrounding tissues. For each component structure looped splines were placed around the active pixels at regular slice intervals and these were then interpolated across all slices, between intervals, to define the structure as an independent object for 3D reconstruction and volumetric analysis (for full segmentation protocol see Chapter 3.2 (Smith, Bernhardt et al. 2016). The tissue segmentation protocol I employed has been shown to provide repeatable and precise volumetric measurements of morphological structures of the bumblebee brain (Smith, Bernhardt et al. 2016). Here I segmented the major components of the left and right mushroom body separately – lobe (Lo), lateral calyx (LC) and medial calyx (MC) – allowing us to explore the potential differential effect of neurotoxic pesticides on each functionally different structure. To calculate volumes I used the voxel count function in SPIERS Edit, and corrected for variation in body size using the inter-tegula width shown to be an accurate proxy for body size (Cane 1987). Inter-tegula width was measured using digital callipers (Workzone®).

4.2.4 Data Analysis

Statistical analyses were conducted in R version 3.0.1 (R Development Core Team 2014) using RStudio version 1.0.143, with mixed effects models using the lme4 and lmerTest packages (Bates, Mächler et al. 2015, Kuznetsova A 2017). Data for neuropil volumes were log

transformed and for MB relative volume comparisons across treatments I used linear mixed-effects models (LMERs). In addition to treatment as a fixed factor, I included worker size (inter-tegula width) to consider any disproportionate effect on small or large workers (Samuelson, Chen-Wishart et al. 2016) and colony was incorporated into the model as a random factor. Separate models (LMERs) for each brain structure were also performed for comparisons between treatments for 12-day workers only. Mean volumes provided in the text are back transformed from model outputs (additional average volumes from raw data are provided in Appendicies) and all percentages differences were calculated as the difference between mean model estimates. For Figure 4.2, all panels represent model estimates of the mean with ±95% BCI back-transformed from an LMM, and jittered points corresponding to the raw data. Spearman's Rank correlation coefficients and associated P values were calculated using the cor.test function of the stats package of R.

4.3 RESULTS

Using only workers that performed the full PER assay (Chapter 2), I produced 3D reconstructions of the separate right and left MB components, along with each left and right antennal lobe for 92 workers (Figure 4.1; *control*=21; *pre-eclosion*=23; *post-eclosion*=24; *continual*=24), representing a sample size far exceeding any µCT study on insect soft tissue to date (Appendix 4.1).

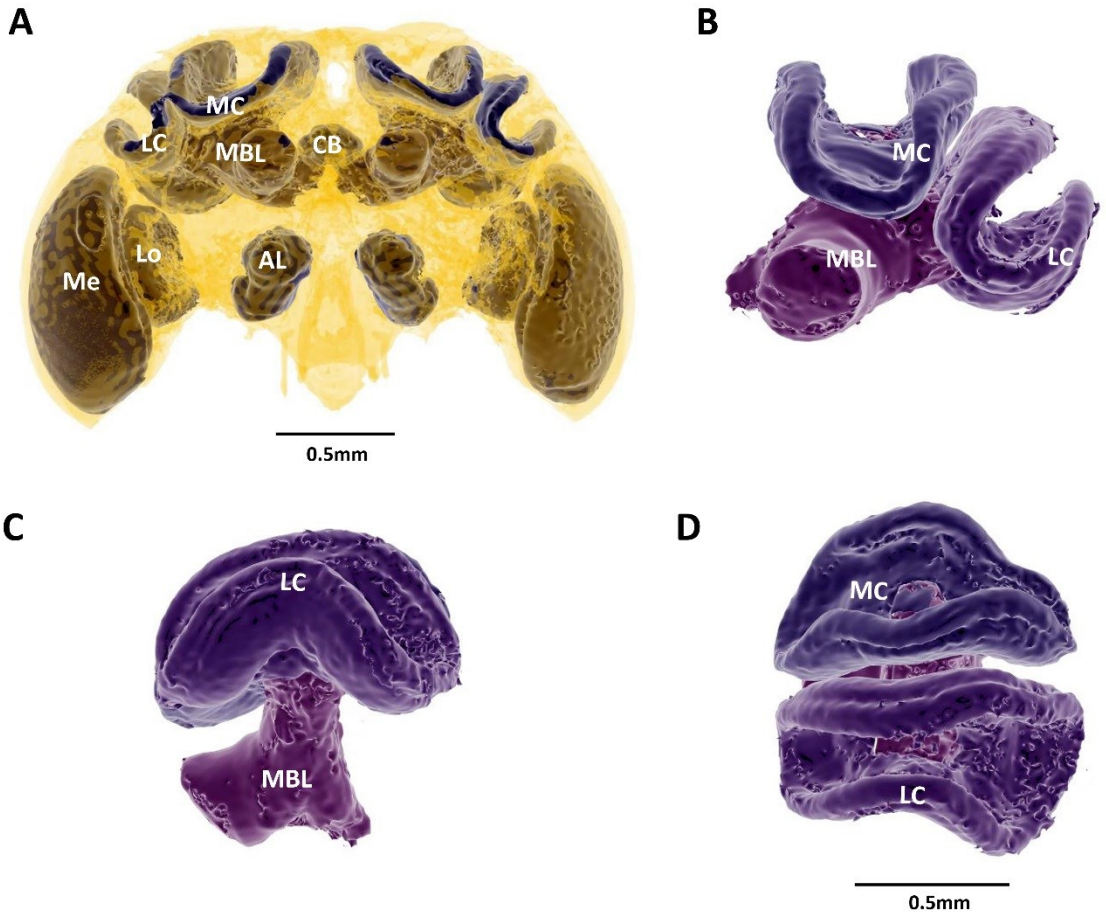


Figure 4.1 3D rendering of a bumblebee brain from µCT imaging. **A**) Specific brain structures shown in dark purple surrounded by remaining brain tissue in transparent yellow; medial calyx (MC), lateral calyx (LC) mushroom body lobe (MBL), antennal lobe (AL), medulla (Me), lobula (Lo) and central body (CB). **B**) Close-up frontal view of a mushroom body (MB) showing the three primary component structures, the mushroom body lobes (MBL), medial calyx (MC) and lateral calyx (LC). **C**) MB lateral view. **D**) MB dorsal view.

4.3.1 Mushroom Body Volume

When correcting for body size variation (see Methods 4.2.3), components of the mushroom body for 3-day workers showed smaller average volumes across all three pesticide treatments compared to *control* exposure (Figure 4.2; Table 4.1; Appendix 4.2, 4.3). Specifically, I detected the largest reductions in *post-eclosion* and *continual* exposure workers in the

following components: *post-eclosion* left and right lateral calyces (mean model estimated %Δ =-16% and -15%; t≥2.51, p≤0.031) and right medial calyx (-21%; t=-2.34, p=0.033); *continual* left lateral calyx (-13%; t=-2.14, p=0.035) and right lateral calyx (-12%; t=-1.79, p=0.077; Table 4.1; Appendix 4.3).

Focusing on *control* exposure workers, there was a significant positive effect of age on the volumes of the right lateral calyx and left and right medial calyces (t≥2.02, p≤0.047), as evidenced by 12 compared to 3-day workers showing a volumetric increase of 17%, 22% and 21% (Table 4.1; Appendix 4.3), demonstrating age-related growth, with increases also seen for the remaining structures; left lateral calyx 14%, left lobes 14% and right lobes15% (Table 4.1; Appendix 4.2). Consistent negative model estimates for the treatment*age interaction across all three pesticide treatments, however, highlighted that pesticide exposure reduced the degree of age-related growth (Figure 4.2; Appendix 4.3). Specifically, significant interactive effects were detected for *pre-eclosion* exposure workers for the right and left lateral calyces (t≥2.07, p≤0.042; Figure 4.2), with a similar pattern exhibited in the right and left medial calyces, and right lobe (t≥1.75, p≤0.084; Appendix 4.3).

Assessing 12-day workers only, I also found the left and right lateral calyces, and left medial calyces, to be significantly smaller in all three pesticide exposure treatment cohorts (a reduction of ≥19%; t≥2.53, p≤0.033). The right medial calyx for *pre-eclosion* exposure workers was significantly smaller (-25%; t=-2.84, p=0.027), and close to signifcantly smaller in *post-eclosion* and *continual* (-23%, -22%; t≥2.05, p≤0.081; Table 4.1; Appendix 4.4).

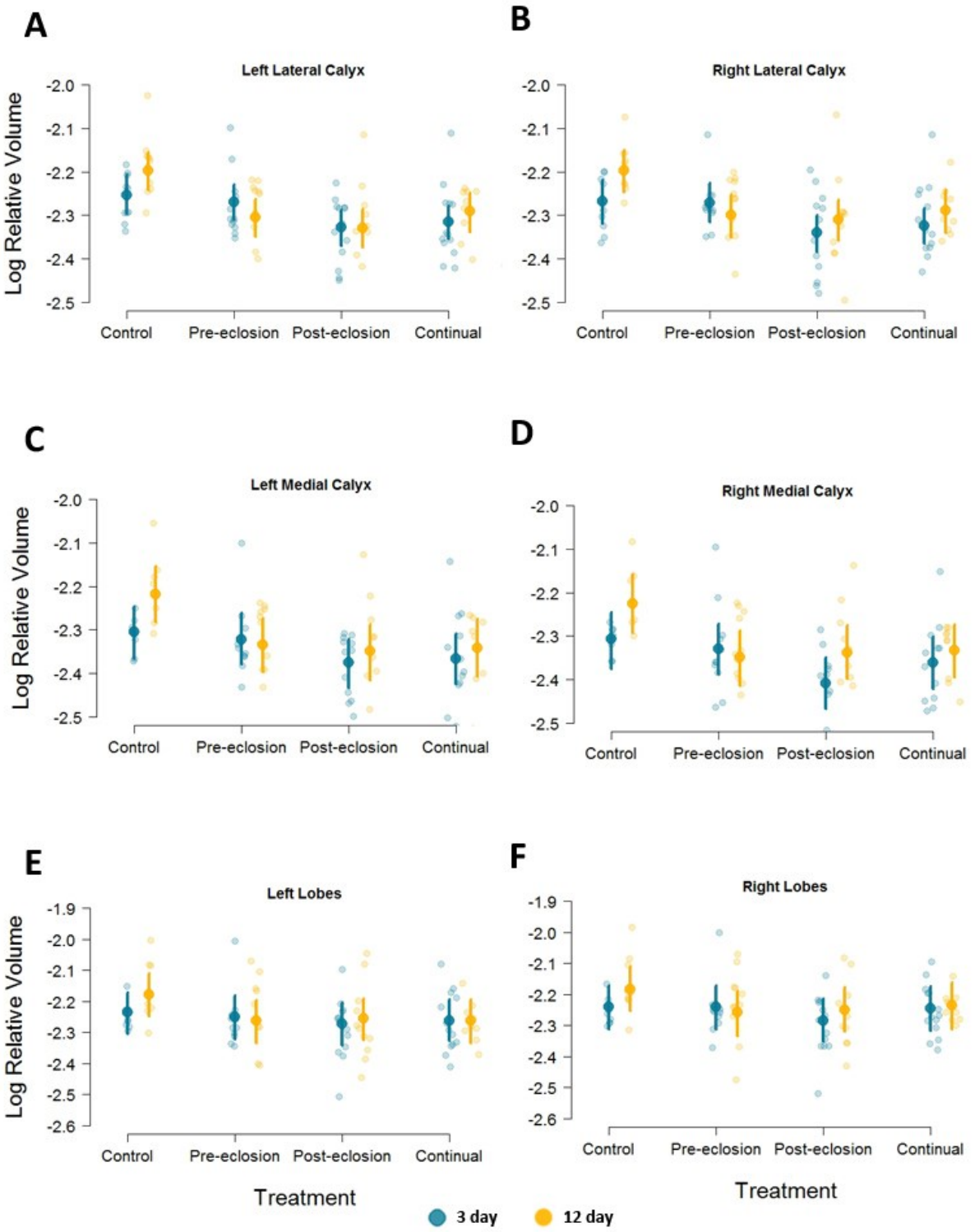


Figure 4.2 Comparison of the volumes (mm3) for each left and right MB of bumblebee workers. a-f, Relative volumes (corrected for worker size) for 3-day (blue) and 12-day (orange) workers have been log-transformed. Large points show model estimates of the mean and bars depict ±95% BCI back-transformed from an LMM, with semi-transparent, jittered points corresponding to the raw data.

Table 4.1 Mushroom body component structure volumes. Back transformed model means from relative volumes, credible intervals and percentage differences for pesticide treatment groups and control age cohorts

	Back transformed model mean	Lower CI	Upper CI	Difference from *Control*	*Control* Age Difference
Left Lateral Calyx					
Control 3 Day	*0.00559*	*0.00501*	*0.00617*		
Pre-eclosion 3 Day	0.00540	0.00490	0.00589	-3%	
Post-eclosion 3 Day	0.00471	0.00427	0.00513	-16%	
Continual 3 Day	0.00484	0.00447	0.00525	-13%	
Control 12 Day	*0.00636*	*0.00575*	*0.00708*		14%
Pre-eclosion 12 Day	0.00497	0.00447	0.00550	-22%	
Post-eclosion 12 Day	0.00469	0.00427	0.00513	-26%	
Continual 12 Day	0.00514	0.00457	0.00575	-19%	
Right Lateral Calyx					
Control 3 Day	*0.00541*	*0.00490*	*0.00603*		
Pre-eclosion 3 Day	0.00538	0.00490	0.00603	-1%	
Post-eclosion 3 Day	0.00459	0.00417	0.00513	-15%	
Continual 3 Day	0.00475	0.00437	0.00525	-12%	
Control 12 Day	*0.00634*	*0.00562*	*0.00708*		17%
Pre-eclosion 12 Day	0.00504	0.00457	0.00562	-21%	
Post-eclosion 12 Day	0.00492	0.00447	0.00550	-22%	
Continual 12 Day	0.00516	0.00457	0.00575	-19%	
Left medial calyx					
Control 3 Day	*0.00497*	*0.00427*	*0.00575*		
Pre-eclosion 3 Day	0.00478	0.00417	0.00550	-4%	
Post-eclosion 3 Day	0.00424	0.00372	0.00490	-15%	
Continual 3 Day	0.00432	0.00372	0.00501	-13%	
Control 12 Day	*0.00605*	*0.00513*	*0.00708*		22%
Pre-eclosion 12 Day	0.00464	0.00398	0.00537	-23%	
Post-eclosion 12 Day	0.00449	0.00389	0.00525	-26%	
Continual 12 Day	0.00458	0.00389	0.00537	-24%	
Right medial calyx					
Control 3 Day	*0.00495*	*0.00427*	*0.00575*		
Pre-eclosion 3 Day	0.00469	0.00407	0.00550	-5%	
Post-eclosion 3 Day	0.00392	0.00339	0.00457	-21%	
Continual 3 Day	0.00439	0.00380	0.00513	-11%	
Control 12 Day	*0.00598*	*0.00513*	*0.00708*		21%
Pre-eclosion 12 Day	0.00449	0.00389	0.00525	-25%	
Post-eclosion 12 Day	0.00462	0.00398	0.00537	-23%	
Continual 12 Day	0.00466	0.00398	0.00537	-22%	
Left MB lobes					
Control 3 Day	*0.00581*	*0.00490*	*0.00692*		
Pre-eclosion 3 Day	0.00564	0.00479	0.00676	-3%	
Post-eclosion 3 Day	0.00535	0.00447	0.00631	-8%	
Continual 3 Day	0.00548	0.00457	0.00646	-6%	
Control 12 Day	*0.00665*	*0.00562*	*0.00794*		14%
Pre-eclosion 12 Day	0.00546	0.00457	0.00646	-18%	
Post-eclosion 12 Day	0.00557	0.00468	0.00661	-16%	
Continual 12 Day	0.00549	0.00457	0.00661	-17%	
Right MB lobes					

Control 3 Day	*0.00572*	*0.00479*	*0.00692*		
Pre-eclosion 3 Day	0.00576	0.00479	0.00692	1%	
Post-eclosion 3 Day	0.00521	0.00437	0.00631	-9%	
Continual 3 Day	0.00570	0.00479	0.00676	0%	
Control 12 Day	*0.00656*	*0.00550*	*0.00794*		15%
Pre-eclosion 12 Day	0.00551	0.00457	0.00661	-16%	
Post-eclosion 12 Day	0.00566	0.00468	0.00676	-14%	
Continual 12 Day	0.00583	0.00479	0.00708	-11%	

4.3.2 Learning Score and Mushroom Body Volume

For each worker that passed the responsiveness test (n= 204) I plotted thorax width against the total number of learnt responses achieved (learning score) by each respective worker (including zero scores). For 3-day adults I found no significant relationship for workers from any of the treatments (Figure 4.3A; Table 4.2). For 12-day adults I also found no significant relationship for workers from pesticide exposed colonies, however, there was a significant negative relationship for workers from *control* colonies (Spearman's rank: -0.67, p=0.034; Figure 4.3B; Table 4.2). This finding indicates that if worker age is not appropriately considered, then body size may not be an appropriate measure when relating to certain behavioural syndromes, as whilst body size does not change after eclosion my findings show that the brain volume does change significantly with age. I next pooled all 3 and 12-day adults per treatment for the workers that had been µCT-scanned and plotted the absolute (uncorrected for body size) and relative (corrected for body size) volume of the combined left and right mushroom bodies against respective learning scores. I found that absolute volume showed no significant relationship with learning score across all treatments, including *control* workers, which in contrast to the findings for thorax width, showed a positive rather than negative slope (Figure 4.3C; Table 4.2). Moreover, when plotting relative brain size, I found a significant positive relationship for workers from *control* colonies (0.62, p=0.019), and yet

there was again no significant relationship for workers from the pesticide exposure colonies (Figure 4.3D; Table 4.2). Critically, this latter finding that suggests that mushroom body function is not only impaired through a reduction in volume of the neuropil but also as a result the physiological composition of the tissue, as despite some pesticide and *control* exposure workers possessing a similar mushroom body size, pesticide exposed workers did not attain the same level of learning performance. However, this must be considered with caution due to the high number of non-learners in the pesticide exposure treatments. I therefore also looked at the relationship between relative MB volume and if a bee was a learner or non-learner. I found no significant effects of MB relative volume on if an individual was a learner or non-learner in any treatment group: *control* ($z=1.55$, $p=0.12$), *pre-eclosion* ($z=0.24$, $p=0.81$), *post-eclosion* ($z=-0.29$, $p=0.77$) and *continual* ($z=-0.15$, $p=0.88$) (Figure 4.4).

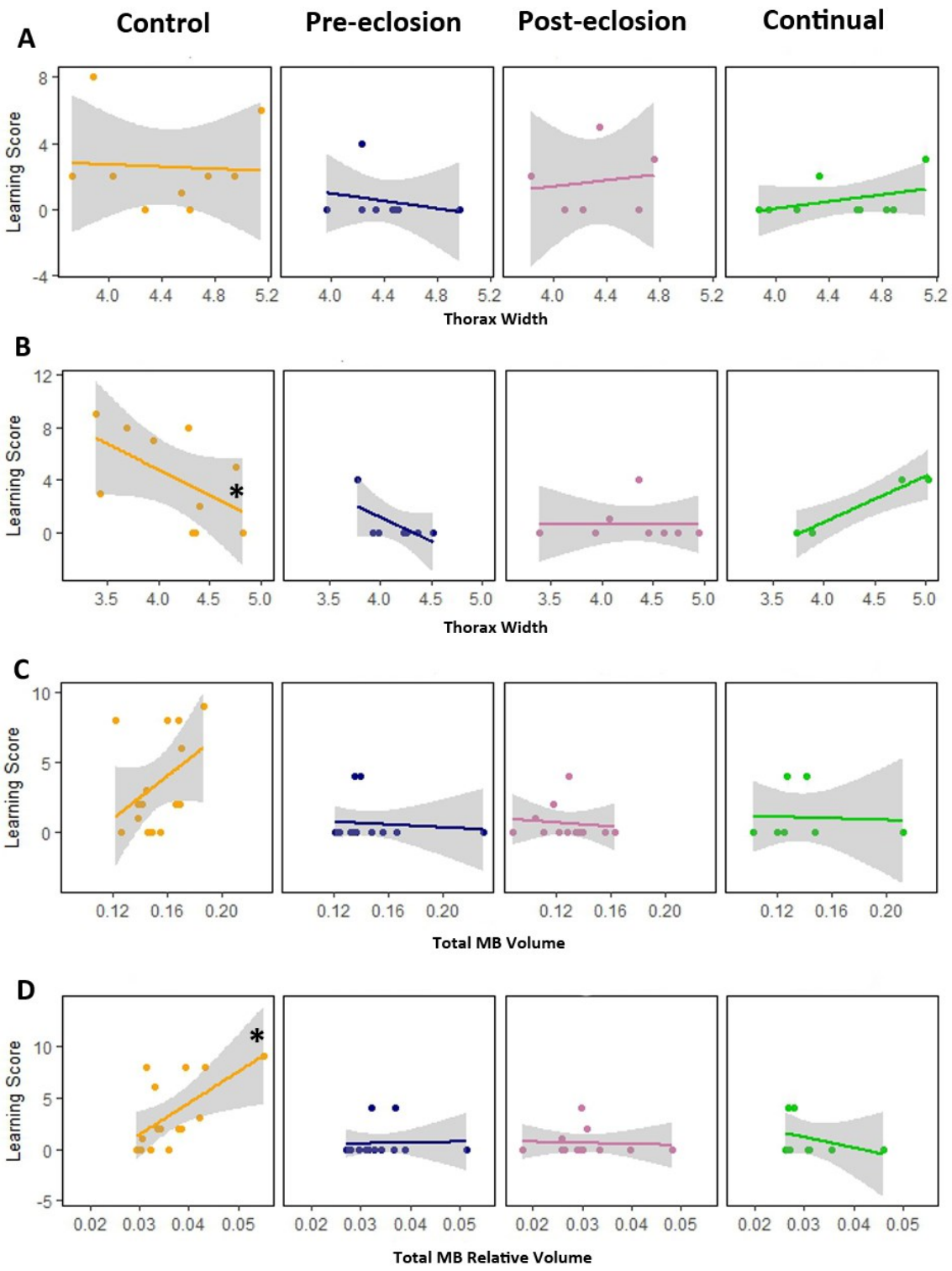


Figure 4.3 Individual body size and brain volumes plotted against respective learning scores. A and B, Thorax width (mm) as a proxy for body size; A) 3-day individuals; B) 12-day individuals. For C and D data points represent 3 and 12-day workers plotted together. C) Total absolute mushroom body (MB) volumes (mm^3) with left and right hemispheres combined; D) Total relative mushroom body (MB) volumes (body size corrected values in mm^3). Asterisks denote significant positive/negative relationships (alpha value = 0.05), and details on slopes and spearman's rank outputs please see Table 4.2.

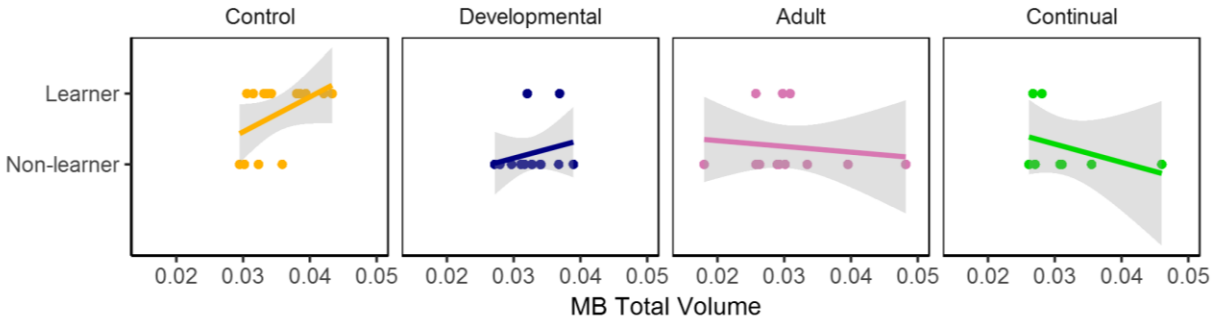


Figure 4.4 Individual mushroom body volume plotted against learners and non-learners. The total relative mushroom body (MB) volumes (body size corrected values in mm3) of learners and non-learners.

MB Component	CONTROL		PRE-ECLOSION		POST-ECLOSION		CONTINUAL	
	SR	p value	SR	p value	SR	p value	SR	p value
Bee Size	-0.433	**0.06405**	-0.432	0.1078	-0.06403	0.8278	0.561517	**0.04584**
Bee Size 3-Day	-0.00874	0.9822	-0.41239	0.31	0.27323	0.6004	0.365148	0.3339
Bee Size 12-Day	-0.67085	**0.03372**	-0.61237	0.1438	-0.2338	0.5773	0.894427	0.1056
Absolute MB volume	0.376003	0.1672	0.101274	0.7305	-0.31208	0.3234	0.251976	0.5472
Relative MB Total	0.651616	**0.0085**	0.202548	0.4874	-0.03672	0.9098	-0.37796	0.3559
Mcal.left	0.54734	**0.0282**	0.101274	0.7305	0.080531	0.7937	-0.6773	**0.04504**
Lcal.left	0.454471	**0.0506**	0.090784	0.7476	0.263821	0.3621	-0.59642	**0.04066**
Mcal.right	0.56948	**0.02669**	0.151911	0.6042	0	1	-0.34816	0.3242
Lcal.right	0.418492	**0.08392**	0.101274	0.7305	0.221462	0.4671	-0.52969	**0.07653**
Calyces.Total	0.622412	**0.01321**	0.151911	0.6042	0.128505	0.6906	-0.37796	0.3559
lobes.left	0.497494	**0.0357**	0.317745	0.2485	-0.15112	0.6061	-0.40134	0.2212
lobes.right	0.472144	**0.04789**	0.151911	0.6042	-0.13063	0.6562	-0.47964	0.1146
MB.left	0.605026	**0.01302**	0.151911	0.6042	0.053688	0.8617	-0.60757	**0.08266**
MB.right	0.651616	**0.008491**	0.101274	0.7305	0.093953	0.7601	-0.34816	0.3242

4.3.3 Antennal Lobes

The antennal lobes were also segmented for each individual (Figure 4.4), in 3-day workers, both the left and right antennal lobes from *post-eclosion* exposure showed a trend of reduction in relative volume compared to the *control* (-21%; t=2.39, p=0.027; -19%; t=2.05, p=0.055), but there was no significant difference for *pre-eclosion* or *continual.* For 12-day workers I found no significant difference between treatments in the relative volumes of the antennal lobes, and this was supported by no significant treatment*age interaction (Figure 4.5; Appendix 4.5).

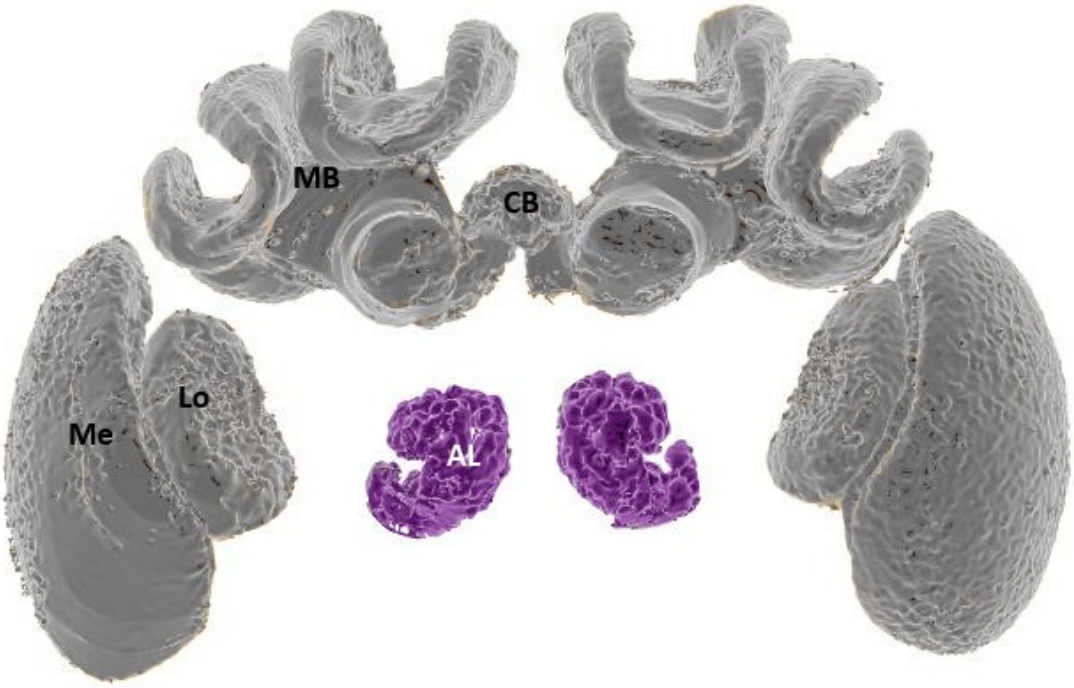


Figure 4.5 3D rendering of bumblebee brain structures from µCT imaging highlighting antennal lobes. Left and right antennal lobes (AL) in purple. Greyed out brain structures show mushroom bodies (MB), medullas (Me), lobulas (Lo) and central body (CB).

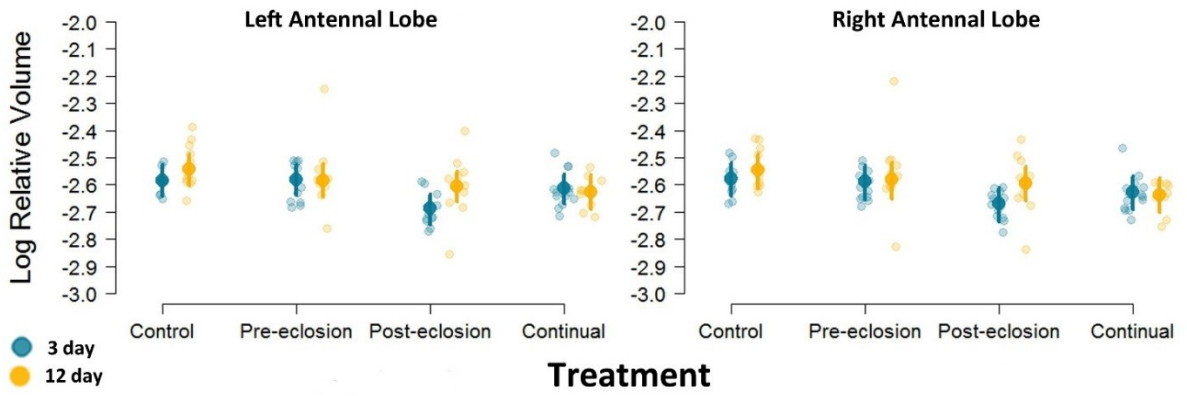


Figure 4.6 Comparison of the volumes (mm3) for each paired left and right antennal lobe, of bumblebee workers. Relative volumes (corrected for worker size) for 3-day (blue) and 12-day (orange) workers have been log-transformed. Large points show model estimates of the mean and bars depict ±95% BCI back-transformed from an LMM, with semi-transparent, jittered points corresponding to the raw data.

4.4 Discussion

In this chapter I investigated how exposure to field-realistic levels (5ppb) of a neonicotinoid pesticide during key developmental stages (brood and early adult development) affected brain neuropil growth of worker bumblebees at different ages (3-day and 12-day). I found that imidacloprid exposure during brood development and in early adulthood can impede growth of the mushroom bodies (MBs) and that these reductions in volume correlate with functional impairments in the form of impaired learning performance.

4.4.1 Age related mushroom body growth

In *control* workers I found consistent age-related growth of the MB calyces and lobes between 3-day and 12-day workers, with the calyces in particular exhibiting large increases in relative volume (lateral mean 16% and medial mean 21%). This age-related growth is consistent with previous findings in bumblebees (*B. impatiens*), although Jones *et al*. (Jones, Leonard et al. 2013) did report a lower ~9% increase in volume of the calyces (total combined) between

these ages. Furthermore, my findings in Chapter 2 showed a significant age enhancement in learning performance in workers, which was also reflected in the age-related volume changes of the mushroom body. My results therefore conform to both previous findings on age-related growth and the expectation that enhanced learning with age would be mirrored by larger MB volume. Based on these findings I would then expect pesticide exposure to impair this age-enhancement.

4.4.2 Pesticide impacts on mushroom body growth

In 3-day workers exposed to pesticide during adulthood (sampled from *post-eclosion* and *continual* treated colonies), I found smaller lateral and medial calyces (avg. -14%) compared to *control*. Indicating that this early period of growth occurring between workers eclosing and ~3 days can be impaired by imidacloprid, even with just 3 days of exposure (*post-eclosion*), highlighting just how sensitive to stressors bumblebees might be during this early developmental phase. This finding is of particular importance, as previous studies have shown that most post eclosion brain growth in bumblebees occurs within these first few days of adulthood, and most notably in the calyces of the MB (Riveros and Gronenberg 2010) (Jones, Leonard et al. 2013). In addition to the reduced level of MB growth in young bees, we found that the age-related volume increase between 3-day and 12-day workers was also inhibited for the lateral and medial calyces, with the relative volumes of 12-day worker calyces being, on average, smaller than that of *control* 12-day workers but also smaller than those of 3-day (*control*) workers. This was evidenced in all three pesticide exposure treatments, including *pre-eclosion,* where despite exposure here being limited to the period of brood development, with no exposure as adults, they exhibited the same response as *post-eclosion* (adult) exposure workers.

For *post-eclosion* and *continual* exposure workers this inhibited plasticity could be explained by neonicotinoids directly affecting neuronal signalling of the Kenyon cells (KCs), which are densely packed to form the MBs (Kenyon 1896). For instance, electrophysiological studies on honeybees have shown that Kenyon cells exposed to neonicotinoids (imidacloprid and clothianidin) undergo tonic depolarisation and (subsequent) effects on sensitivity to acetylcholine (Ach) (Palmer, Moffat et al. 2013, Moffat, Pacheco et al. 2015). And neurotransmitters such as Ach have been linked with roles in regulation of experience-dependent neuropil growth (Ismail, Robinson et al. 2006, Dobrin, Herlihy et al. 2011). It is these resultant non-functional cells that likely lead to the previously reported impaired MB function, that is the integration of multimodal signals, learning and memory (Aliouane, el Hassani et al. 2009, Williamson and Wright 2013, Tan, Chen et al. 2015, Wright, Softley et al. 2015) potentially through this inhibited plasticity of the MB calyces. Indeed synaptic structures of the calyces, microglomeruli, have been shown to increase in density with learning and memory performance (Hourcade, Muenz et al. 2010, Li, MaBouDi et al. 2017) and synaptic organization and structural changes in the calyces have been linked to age (Groh, Lu et al. 2012, Fahrbach and Van Nest 2016) sensory experience (Stieb, Hellwig et al. 2012), behavioural changes (Krofczik, Khojasteh et al. 2008) and foraging (Farris, Robinson et al. 2001). That *pre-eclosion* workers were affected indicates that neonicotinoid exposure could be impeding neurogenesis - preventing neuronal precursor cells from giving rise to Kenyon cells, which occurs only during pre-eclosion development and not in adults (Fahrbach, Strande et al. 1995, Farris, Robinson et al. 1999). For example, thermal stress during larval and pupal development of *Drosophila melanogaster* has been shown to reduce the number of KCs in the MB through preventing KC proliferation (Wang, Green et al. 2007). Alternatively, reduced volume could be a result of *pre-eclosion* exposure affecting KC size and dendritic growth, as

has been shown from exposure experiments on bumblebee Kenyon cell cultures and extractions (Wilson, Velarde et al. 2013).

The neuronal development of bees can be highly susceptible to stressors. Even small variations in temperature, just 1°C deviation from the usual rearing temperature, during brood development has been shown to affect microglomeruli density at the olfactory input region of the calyces, with these effects persisting into adulthood (Groh, Tautz et al. 2004). Bees may be similarly vulnerable to neonicotinoid exposure during this period resulting in comparable developmental restrictions. However, in contrast to my prediction that developmental impairment would be most prominent in younger newly emerged workers, *pre-eclosion* 12-day bees were more severely affected than 3-day bees. It could be that brood and age-only related developmental growth are relatively unchanged but the impaired neuronal function in the KCs inhibits the experience-dependent plasticity (Riveros and Gronenberg 2010, Jones, Leonard et al. 2013) that manifests with age through increased sensory stimuli and behavioural experiences, resulting in the effects of *pre-eclosion* exposure being more pronounced in older workers, similar to what has been reported previously (Tomé, Martins et al. 2012, Wilson, Velarde et al. 2013).

Distinguishing between the MB components, revealed the differential effects of exposure on volume in the calyces and MB lobes. Unlike the MB calyces where large volume reductions were found, I found no significant effects on volume in the MB lobes. This represents an interesting finding given calyces are the predominant input regions, supplied by afferent neurons carrying nerve impulses from sensory stimuli for processing, whereas the lobes serve predominantly as the output regions with efferent neurons carrying neural impulses away from the MBs. These localized differences in effects may be a reflection of the level of plasticity in these two components of the MB, with the calyces seemingly exhibiting the most

of the brain structures asessed (Durst, Eichmüller et al. 1994, Kühn-Bühlmann and Wehner 2006, Riveros and Gronenberg 2010, Cabirol, Cope et al. 2018).

Whether induced through adult or brood exposure to neonicotinoids, impaired adult plasticity of the MB by imidacloprid, given the strong association between MB plasticity and task performance (Durst, Eichmüller et al. 1994, Gronenberg, Heeren et al. 1996, Kühn-Bühlmann and Wehner 2006, Withers, Day et al. 2008, Riveros and Gronenberg 2010, Cabirol, Cope et al. 2018), will likely have severe consequences for overall adult worker behaviour and their ability to contribute to colony function. Furthermore, that it may only take exposure during part of brood development (regardless of adult exposure) to affect adult brain plasticity indicates bumblebee colonies may be susceptible to even small windows of pesticide exposure.

4.4.3 Mushroom body growth was associated with increased learning performance

Following on from the behavioural tests detailed in Chapter 2, which assessed olfactory associative learning, I looked to see whether neuropil volume or indeed body size was positively correlated with learning performance for *control* exposure workers to test the 'bigger-is-better' hypothesis. The relative volume of the mushroom body significantly increased with age, showing a large degree of apparent innate developmental plasticity over the nine days between 3 and 12-day workers. Importantly, the larger mushroom bodies in 12-day workers was associated with this age cohort also showing higher learning capability, providing support that bigger is indeed better for conditioned olfactory learning as tested in this study (Chapter 2). Moreover, relative volume of all component structures of the MB showed a significant positive correlation with learning score. However, the same relationships were not found for absolute MB volume or indeed body size, with absolute MB volume

showing a positive but non-significant correlation and thorax width showing a negative correlation with learning scores. For bumblebees this suggests that it is relative brain size rather than absolute brain or body size that reflects learning performance, similarly it has been previously reported that learning performance is indeed not dependent on body size in bumblebees (Riveros and Gronenberg 2009, Evans and Raine 2014).

Larger bees, although more likely to forage and forage more efficiently (Goulson, Peat et al. 2002, Spaethe and Weidenmüller 2002) still perform other in nest tasks (Cameron and Robinson 1990) and many workers (regardless of size) will forage in their lifetime (Free 1955), suggesting behavioural plasticity is important to colony function. Unlike honeybees, bumblebees are thought to exhibit a low level of age-based polyethism (Cameron 1989, O'Donnell, Reichardt et al. 2000) with instead, individuals showing patterns of weak specialization on different tasks (Jandt, Huang et al. 2009, Jandt and Dornhaus 2011). Adult workers have been known to carry out foraging behaviour as young as two days after emergence (Yerushalmi, Bodenhaimer et al. 2006, Gill and Raine 2014) but also may not begin foraging until after 30 days (O'Donnell, Reichardt et al. 2000). They can also exhibit olfactory learning, comparable to that of older bees, just 2 hrs after emergence (Riveros and Gronenberg 2009). However, multiple studies report that younger bees are more likely to perform in nest tasks and more mature bees are more likely to forage (Free 1955, Cameron 1989, Jandt, Huang et al. 2009). This indicates that bumblebees emerge developmentally ready to perform the learning required for task performance, requiring only a short period before being able to forage.

Despite body size having no effect on the proportion of learnt responses (Appendix 2.2) and no significant relationship with learning score (or negative for 12-day workers, Figure 4.3;

Table 4.2), body size did have a significant positive effect on probability of a worker being a learner (Appendix 2.2). Given that individuals in this study were only given 10 trials in the learning assay, if and when workers would have eventually shown a learnt response could not be tested, it may be that size influences when workers first learn the association rather than learning score once they have. Does this tell us that size might determine when or if you become a forager, but once you forage size does not predict foraging performance, rather the adult development of the brain (manifest as relative volume) determines performance. And furthermore, it is this observed large, early age-related adult brain development that is vital in preparing workers for the increased complexity of foraging or the ability to switch between tasks in response to colony demands.

4.4.4 Pesticide induced reduction in mushroom body development resulted in poorer learners

With these findings I was able to test the prediction that the impaired learning behaviour observed in pesticide exposure workers was associated with a reduction in mushroom body volume. Indeed, pesticide exposure workers did show reduced mushroom body volumes in a pattern paralleled by our observations of impaired learning behaviour. This was evidenced by 12-day workers from all pesticide exposure treatments exhibited both smaller mushroom body relative volumes than *control* and reduced ability to produce a learnt response, with *post-eclosion* and *pre-eclosion* also showing lower learning scores. Additionally, 3-day workers from *post-eclosion* and *continual* treatments had smaller relative MB volume, and although this was not reflected in reduced learning performance, workers from these pesticide treatments did also exhibit reduced responsiveness.

Plotting each individual's mushroom body relative volume against their learning score revealed a positive correlation for *control* workers, demonstrating that disproportionately

large mushroom bodies for the individual's size performed better in the learning assay. Yet, critically, this relationship was not found for workers from all three pesticide exposure treatments. Furthermore, despite some of the pesticide exposure workers possessing similar relative mushroom body volumes to *control* workers, pesticide exposure workers still demonstrated a lower learning score (in fact often zero). Indicating that not only is reduced relative volume associated with impaired learning, but also that neuronal functioning of this region must have been impaired. This reiteration of the developmental susceptibility of young (callow) workers to pesticide exposure, may be explained when considering the rates of brain growth following adult emergence. As previously highlighted as much as 12% of adult MB calyces growth can occur during the first 72hrs (Jones, Leonard et al. 2013) and this study found an avg. 18% increase between 3-day and 12-day workers. That workers usually undergo such high rates of brain development during these early days post eclosion may explain why stress during this phase produced the behavioural effects I observed. These results taken together present a strong link between pesticide exposure, MB plasticity and subsequent function.

4.4.5 The effect of pesticide exposure on Antennal lobe growth

The antennal lobes showed an avg. 9% volume increase between 3-day and 12-day workers in contrast to the ~18% increase reported by Jones *et al* (Jones, Leonard et al. 2013), however with the caveat of differences between studies in the bumblebee species, sensory experience of individuals and volume estimation. Unlike in the MBs, the effects of pesticide exposure on AL volume were restricted to 3-day workers and observed only in the *post-eclosion* pesticide treatment suggesting developmental exposure does not translate to adult impairment. I

found no significant difference in volume in any pesticide treatment for 12-day workers compared to 12-day *control* individuals.

The antennal lobes are the first order olfactory neuropil, where olfactory information is initially detected and then processed in the bee brain (Hansson and Anton 2000) and usually undergo a burst of growth in the first 3 days after eclosion (Jones, Leonard et al. 2013). I expected that my findings of the impairment of this early adult development in the AL would correlate with the reduced responsiveness to a sucrose reward as evidenced in Chapter 2. Given that olfactory function of the antennal lobe is linked to (AL) glomeruli volume, with increases associated with improved learning performance (Sigg, Thompson et al. 1997) long-term olfactory memory (Hourcade, Perisse et al. 2009) and foraging experience (Brown, Napper et al. 2004). However, this was not the case, in fact the treatment group in which a reduction in AL volume was observed was one in which no significant reduction in learning performance was found. However, responsiveness was significantly reduced in 3-day *post-eclosion* workers, the same as those with reduced AL volume. This impairment of the ALs may be associated with the reduced sucrose responsiveness of these workers I found in chapter 2, through the action of the neonicotinoid (agonists of nicotinic acetylcholine receptors (nAChRs)), honeybees whose antennal lobes were injected with nicotinic antagonists showed changes in sucrose sensitivity (Thany and Gauthier 2005). Although to fully assess changes in the brain associated with sucrose responsiveness, a study of the volume of the subesophageal ganglion – where the central processing of gustatory information occurs (Mitchell, Itagaki et al. 1999, de Brito Sanchez 2011), is required. In addition to direct effects, pesticide action on the ALs could have knock-on effects to further brain neuropils. The AL neurons project into the MB via the input regions of the calyces (Kirschner, Kleineidam et al. 2006), and so effects of neonicotinoid action on cellular function here at the initial site of olfactory information

processing could impair the information subsequently input to the MB calyces. With this potentially further preventing experience-dependent growth and compounding the direct effects of neonicotinoid action in the MBs.

4.4.6 Implications

To conclude, my findings in chapter 2 showed that brood and early adult exposure to neonicotinoids can have both immediate and delayed effects on later adult behavioural performance. Here I show that exposure during these two key developmental phases also impedes the growth of the mushroom bodies (MBs) and antennal lobes of the brain and that these volumetric reductions correlate with functional impairments of reduced responsiveness and learning performance. Bumblebee colonies are reliant on newly emerging cohorts of workers to be effective task performers, with adult workers shown to carry out foraging behaviour as young as two days after emergence (Yerushalmi, Bodenhaimer et al. 2006, Gill and Raine 2014). My results show that pesticde exposure during brood development or the first few days of adulthood can lead to workers with developmentally and functionally impaired regions of the brain, suggesting colonies may be producing an impaired workfore, sending out foragers that are physiologically ill-prepared for the task. (Perry, Søvik et al. 2015). And whilst a colony can withstand the functional loss of some adult workers, increasing impairment of newly emerging individuals will put a strain on the rest of the workforce, eventually leading to an impaired colony (Bryden, Gill et al. 2013).

Chapter 5

EFFECTS OF PESTICIDE EXPOSURE ON THE MORPHOLOGY OF FURTHER BRAIN STRUCTURES – THE OPTIC LOBES AND CENTRAL BODY

5.1 INTRODUCTION

Bumblebees forage in large, complex landscapes requiring their brains to rapidly detect and respond to numerous diverse stimuli in a wide range of sensory modalities including chemical, visual, auditory and somatosensory (Collett and Collett 2002, Wehner 2003, Collett, Graham et al. 2006, Kulahci, Dornhaus et al. 2008, Merkle and Wehner 2008, Lihoreau, Chittka et al. 2010, Philippides, Baddeley et al. 2011, Lihoreau, Chittka et al. 2012, Lihoreau, Raine et al. 2012, Collett, Chittka et al. 2013, Rössler and Brill 2013). The ability to distinguish and integrate an array of sensory cues while physically mobilising themselves around obstacles across the landscape in fast flight is vital. For example multimodal signals have been shown to be important for the accuracy of decision making in foraging (Kulahci, Dornhaus et al. 2008, Leonard, Dornhaus et al. 2011). Beyond the mushroom bodies, multiple other neuropils are responsible for the integrated processing of information that enable bees to carry out the repertoire of behaviours needed for foraging. Therefore, it is important to understand the brain structures that facilitate these processes and how their morphological development and

subsequent function could be affected by exposure to pesticides. Here I focus on the medulla and lobula (the optic lobes) along with the central body.

5.1.1 Central Body

The central body is largely considered to be involved in complex motor function, with a role in the control of walking, spatial orientation and providing general motor control in insects (Strausfeld 1999, Strauss 2002). It could be considered analogous to the vertebrate motor control centres in the brain, the cerebellum or the basal ganglia. In bees it is suggested that the central body processes multisensory information, with studies showing neurons respond to visual, olfactory and mechanical stimuli (Homberg 1985, Milde 1988). Further studies also suggest a role in the formation of visual memory and visual processing (Liu, Seiler et al. 2006, Homberg 2008, Heinze, Gotthardt et al. 2009). And studies in *D. melanogaster*, using mutants with structural defects of the central body, suggest a role in learning and memory with regards to visual (Liu, Seiler et al. 2006, Ofstad, Zuker et al. 2011), olfactory (Heisenberg, Borst et al. 1985), and gustatory (Bouhouche, Vaysse et al. 1993) spatial learning paradigms. Given the array of associated functions of the CB, impaired development and function could have serious implications for the ability of workers to carry out colony tasks – most notably, foraging.

Indeed, previous studies have indicated that pesticides can alter motor function in bees. Neonicotinoids have been shown to impair honeybee performance of the waggle dance – with significantly fewer circuits of the waggle dance performed in honeybees 24 hours after being exposed to imidacloprid (Eiri and Nieh 2012). Bees orally exposed to imidacloprid for 24 hours also spent more time on their back and were more likely to fail to right themselves (Williamson, Willis et al. 2014). And at low doses (1.25 ng/bee) imidacloprid has been found

to induce increased motor function in honeybees (Lambin, Armengaud et al. 2001). Furthermore, pesticide exposure has also been linked to effects on orientation; Henry *et al*. (Henry, Beguin et al. 2012) showed that sub-lethal doses of the neonicotinoid thiamethoxam decreased the chance of honey bee (*Apis mellifera*) foragers locating their colony leading to increased mortality. Similar results for honeybees were found by Fischer *et al*. (Fischer, Müller et al. 2014), showing individuals exposed to imidacloprid, clothianidin or thiacloprid all exhibited significantly lower successful return flights in catch and release experiments, along with reduced probability of responding to a salient landscape structure and less directed flights during homing. Taken together, these results suggest an interaction between neonicotinoids and the central body – potentially giving rise to this impaired function. However, whether these impairments are linked to the development or growth of the CB is yet untested.

5.1.2 Optic Lobes

In order to forage effectively, bumblebee workers must visualise colour, recognise and distinguish patterns and detect motion allowing them to locate, identify and navigate towards suitable flower patches (Ney-Nifle, Keasar et al. 2001, Lotto and Chittka 2005, Dyer, Spaethe et al. 2008, Dyer, Paulk et al. 2011, Foster, Sharkey et al. 2014). Bees possess true colour vision (Peitsch, Fietz et al. 1992) and display an impressive capacity for visual learning (Srinivasan 1994, Zhang and Srinivasan 1994, Srinivasan and Zhang 1998, Strang and Sherry 2014). They can use colour cues to learn patterns and shapes (Zhang, Srinivasan et al. 1995, Hempel de Ibarra, Giurfa et al. 2002, Hempel de Ibarra, Vorobyev et al. 2014) and achromatic cues to detect motion and optic flow along with orientation (Zhang and Srinivasan 1994, Giger and Srinivasan 1996, Lehrer 1997, Chittka and Tautz 2003). The brain neuropils that are

responsible for detection and initial processing of visual information in insects are the optic lobes which include the paired structures of the medulla and lobula that connect to the eyes. The flow of visual information in the bee eye begins where visual signals are detected by photoreceptors in the retina, these input to the visual processing centres, the lamina, medulla (Me) and lobula (Lo) of the brain (Strausfeld 1976). Most neurons of the medulla project into the lobula from which neurons project to the calyces of the MB - although additionally some neurons circumvent the lobula outputting directly to the central brain (Strausfeld 1976, Ribi and Scheel 1981).

As with other neuropils of the bumblebee brain the optic lobes have been reported to exhibit age and experience dependent changes in volume (Riveros and Gronenberg 2010, Jones, Leonard et al. 2013). Additionally, volume changes in the optic lobes of other eusocial insects that possess similar brain structures to bumblebees, have been shown to correlate with behavioural changes: in *Messor pergandei* ant queens, there is a volume reduction of the medulla correlated with a change from being attracted to the light and open spaces prior to mating, to being attracted to the dark once mating has taken place (Julian 2002). And in ants *Cataglyphis bicolor*, increased volume of the medulla optic lobe was associated with foraging experience (Kühn-Bühlmann and Wehner 2006).

Exposure to neonicotinoid pesticides has been linked to effects on the visual learning capacity of bees; Honeybees orally exposed to imidacloprid exhibited reduced visual learning capacities in T-tube maze evaluation (Han, Niu et al. 2010). Studies have also indicated that neonicotinoid exposure in bees may impair their ability to recognize visual cues during foraging, for example Fischer *et al*. (Fischer, Müller et al. 2014) found that imidacloprid, clothianidin or thiacloprid each reduced the ability of homing bees to respond to structural

landscape cues. With further studies indicating reduced navigation and orientation, honeybee foragers exposed to sub-lethal doses of the neonicotinoid thiamethoxam were less likely to locate their colony, leading to increased mortality (Henry, Beguin et al. 2012). Whether such behavioural impairments are reflected in changes to the developmental or adult growth of the optic lobes is yet to be investigated.

5.2 METHODS

For methods of experimental set-up and exposure see Chapter 2 (pages 57-67), and for micro-CT scanning and image analysis see Chapters 3 and 4 (pages 87-94 and 117-119, respectively).

5.3 RESULTS

From the bees that performed the full PER assay, I reconstructed each right and left medulla and lobula along with the central body for 92 workers (*control*=21; *pre-eclosion*=23; *post-eclosion*=24; *continual*=24; Figure 5.1). Correcting for body size variation, I measured relative volumes for each independent structure.

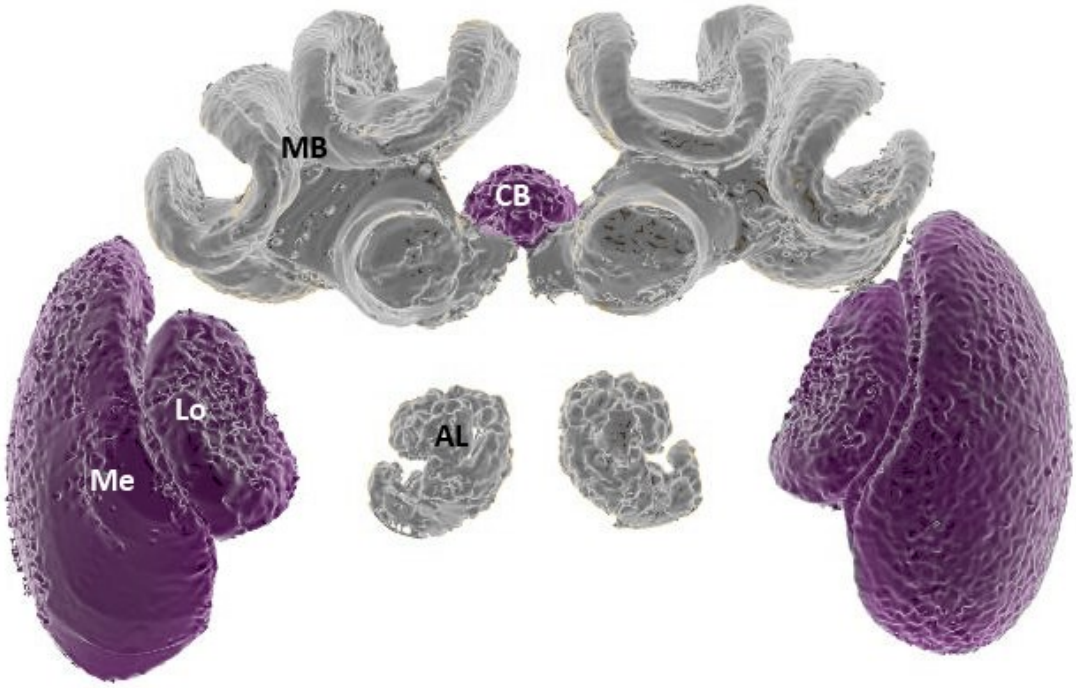


Figure 5.1 3D rendering of bumblebee brain structures from µCT imaging highlighting the optic lobes and central body. Left and right optic lobes; medulla (Me) and Lobula (Lo), and central body (CB) in purple. Greyed out brain structures show mushroom bodies (MB) and antennal lobes (AL).

5.3.1 Central Body

In the central body (CB) I detected a slight average volumetric increase from 3 to 12 days workers from *control* colonies, but this effect of age on relative volume was not found to be significant (mean model estimated %Δ = 5%; t=0.118 p=0.906)

Considering the pesticide exposure treatment groups, I found no significant effect on volume compared to *control* for either age cohort (Figure 5.1, Appendix 5.1). However, there were consistently smaller relative volumes for each pesticide exposure treatment compared to *control* (Table 5.1) with the largest decreases found in *pre-eclosion* workers. This is perhaps to be expected given that the CB exhibits relatively little adult plasticity and the majority of

its development will occur during pupal development, in conjunction with the *pre-eclosion* exposure period.

In line with previous findings I also found a negative effect of size (thorax width) on relative volume of the central body (t=-3.294; p=0.00157).

	Back transformed log model mean	Lower CI	Upper CI	Difference from *Control*	Age Difference
Central Body					
Control 3 Day	0.000608184	0.000521125	0.000695243		
Pre-eclosion 3 Day	0.000548572	0.000476697	0.000620447	-10%	
Post-eclosion 3 Day	0.000568997	0.000497573	0.000640421	-6%	
Continual 3 Day	0.000596425	0.000524406	0.000668444	-2%	
Control 12 Day	0.000635569	0.000544338	0.000726801		5%
Pre-eclosion 12 Day	0.000540898	0.000457296	0.0006245	-15%	
Post-eclosion 12 Day	0.000548812	0.000474077	0.000623546	-14%	
Continual 12 Day	0.000584681	0.000496678	0.000672684	-8%	

5.3.2 Optic Lobes

For 3-day workers I found no significant effect on relative volume of any of the optic lobes, the (left and right) Lobula and medulla, in any pesticide treatment group (Figure 5.2, Appendix 5.1), there were if anything, slight increases in the *continual* treatment. Considering 12-day workers only, there was also no significant effect of pesticide exposure (*Pre-eclosion*, *post-eclosion* or *continual*) on relative volume (Appendix 5.2): I observed slight reductions in relative volume in the medullas of *pre-eclosion* workers, slight increases in the lobulas, with larger increases in the medullas for *post-eclosion* and slight reductions in relative volume in each optic lobe component for *continual* (Table 5.2).

I detected a consistent increase in relative volume of the optic lobes with age; left and right Lo (%Δ =12% for both), left and right Me (%Δ =10% and 9% respectively, Figure 5.1, Table 5.2), however I found no significant effect of age (Appendix 5.1).

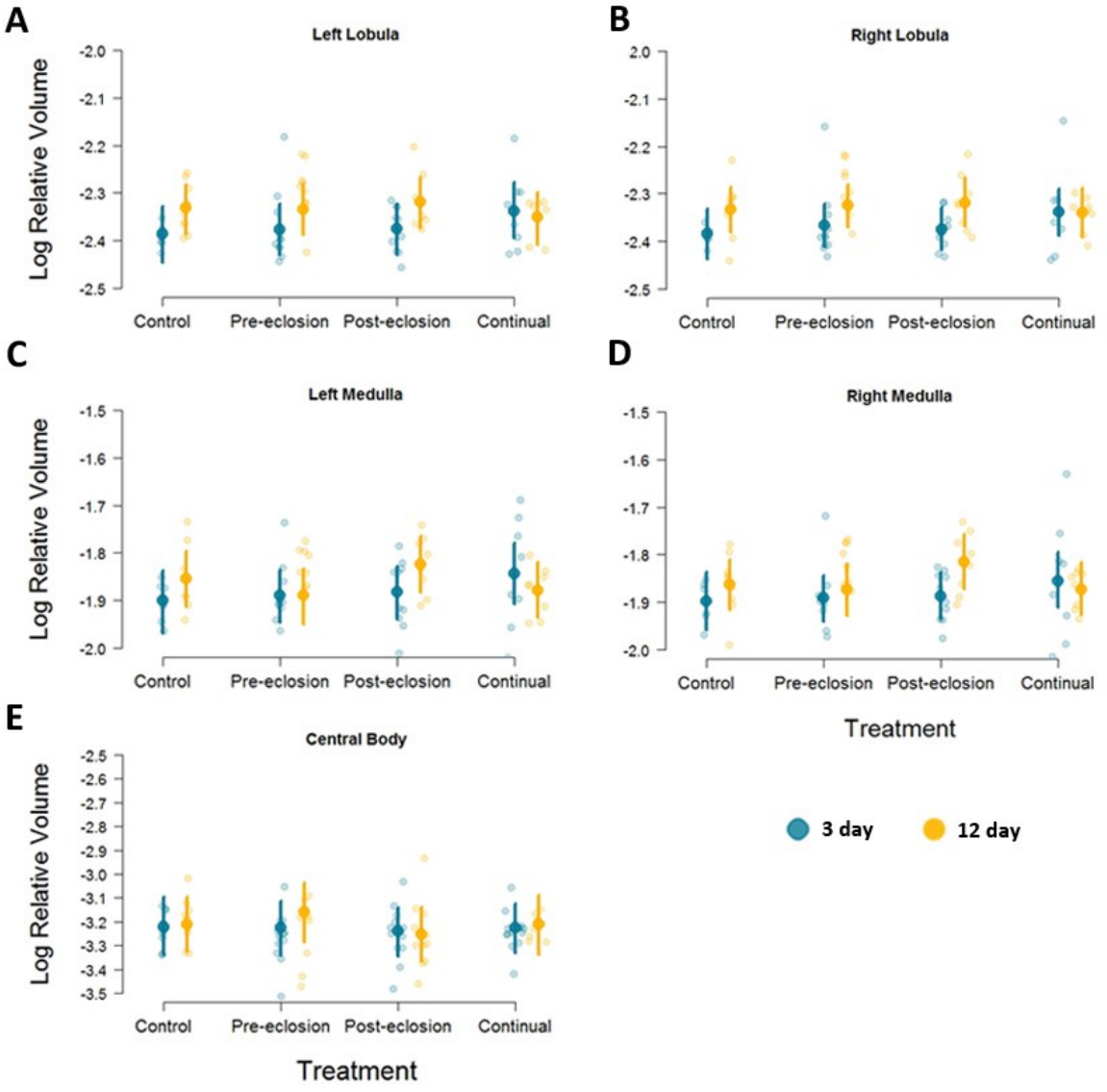


Figure 5.2 Comparison of the volumes (mm3) for each paired left and right lobula and medulla, and the central body, of bumblebee workers. Relative volumes (corrected for worker size) for 3-day (blue) and 12-day (orange) workers have been log-transformed. Large points show model estimates of the mean and bars depict ±95% BCI back-transformed from an LMM, with semi-transparent, jittered points corresponding to the raw data.

	Back transformed log model mean	Lower CI	Upper CI	Difference from *Control*	Age Difference
Left Lobula					
Control 3 Day	*0.00412*	*0.00355*	*0.00469*		
Pre-eclosion 3 Day	0.00421	0.00368	0.00474	2%	
Post-eclosion 3 Day	0.00423	0.00370	0.00477	3%	
Continual 3 Day	0.00459	0.00396	0.00522	11%	
Control 12 Day	*0.00463*	*0.00404*	*0.00522*		12%
Pre-eclosion 12 Day	0.00464	0.00405	0.00524	0%	
Post-eclosion 12 Day	0.00482	0.00418	0.00545	4%	
Continual 12 Day	0.00449	0.00389	0.00509	-3%	
Right Lobula					
Control 3 Day	*0.00414*	*0.00361*	*0.00467*		
Pre-eclosion 3 Day	0.00430	0.00382	0.00478	4%	
Post-eclosion 3 Day	0.00421	0.00373	0.00469	2%	
Continual 3 Day	0.00458	0.00401	0.00515	11%	
Control 12 Day	*0.00462*	*0.00409*	*0.00516*		12%
Pre-eclosion 12 Day	0.00475	0.00420	0.00530	3%	
Post-eclosion 12 Day	0.00481	0.00423	0.00538	4%	
Continual 12 Day	0.00454	0.00399	0.00509	-2%	
Left Medulla					
Control 3 Day	*0.01265*	*0.01070*	*0.01461*		
Pre-eclosion 3 Day	0.01288	0.01112	0.01463	2%	
Post-eclosion 3 Day	0.01312	0.01131	0.01493	4%	
Continual 3 Day	0.01433	0.01215	0.01650	13%	
Control 12 Day	*0.01397*	*0.01201*	*0.01593*		10%
Pre-eclosion 12 Day	0.01291	0.01110	0.01471	-8%	
Post-eclosion 12 Day	0.01504	0.01285	0.01724	8%	
Continual 12 Day	0.01321	0.01127	0.01516	-5%	
Right Medulla					
Control 3 Day	*0.01261*	*0.01080*	*0.01442*		
Pre-eclosion 3 Day	0.01285	0.01128	0.01442	2%	
Post-eclosion 3 Day	0.01297	0.01136	0.01458	3%	
Continual 3 Day	0.01399	0.01205	0.01593	11%	
Control 12 Day	*0.01373*	*0.01197*	*0.01548*		9%
Pre-eclosion 12 Day	0.01341	0.01171	0.01511	-2%	
Post-eclosion 12 Day	0.01526	0.01321	0.01731	11%	
Continual 12 Day	0.01342	0.01160	0.01523	-2%	

5.4 DISCUSSION

In contrast to the significant volumetric reductions that were detected in the mushroom bodies and antennal lobes in pesticide exposure treatment groups, there were no detectable

significant effects on the volume of the optic lobes or central body. Whilst my study only considered volume and not composition of the neuropil, it seems as though the MBs are disproportionately affected. This finding suggests that it is the primary effect on MB developmental plasticity, rather than other neuropils, that is the mechanism for explaining issues with behavioural function from pesticide exposure.

5.4.1 Potential explanations for this disproportionate effect?

I suggest three (non-mutually exclusive) explanations for the disproportionate effect on the MB's and AL's over the optic lobes and CB:

(1) It may be that the optic lobes and central body growth were affected by pesticide exposure, but the volume change was too subtle for our µCT technology or experimental sample sizes to detect this. For example, although not significant, there was a consistent trend of lower CB volumes in all pesticide exposure treatments compared to *control.*

(2) Neonicotinoids are an agonist of the nicotinic acetyl-choline receptors found in large numbers in bee Kenyon cells that, compared to the other neuropils, are in much higher density in the mushroom bodies (Rössler and Groh 2012). This could suggest that the impaired visual learning and motor function previously reported manifests through distribution in the MB where multimodal information from optic lobes (and other neuropils) is integrated as opposed to impairment of the initial processing in the medulla, lobula or CB itself. Further to this, the role of the mushroom bodies in the formation of memory denotes a high level of synaptic plasticity, and therefore a higher rate of growth is still expected during adulthood (Farris, Robinson et al. 2001, Heisenberg 2003, Jones, Leonard et al. 2013). In contrast, based on the findings here and previous studies the CB exhibits little or no adult growth, whilst the optic lobes exhibit less plasticity than the MB's and AL's (Riveros and Gronenberg 2010, Jones,

Leonard et al. 2013). Therefore, functional impairment of these neuropils may not be reflected in volume changes as appears the case in the MB's.

(3) Whilst my experimental setup likely prevented visual stimuli, olfactory stimuli could not be fully excluded without isolating individuals. Within a colony nest there are numerous volatile and non-volatile odours stemming from the pollen and wax, as well as hydrocarbon profiles of colony members detected though antennal contact. It is therefore possible that MB and AL growth with age were influenced or enhanced to some degree by these olfactory stimuli or by the lack of visual stimuli in comparison to the optic lobes. Indeed, bees raised without visual stimuli were shown to have increased MB and AL volume – suggesting an investment in olfactory processing to compensate for a lack of visual information (Jones, Leonard et al. 2013). The inverse of which however was not found, with the optic lobe volume not affected by the absences of olfactory stimuli, although as with my study olfactory stimuli could not be fully removed from the experiment setup. It is possible that the effects of pesticide exposure are acting to reduce this experience (as discussed in Chapter 4), or compensatory growth and thus the lack of experience related plasticity in the optic lobes influenced by their environmental conditions masked the effects of pesticide exposure.

In order to fully explore the effects of pesticide exposure on the optic lobes and central body would requiring coupling the volume estimates with relevant behavioural tests such as those assessing visual learning performance and motor control. Despite the lack of effects on the growth of the optic lobes and CB in pesticide exposure treatments I would expect reduced performance in such behavioural tests, driven either by functional impairment in these structures or through the impaired developmental plasticity of the MB's that I report. Furthermore, given the findings I report it would be important to re-assess the effects of pre

and post-eclosion pesticide under visually stimulating conditions to determine whether experience related growth of the optic lobes is what is in fact impeded.

Chapter 6

GENERAL DISCUSSION

6.1 SUMMARY OF FINDINGS

In Chapter 2 I found that individual worker bumblebees exposed to sub-lethal, field realistic levels of a major neonicotinoid pesticide during two key developmental phases suffered impaired olfactory learning as adults. I show that older 12-day workers (rather than 3-day workers) exhibited a reduced ability to learn and overall learning level, whether exposed as adults or during brood development indicating that the observed age-enhancement in learning performance is also impeded. Previous studies have shown that larval exposure to neonicotinoids can impair adult olfactory learning in honeybees (Yang, Chang et al. 2012, Tan, Chen et al. 2015), however these studies are limited by artificial exposure methods or high doses of neonicotinoids unlikely to be encountered in the field. My results show for the first time, that bumblebees are also susceptible to neonicotinoid exposure during brood development and that these effects can persist into later adulthood. Furthermore, I showed that responsiveness to sucrose was also reduced in young (3-day) bees when exposed to neonicotinoids as adults but not when exposed during brood. The reduction in both olfactory learning performance and responsiveness to sucrose stemming from exposure to field-realistic levels of a neonicotinoid pesticide can provide a mechanistic explanation for

previously reported effects on colony function, such as decreased foraging efficiency (Gill, Ramos-Rodriguez et al. 2012, Feltham, Park et al. 2014, Gill and Raine 2014). Furthermore, the finding that workers reared in neonicotinoid contaminated nests can emerge with a reduced capacity to learn and less able to improve with age, means that colonies foraging on neonicotinoid treated food sources may be producing a behaviourally impaired workforce that are less able to efficiently contribute to the colony.

In chapter 3 I presented the development and validation of a new protocol to explore variation in tiny soft tissue structures using micro-CT and image analysis that allowed me to investigate changes to bumblebee brain morphology. The majority of past studies investigating variation in insect brain morphology have required the application of histological techniques (Durst, Eichmüller et al. 1994, Withers, Fahrbach et al. 1995, Molina and O'Donnell 2007, Riveros and Gronenberg 2009, Gronenberg and Couvillon 2010, Riveros and Gronenberg 2010, Jandt, Bengston et al. 2014). Such studies however can require invasive dissection, time-consuming fixing and physical tissue slicing using a microtome (Friedrich and Beutel 2008), leading to tissue distortion, desiccation and permanent damage to the sample, and crucially suffer biased measurements that impede accurate quantification of morphology (Andersen and Gundersen 1999, Dorph-Petersen, Nyengaard et al. 2001, Ju, Warren et al. 2006, Simmons and Swanson 2009). My method facilitated the production of high quality 3D reconstructions of 19 bumblebee brains with high resolution and precision, reduced error (compared to existing methods) and that can be revisited for repeated new exploration and comparative analyses of tissue structure without the need for additional sample preparation. Moreover, this technology and the applied methodology can be used to look at soft tissue trauma, allowing the exploration of post-exposure response to chemicals or any stressor in question (i.e. temperature).

In Chapter 4, I applied this new protocol for visualizing and evaluating the bee brain to estimate differences in volume of specific structures of the brain associated with olfactory learning and memory of individual worker bees. Following on from their experimental exposure detailed in chapter 2, I found that the volume of the MB increases with age and correlates positively with learning performance but pesticide exposure during brood or early adult development impairs this growth which in turn correlated with reduced olfactory learning performance. The mushroom bodies (MBs) are the centres for sensory integration, learning and memory in insects (Erber, Homberg et al. 1987, Heisenberg 1998, Zars 2000) and these functions are dependent on their development and functional and structural plasticity (Farris, Robinson et al. 2001, Withers, Day et al. 2008, Riveros and Gronenberg 2010, Cabirol, Cope et al. 2018). A large amount of post eclosion brain growth in bumblebees occurs within the first few days of adulthood (Riveros and Gronenberg 2010, Jones, Leonard et al. 2013), my results show that young workers exposed to a neonicotinoid during this key development phase have (-14%) smaller MB calyces. That just 3 days of exposure (*post-eclosion*) can lead to these levels of impaired growth highlights just how sensitive to stressors bumblebees are during this early stage. Furthermore, mirroring the effects of learning in chapter 2, the age-related volume increase of the MB's between young and old workers was inhibited by pesticide exposure during brood or early adult development. In addition, I found that the larger mushroom bodies of older workers correlated with this age cohort also showing greater learning performance. And in support of my predictions I showed that pesticide exposure workers that exhibited impaired learning also had on average reduced mushroom body volumes. In this chapter I also revealed an effect on the volume of the antennal lobes in young 3-day bees following exposure to pesticide as adults, interestingly in a similar pattern seen in effects on responsiveness shown in chapter 2. Finally, in Chapter 5 I found that further

structures of the bumblebee brain – the Optic lobes and the central body exhibited a differential response to both pesticide exposure and age compared to that in the MB's and AL's, detecting no effects on initial development or age-enhanced growth. That I didn't find the same pattern of effects in these other neuropils suggests that MBs are disproportionately affected and therefore could provide the mechanistic explanation of that it is primarily impaired MB developmental plasticity that is causing the behavioural issues seen in this and other studies.

6.2 Implications for bumblebees and other insect pollinators

Recent studies investigating the effect of pesticide exposure on the growth of social bee colonies have reported effects being manifested as late as 2-3 weeks from the onset of exposure (Gill, Ramos-Rodriguez et al. 2012, Rundlof, Andersson et al. 2015, Arce, David et al. 2017, Tsvetkov, Samson-Robert et al. 2017), and even lag effects of a few weeks after a short period of initial exposure (Whitehorn, O'Connor et al. 2012, Siviter, Koricheva et al. 2018). The findings presented here of early exposure to neonicotinoids having both immediate and delayed effects on later adult behaviour can therefore provide a mechanistic explanation for these previous reports. With eusocial bee colonies having overlapping generations, colonies are reliant on newly emerging cohorts of workers to be effective task performers. Whilst the death of a few adult workers may not necessarily be an issue, if a colony's future generations of workers are predisposed to be a less efficient functioning cohort this can have serious implications for the future fate of the colony. Bryden *et al*. (Bryden, Gill et al. 2013) modelled how increasing proportions of impaired individual workers requires colony members to pick-up the slack leading to a density dependent build-up of colony level impairment leading to a higher risk of causing colony collapse. Bumblebees, although they exhibit a low level of age-

based polyethism (Cameron 1989, O'Donnell, Reichardt et al. 2000), adult workers have been known to carry out foraging behaviour as young as two days after emergence (Yerushalmi, Bodenhaimer et al. 2006, Gill and Raine 2014). Yet I show that pesticde exposure leading up to, or during, the first few days of adulthood means colonies may be sending out foragers that possess functionally impaired regions of the brain and of a volume that may be representative of a younger worker effectively representing a physiologically ill-prepared precocious forager (Perry, Søvik et al. 2015). Furthermore, our results suggest that even if workers were to adaptively respond and delay performance of a task in order to try and developmentally recover, this strategy may be futile as 12-day workers that were exposed during brood but not as an adult still showed the same learning capability as 3-day workers experiencing the same treatment. Exposure during brood development can therefore affect at least half a worker's life-span, and further prolonged experiments could elucidate whether it causes permanent damage. Indeed, if bumblebees are be unable to recover from early developmental exposure then irrespective of the timing of exposure, be it during brood development or as adults or whether exposure is reduced or ceases during the colony lifetime, the damage is already done. For example, oilseed rape, a major neonicotinoid treated crop frequently foraged on by bees (Stanley, Gunning et al. 2013) has mass, pulsed flowering, thus once flowing subsides pesticide exposure of surrounding colonies may be reduced or cease. An ability of individuals to recover from this point forward could reverse the impact of these effects on the colony (Laycock and Cresswell 2013), however my findings suggest that at this point it may be too late due to irrecoverable effects from early developmental exposure.

6.3 LIMITATIONS

6.3.1 Chapter 2 limitations

I assessed the learning performance of bumblebees using the PER learning assay as previously performed in past studies (Riveros and Gronenberg 2009, Stanley 2015). However, I used a lower number of learning trials (10), than are typically implemented for studies on bumblebees which have been shown to exhibit lower rates of success in PER conditioning studies than honeybees (Honeybee: (Bitterman, Menzel et al. 1983, Laloi, Gallois et al. 2001, Wright, Carlton et al. 2009) Bumblebee: (Riveros and Gronenberg 2009, Sommerlandt, Rössler et al. 2014)). Studies therefore often use 15 or more rewarding trials in olfactory PER conditioning for bumblebees (Riveros and Gronenberg 2009, Smith and Raine 2014, Stanley 2015), and doing so in my study would have allowed a more robust investigation into how learning performance correlates with volume changes in the bee brain. However, as the experimental set up meant a trade-off between the number of learning trials and the overall number of individuals that could be tested, I opted for 10 trials, with previous studies indicating most bees will learn the association within this number of trials (Riveros and Gronenberg 2009). Further learning trials would have potentially shown either a larger effect on learning between treatments or that pesticide exposed bees would have eventually learnt, with effects manifesting as an effect on learning speed. Furthermore, across all three pesticide exposure treatment groups, just 33% of individuals learnt the association, meaning that the comparative analysis of learning performance over ten trials for each treatment and age-cohort should be taken with a degree of caution. An alternative explination for the lower level of learning observed is the conditions of the experiemental setup in which in order to minimize experience related changes in the brain workers were restricted to the nest, with

no olfacotry stimuli beyond that within the nest box. This lack of experience could have affected the olfactory learning performance given that foraging experience has been shown to be correlated with performance in lab based learning assays (Raine and Chittka 2008, Riveros and Gronenberg 2009). That is to say that the workers tested from all colonies, given their restricted access to olfactory and visual stimuli could be considered to be developmentally naïve and thus exhibiting an overall lower behavioural performance than expected. Finally, it must also be considered that the effects on learning could also have been influenced by pesticide effects on motor function (control of the proboscis extension and hyperactivity), at low doses (1.25 ng/bee) imidacloprid has been found to induce increased motor function in honeybees (Lambin, Armengaud et al. 2001, Williamson, Willis et al. 2014). Or alternatively further unknown effects appetite or the ability to detect odour.

An interesting finding and one contrary to my initial predictions was that the *continual* treatment (in which workers were exposed throughout brood development and adulthood) did not perform worst regarding the ability to learn or overall learning performance. This exposure group did however produce the fewest bees to be harnessed for PER testing due to colony performance during the experimental period and the fewest responsive bees (whereby non-responsive bees drop out of further testing) compared to all other treatments (Table 2.3 and 2.4) and it is perhaps at this stage that the severity of this level of exposure was observed, with the severity of impact of continual prolonged exposure selecting for individuals who are least affected.

6.3.2 Chapter 3 limitations

To validate the micro-CT and image analysis protocol I compared our volumetric measures of bumblebee brain structures to those previously reported in two select studies Mares et al.

(Mares, Ash et al. 2005)and Jones et al. (Jones, Leonard et al. 2013), predicting that these studies had overestimated the volumes of each of the brain structures. Whereas, being able to differentiate tissues based on density, excluding extraneous internal tissue and considering the textures, pits and hollows, my approach facilitated a more realistic representation of these complicated structures, and thus I argue has produced consistently lower volume estimations in comparison. However, alternative explanations for these smaller estimated structural volumes must be considered: 1) that the previous case-studies examined brain volumes in a different bumblebee species, *B. impatiens*, 2) that the brains I sampled were not a mature adult size given they were of very young bees (only four days old) with little experience and furthermore, I did not compare like for like ages between studies, which is important as brain structure volumes have been reported to increase with age by as much as 37% (Jones, Leonard et al. 2013). My study would have therefore benefitted from performing a proof of concept on multiple (at least three) different ages to consider age effects in my comparisons. Although histological methods can be considered considerably more invasive than the method I present here, there are still elements of my protocol that could potentially lead to error and variation in volume estimations. Heads being preserved in ethanol and the staining conditions 0.5% PTA solution (0.5mg/ml concentration in 70/30% ethanol/water solution) could both potentially lead to shrinkage (Buytaert, Goyens et al. 2014). I feel that this protocol of imaging, segmentation and image analysis has great potential for application to further studies of soft tissues but could be streamlined in regard to the image segmentation process. My study utilized SPIERS software which is both freely available and has low system requirements, however despite some semi-automated features the predominately manual tracing form of image segmentation applied is relatively time consuming. For future studies I suggest the development and implementation of an automated segmentation procedure

would save both time and money as well as removing inconsistencies both within and between individuals performing the segmentation (Morey, Petty et al. 2009). Moreover, the consistent repeatability of an automated method would be of particular value to comparative studies and make cross study comparisons more reliable.

6.3.3 Chapter 4 and 5 limitations

I report age-related increases in the volume of the mushroom bodies and the antennal lobes however these were not found in the optic lobes. Bees were reared in the colony nest box, inside controlled environment conditions, under red light and with no foraging or additional sensory experiences in order to reduce experience related variation in brain development. Although visual stimuli will have been largely prevented, without isolating individuals, olfactory stimuli could not be fully prevented due to an array of volatile and non-volatile odours stemming from the pollen and wax, as well as hydrocarbon profiles of colony members detected though antennal contact. Therefore, this apparent discrepancy in age-related growth, as well treatment effects between the MB's & AL's and those observed in the optic lobes may be a product of an imbalance in the olfactory vs visual experiences workers had encountered. And given the relationship between the volume of these structures related to olfactory processing, this limitation may also have influenced the olfactory learning performance of workers. However, any such effects of difference between visual and olfactory stimuli should not affect my comparable findings between treatments, only raise questions around the level of confidence that can be had in the degree of innate growth I find.

6.4 FUTURE RESEARCH

The research presented in my thesis provides numerous insights but also raises many questions, here I will discuss the research ideas and directions that could be taken from each chapter for future exploration:

6.4.1 Effects of developmental pesticide exposure on other aspects of learning and behaviour

My findings in Chapter 2 showed that learning performance in bumblebees can be impeded by both brood and adult neonicotinoid exposure, however this as with many studies, focused on olfactory associative learning (assessed using the proboscis extension reflex learning paradigm) (Decourtye, Devillers et al. 2004, El Hassani, Dacher et al. 2008, Aliouane, el Hassani et al. 2009, Han, Niu et al. 2010, Williamson and Wright 2013, Tan, Chen et al. 2015, Wright, Softley et al. 2015). Given the importance of other sensory modalities and multimodal signals in task performance (Collett and Collett 2002, Kulahci, Dornhaus et al. 2008, Leonard, Dornhaus et al. 2011, Collett, Chittka et al. 2013, Rössler and Brill 2013), it would be interesting to assess how pre-eclosion exposure affects other behaviours such as visual learning or motor control. Following which there is the potential to then explore the relationship between behavioural performance and the morphology of relevant associated brain structures as investigated in chapters 4 and 5. Such as visual learning and the optic lobes or MB (Strausfeld 1976) (Zars 2000) and motor control and the central body (Strausfeld 1999, Strauss 2002).

Alongside bumblebees, honeybees and solitary bees contribute significantly to pollination (Calderone 2012, Woodcock, Edwards et al. 2013) and a diversity of bee species is important for pollination (Klein, Steffan–Dewenter et al. 2003, Garibaldi, Steffan-Dewenter et al. 2013, Winfree, Reilly et al. 2018). It is therefore important to consider how such species would also

respond to these pesticide exposure treatments, however the results of this study cannot simply be used to infer the same response. For example, bumblebees and honeybees vary substantially in their life history traits, behavioural repertoire and physiology, crucially including the efficiency with which they metabolize pesticides (Cresswell, Robert et al. 2014, Manjon, Troczka et al. 2018). Moreover, honeybees and bumblebees vary appreciably in their crop foraging behaviour and visitation (Woodcock, Edwards et al. 2013). And in regard specifically to the study I present here, they also differ in their brain size and development, exhibiting smaller relative brain size overall compared to bumblebees including significantly smaller (-25%) relative mushroom bodies in honeybees compared to bumblebee workers of the same body size (Mares, Ash et al. 2005) and differ in olfactory learning performance (Bitterman, Menzel et al. 1983, Laloi, Gallois et al. 2001, Wright, Carlton et al. 2009). Therefore, to really evaluate the response of other key bee pollinators would require further independent studies of honeybees and solitary bee species, which would allow the identification of differential sensitivities or vulnerabilities to early developmental exposure to neurotoxic pesticides.

In Chapter 2 I found both effects of age and of adult pesticide exposure treatment on worker responsiveness to sucrose. However, this test was limited to a single, high sucrose concentration (50%) and therefore does not tell us how their sucrose response threshold may have been altered. To fully elucidate these potential age and pesticide induced changes is responsiveness requires a more rigorous investigation under these conditions, performing a full sucrose response threshold test (thus considering multiple different sucrose concentrations). Do bumblebees exposed to neonicotinoid pesticides require higher concentrations of sugars than usual to be stimulated to feed? A more comprehensive investigation is important given the role of sucrose concentration in flower choice (Cnaani,

Thomson et al. 2006) and thus the potential for changes in responsiveness altering the foraging preferences of bees (Pankiw, Waddington et al. 2001, Scheiner, Barnert et al. 2003, Scheiner, Page et al. 2004).

6.4.2 Further exploration of the effects of pesticide exposure on bumblebee brain morphology

I found that pesticide exposure during brood development alone leads to impaired adult brain volumetric development of workers up to 12 days old, suggesting these are irrecoverable effects. However, this study was limited to 3-day and 12-day bees, whether in time older bees, either through age/experience dependent growth can recover to the expected size of mushroom bodies and with that potentially the same learning capacity should also be investigated to elucidate whether these truly are irrecoverable or not. Furthermore, a similar question can be asked of adult exposure. For example, if workers are exposed for only a short period of early adult life, such as for 3 days following eclosion and then tested on their learning performance and mushroom body growth at a later age, can the adult level of plasticity allow full, or some level of recovery? Indeed, it has been shown that bumblebees can clear imidacloprid from their body in 48 hours and recover previously impaired behavioural performance (Cresswell, Robert et al. 2014).

The application of Micro-CT allowed me to assess, in fine detail, the relationship between learning performance, pesticide exposure and age with the volume of components of the bumblebee mushroom body – the lateral and medial calyces and the MB lobes. However, these structures themselves have functional distinct sub-compartments associated with different sensory modalities; the lip and collar of the calyx are considered to handle input from olfactory and visual neuropils respectively, and the basal ring receiving input from each

(Abel, Rybak et al. 2001, Gronenberg 2001). And plasticity of the MB's does not always manifest as a global volume increase but rather a reorganization with localized plasticity of these sub-compartments (Durst, Eichmüller et al. 1994, Maleszka, Barron et al. 2009). Looking further into how changes in these sub-regions relate to pesticide exposure and learning performance may shed light on the specific nature of how they lead to the observed behavioural changes this and other studies report. My application of Micro-CT and the creation of virtual 3D representations of each individual in this study allows me to go back and further explore the specific regions of the MB in more detail in the future.

Further to investigating the volume of brain structures at finer detail it would also be of interest to explore these effects at the neuronal level – to assess what underlying changes occur that results in the morphological differences I find. For example, electrophysiological studies on honeybee Kenyon cells exposed to neonicotinoids indicate that they undergo tonic depolarisation with subsequent effects on acetylcholine (Ach) sensitivity (Palmer, Moffat et al. 2013, Moffat, Pacheco et al. 2015). Given the involvement of Ach in experience-dependent neuropil growth (Ismail, Robinson et al. 2006, Dobrin, Herlihy et al. 2011) this could therefore be a contributing factor to the reduced volumes I observed but further studies to make such links are required.

The volume reductions I find in the MBs following *pre-eclosion* exposure may be a result of impaired neurogenesis - preventing neuronal precursor cells from giving rise to Kenyon cells in developing bees (Fahrbach, Strande et al. 1995, Farris, Robinson et al. 1999). For example, in *Drosophila melanogaster,* thermal stress of developing larvae and pupae reduced the number of Kenyon cells in the MB through preventing proliferation (Wang, Green et al. 2007). To test whether a similar effect is caused by pesticide exposure in bees would require

estimating the number of kenyon cells that comprise the MB rather than just the overall volume. It would also be of interest to assess, along with the number of cells, the individual Kenyon cell size and dendritic growth which, as shown by Wilson *et al*. (Wilson, Velarde et al. 2013) can be affected by pesticide exposure in cell cultures. Together such additional studies could reveal if effects on this level of cell growth and proliferation could therefore translate to large volumetric changes I report.

The initial scope of this study was to investigate whether pesticide exposure could lead to asymmetrical development. Signs of higher asymmetry, in particular fluctuating asymmetry can indicate stress during development (Vishalakshi and Singh 2007, Beasley, Bonisoli-Alquati et al. 2013). Fluctuating asymmetry is an established method for measuring the phenotypic response to environmental stress (Markow 1995, Chapman and Goulson 2000, Stoks 2001, Leamy and Klingenberg 2005) and is defined as random deviations from perfect symmetry of bilateral traits due to variations in the developmental environment (Knopper 2003, Palmer and Strobeck 2003). Some preliminary analysis was conducted to explore asymmetry of the left/right mushroom bodies and has been included as appendix 6.1 However, given time, a furthermore robust analysis of symmetry would be worth undertaking, using fluctuating asymmetry to assess the effects of brood developmental pesticide stress.

Chapters 4 and 5 focused specifically on how pesticides affect volumetric measures of specific structures in the bumblebee brain. However, the application of micro-CT and 3D virtual reconstructions of the brain allows for further aspects of morphology to be investigated. Other than size, structural differences in the brain may be represented by differences in shape. Indeed, the plasticity of the mushroom bodies can manifest not only as a global volume increase but rather a reorganization, with localized plasticity of specific regions (Durst,

Eichmüller et al. 1994, Maleszka, Barron et al. 2009). The structural anatomy of the brain may influence brain function because the processing of neural information is dependent on the size, configuration and alignment of the individual neurons. Which in turn determines the type and amount of synaptic connections that can be formed and the ways they can interconnect surrounding neuropils (Zatorre, Fields et al. 2012). Furthermore, shape differences in specific structures could also be a sign volumetric differences in the surrounding structures or tissues. Developmental stress has been linked to morphological changes beyond volume of both internal and external morphology (Bookstein, Sampson et al. 2001, Hoffmann, Collins et al. 2002, Sowell, Thompson et al. 2002, Cook and Wellman 2004) and as such could lead to impaired function. Therefore, using 3D Landmark-based geometric morphometrics (Webster and Sheets 2017) I began a preliminary investigation into the effects of pesticide exposure on the shape of a complex component structure of the MB – the lateral calyx the details and results of which can been seen in Appendix 6.2.

Appendices

sucrose consumption (ml) as the experiment progressed.

Treatment	Colony	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39	41	43
control	1	9	15	25	15	18	21	20	22	28	34	15	40	30	35	37	37	31	36	35	33	33	37
control	2	14	20	29	17	20	20	29	28	27	33	13	28	28	30	30	34	26	32	35	38	37	35
control	3	10	10	19	14	20	20	21	22	23	30	14	30	28	38	37	38	30	37	35	37	39	39
pre-eclosion	4	7	10	13	10	13	14	15	12	16	19	9	20	17	20	16	17	16	21	21	22	25	26
pre-eclosion	5	13	12	24	18	24	22	20	20	23	26	12	27	25	30	31	32	28	32	30	33	35	35
pre-eclosion	6	25	21	31	24	33	33	31	31	29	39	14	41	34	39	37	40	28	35	37	35	37	45
post-eclosion	7	13	10	29	13	17	18	16	17	20	26	12	26	37	45	46	43	36	43	48	48	54	59
post-eclosion	8	29	22	32	21	24	24	29	23	30	37	14	30	34	39	39	38	32	42	57	42	45	52
post-eclosion	9	18	17	30	23	28	28	35	39	35	39	21	46	42	48	53	52	36	46	52	48	55	55
continual	10	19	18	25	20	22	25	27	28	33	35	13	35	35	40	42	46	27	40	36	40	40	35
continual	11	20	15	22	24	25	25	28	26	28	32	15	35	35	39	36	46	36	38	35	35	33	32
continual	12	5	15	17	12	15	15	21	16	18	25	11	24	1	29	25	28	25	31	33	36	40	41
control	13	17	17	17	23	21	25	21	26	24	28	15	32	30	36	33	35	38	40	39	40	40	41
control	14	15	16	14	17	13	15	16	20	16	21	12	25	23	34	30	31	29	33	33	35	32	34
pre-eclosion	15	17	19	23	26	25	29	24	29	25	29	17	37	28	33	31	31	30	30	26	31	29	31
pre-eclosion	16	17	18	14	23	17	22	18	22	20	24	14	30	27	29	25	25	23	23	22	24	23	23
pre-eclosion	17	13	14	11	19	16	21	19	22	18	20	11	25	21	23	23	23	23	23	21	21	17	19
post-eclosion	18	15	15	15	22	18	22	29	30	19	24	14	28	23	27	36	32	30	28	32	25	27	27
post-eclosion	19	23	23	23	29	25	31	26	36	34	36	21	38	33	38	29	34	34	36	36	34	34	33
post-eclosion	20	15	15	20	26	20	23	22	26	21	26	13	23	22	26	25	29	30	31	28	27	26	26
continual	21	18	19	19	26	25	29	29	37	30	39	21	42	35	39	41	43	45	46	46	47	45	46
continual	22	13	13	10	13	10	12	9	10	7	10	4	10	7	12	12	12	12	13	15	16	9	11
continual	23	14	15	15	21	13	16	16	19	16	19	12	24	22	25	25	25	23	25	24	25	24	26

Appendix 2.2 Statistical comparisons of responsiveness, learners and learning level. Statistical outputs from binomial Generalized Linear Mixed Effects models in R (GLMER). Exposure treatments are comparsons to control workers ('intercept') with significant differences (alpha value of 0.05) highlighted in bold red and near significant (alpha value below 0.1) highlighted in bold black.

Responsive				
GLMER – responsive(y/n) ~ treatment * age + size + (1|colony), family = binomial				
	Estimate	**Std. Error**	**z value**	**Pr(>|z|)**
(Intercept)	-1.44837	0.94805	-1.528	0.1266
pre-eclosion	-0.40216	0.36208	-1.111	0.2667
post-eclosion	-0.94856	0.38551	-2.461	**0.0139**
continual	-0.82279	0.39879	-2.063	**0.0391**
age	0.50103	0.3777	1.326	0.1847
size	0.34516	0.21269	1.623	0.1046
pre-eclosion 12-day	0.05735	0.52859	0.108	0.9136
post-eclosion 12-day	0.47278	0.5483	0.862	0.3885
continual 12-day	0.24245	0.59184	0.41	0.6821

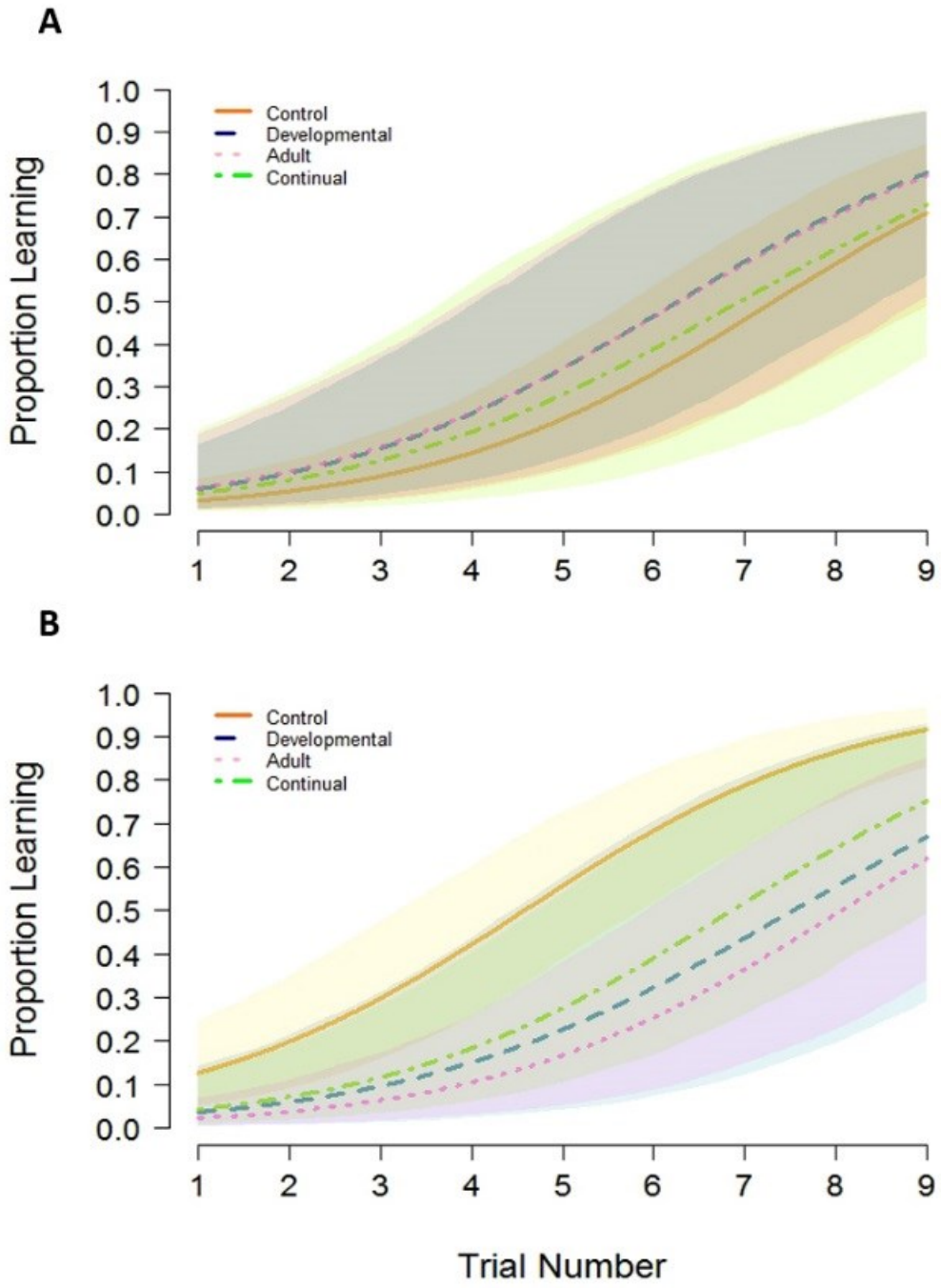
Learners				
GLMER - Learner(y/n) ~ treatment * age + size + (1|colony), family = binomial				
(Intercept)	-4.1961	1.9677	-2.132	0.033
pre-eclosion	-1.0568	0.6586	-1.605	0.1086
post-eclosion	-1.0173	0.7401	-1.375	0.1693
continual	-1.4028	0.8104	-1.731	**0.0835**
age	1.3939	0.6551	2.128	**0.0334**
size	0.9327	0.4341	2.148	**0.0317**
pre-eclosion 12-day	-2.2096	1.0099	-2.188	**0.0287**
post-eclosion 12-day	-1.359	0.9916	-1.371	0.1705
continual 12-day	-0.1863	1.0699	-0.174	0.8617

Learning Level				
GLMER - outcome ~ treatment * age + size + trial + (trial)|colony/id), family = binomial				
(Intercept)	-3.91251	2.40673	-1.626	0.104
pre-eclosion	0.6716	0.77648	0.865	0.3871
post-eclosion	0.56068	0.87817	0.638	0.5232
continual	0.14896	1.02408	0.145	0.8843
age	1.40962	0.65702	2.145	**0.0319**
size	-0.19258	0.51876	-0.371	0.7105
trial	0.58647	0.07343	7.987	**1.38E-15**
pre-eclosion 12-day	-2.32555	1.26426	-1.839	**0.0658**
post-eclosion 12-day	-2.40236	1.16619	-2.06	**0.0394**
continual 12-day	-1.31406	1.21753	-1.079	0.2805

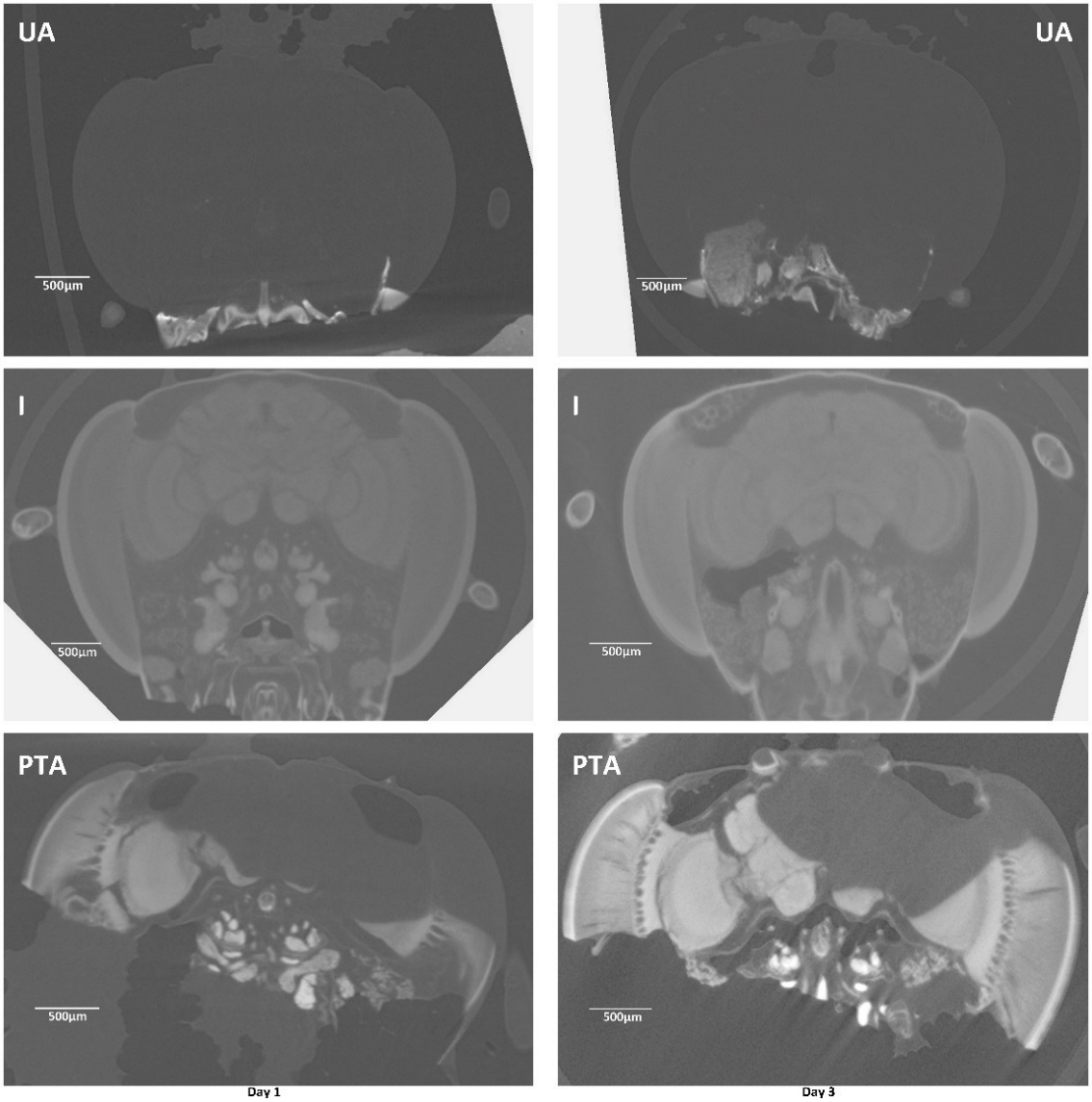
Appendix 2.3 Statistical comparison of responsiveness, learners and learning level for only 12-day bees. Statistical outputs from binomial Generalized Linear Mixed Effects models in R (GLMER). Exposure treatments are comparisons to control workers ('intercept') with significant differences (alpha value of 0.05) highlighted in bold red.

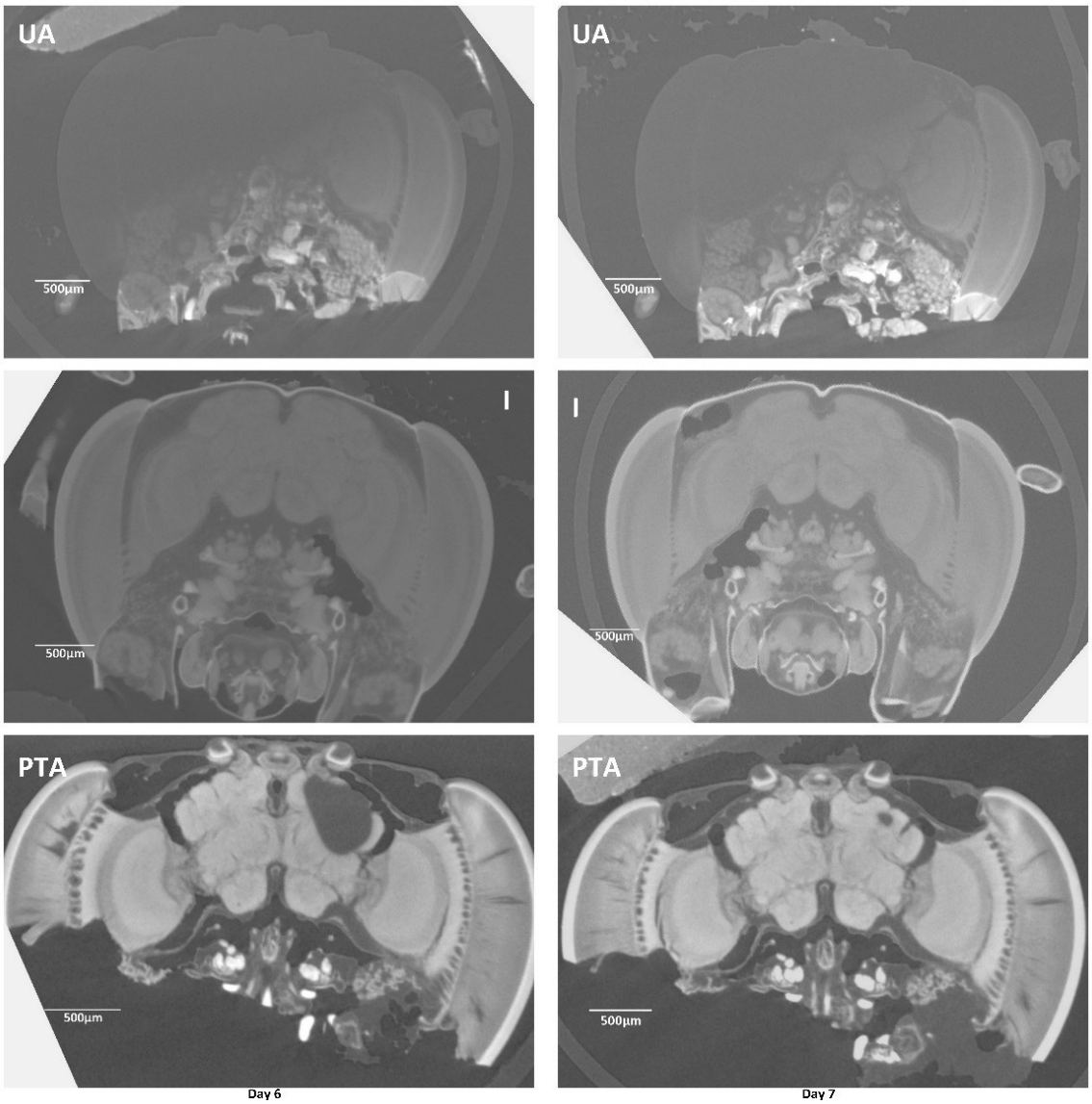
Responsive 12 day only				
	Estimate	**Std. Error**	**z value**	**Pr(>|z|)**
(Intercept)	0.58255	1.67351	0.348	0.728
Pre-eclosion	-0.54509	0.65001	-0.839	0.402
Post-eclosion	-0.60215	0.64573	-0.932	0.351
continual	-0.43481	0.7174	-0.606	0.544
size	0.04113	0.37922	0.108	0.914
Learners 12 day only				
(Intercept)	-1.6191	2.6124	-0.62	0.53541
Pre-eclosion	-3.2712	0.8213	-3.983	**6.80E-05**
Post-eclosion	-2.304	0.7182	-3.208	**0.00134**
continual	-1.5036	0.75	-2.005	**0.04498**
size	0.6477	0.6143	1.054	0.29171
Learners 12 day only				
(Intercept)	-0.1072	2.9042	-0.037	0.9706
Pre-eclosion	-1.8008	1.03	-1.748	**0.0804**
Post-eclosion	-1.7822	0.8306	-2.146	**0.0319**
continual	-0.9119	0.8358	-1.091	0.2753
size	-0.7914	0.678	-1.167	0.2431
trial	0.6351	0.101	6.288	**3.21E-10**

Appendix 2.4 Comparisons between treatments of olfactory learning performance using proboscis extension reflex (PER) conditioning - learning curves with 95% credible intervals. (A-B) Learning curves showing the mean estimates with 95% credible intervals of the proportion of learnt responses over 10 conditioning trials of all bees showing at least one learnt response for (A) 3-day and (B) 12-day cohorts. Lines represent the model fit by treatment from a Binomial GLMM.

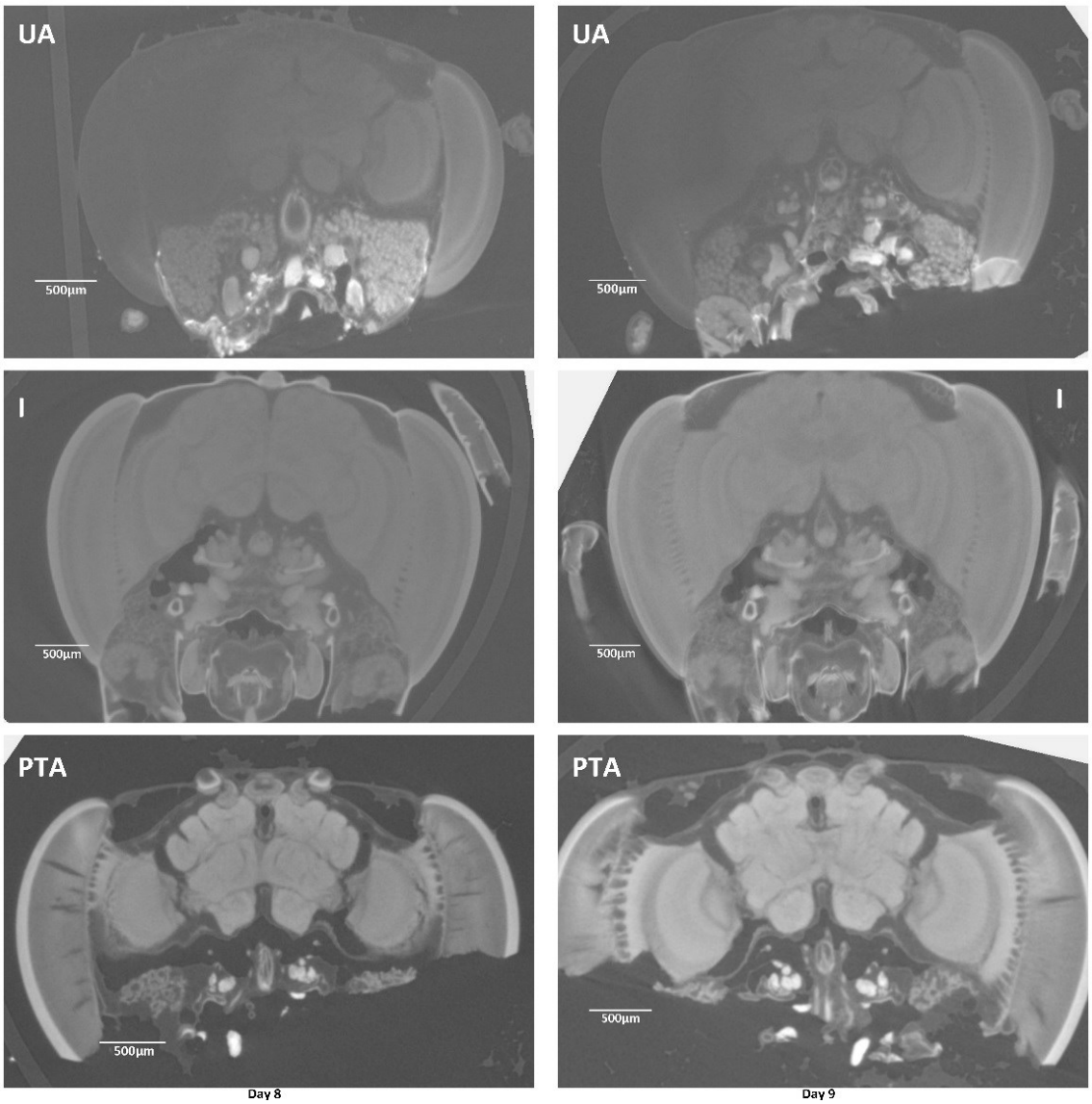


Appendix 3.1 Scanned images from the stain optimization test. Each scan slice shows the degree of staining for each day (days 1, 3, 6, 7, 8 and 9) and stain (Uranyl acetate (UA), Iodine (I) and phosphotungstic acid (PTA)). Progressive staining across days is illustrated by the receding dark area revealing more brain tissue over the staining period. Each stain perfused at different rates reaching their optimum at days 1 and 7 for Iodine and PTA respectively, with UA not fully penetrating the whole brain after 9 days (showing a very slow perfusion rate). Each image shows a slice that best represents the level of perfusion through the brain whilst attempting to show all the brain structures of interest.



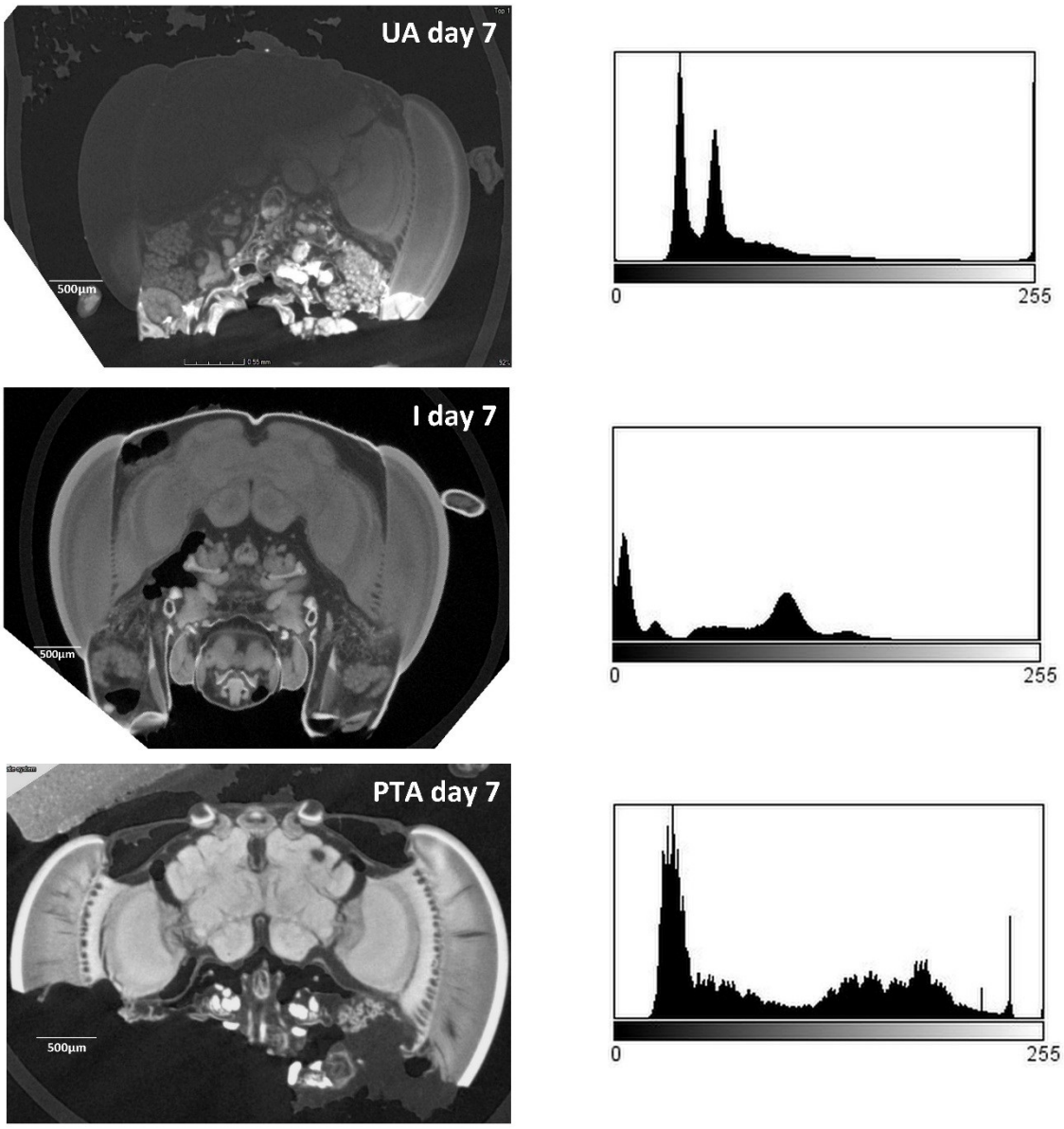


Day 6 Day 7

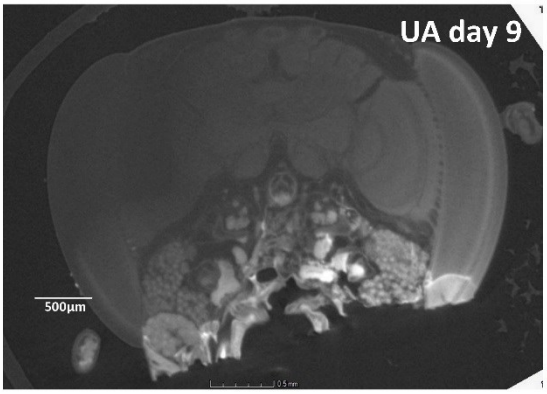


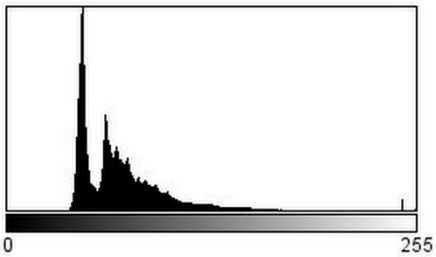
Day 8 Day 9

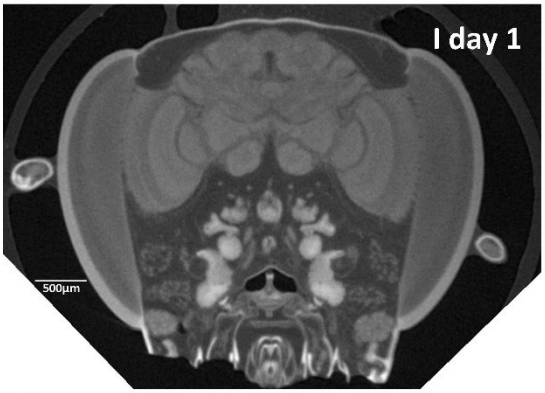
Appendix 3.2 Day 7 scan images and contrast histograms. Slice images for each stain UA, I and PTA are aligned to compare and illustrate the level of contrast enhancement when adjusted for optimum window width and level, with the associated histograms for each stain showing the distribution of the pixel values on the greyscale (0-255). Each slice is representative of the staining seen throughout the brain when comparing all slices. A wider pixel distribution across the greyscale indicates greater tissue differentiation for identifying and segmenting individual structures and regions. Comparison of histograms shows that PTA stain gives the best contrast enhancement.

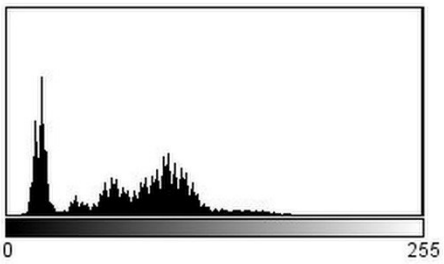


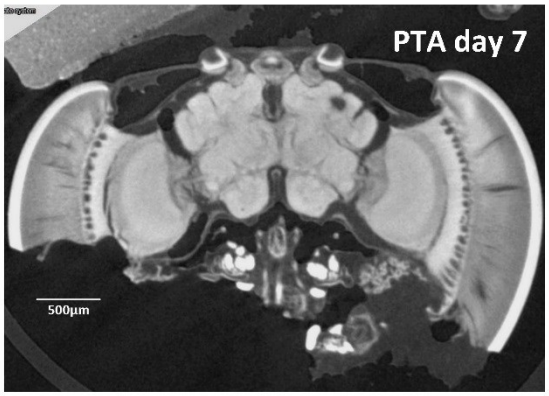
Appendix 3.3 Comparison of scan images and contrast histograms at the days showing the greatest contrast enhancement when adjusted for optimum window width and level.

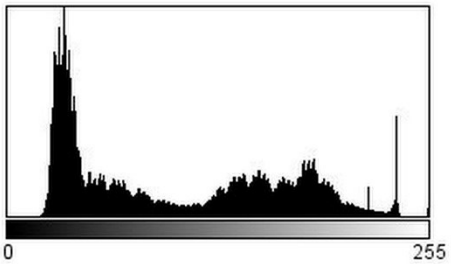












Appendix 3.4 Metris X-Tek HMX ST 225 Micro-CT Scanner at the *Imaging and Analysis Centre*, London Natural History Museum (image courtesy of Dan Sykes and Farah Ahmed).

which structures were successfully (x) and unsuccessfully (-) the 28 bee brains scanned the five sampled colonies. Any structure unsuccessfully stained were excluded from final shows the number of CT scan slices that were obtained from

Colony	Worker ID	No. Scan Slices	MBLobes		MBCalyces		CB	AL	Medulla		Lobulla		Used in Volumetric analysis
			L	R	L	R		R	L	R	L	R	
C11	R1	448	x	-	x	-	x	x	x	x	x	x	N
C11	R13	336	x	x	x	x	x	x	x	x	x	x	Y
C11	R19	258	-	x	-	x	x	-	x	x	x	x	N
C11	R21	361	x	x	x	x	x	x	x	x	x	x	Y
C11	R8	364	-	x	-	x	x	x	x	x	x	x	N
C11	R9	314	-	x	-	x	-	x	x	x	x	x	N
C12	G1	323	x	x	x	x	x	x	x	x	x	x	Y
C12	G11	428	x	x	x	x	x	x	x	x	x	x	Y
C12	G12	335	x	x	x	x	x	x	x	x	x	x	Y
C12	G13	294	x	x	x	x	x	x	x	x	x	x	Y
C12	G16	362	x	x	x	-	x	x	x	x	x	x	N
C12	G27	420	-	-	-	-	-	x	x	x	x	x	N
C12	G28	343	x	x	x	x	x	x	x	x	x	x	Y
C2	G27	339	x	x	x	x	x	x	x	x	x	x	Y
C2	G29	454	x	x	x	x	x	x	x	x	x	x	Y
C2	G3	366	x	x	x	x	x	x	x	x	x	x	Y
C2	G8	580	x	x	x	x	x	x	x	x	x	x	Y
C3	W1	434	x	x	x	x	x	x	x	x	x	x	Y
C3	W25	319	x	x	x	x	x	x	x	x	x	x	Y
C3	W41	372	x	x	x	x	x	x	x	x	x	x	Y
C3	W5	420	x	x	x	x	x	x	x	x	x	x	Y
C3	W6	241	x	x	-	x	x	x	x	x	x	x	N
C3	W7	340	x	x	x	x	x	x	x	x	x	x	Y
C6	W1	353	x	x	-	x	x	x	x	x	x	x	N
C6	W11	469	x	x	x	x	x	x	x	x	x	x	Y
C6	W12	323	x	x	x	x	x	-	x	x	x	x	N
C6	W13	296	x	x	x	x	x	x	x	x	x	x	Y
C6	W52	353	x	x	x	x	x	x	x	x	x	x	Y

Table 1. Numbers of scanned bees and segmented MB component structures.

	Colony	1	2	3	4	5	6	7	8	9	10	11	12	Totals
	Treatment	Control	Control	Control	Pre-eclosion	Pre-eclosion	Pre-eclosion	Post-eclosion	Post-eclosion	Post-eclosion	Continual	Continual	Continual	
	Scanned	8	8	9	7	13	6	6	11	10	10	11	6	105
Segmented	Left Medial Calyx	7	6	5	4	12	6	4	10	8	9	9	3	83
	Right Medial Calyx	7	6	4	4	12	6	4	11	7	10	7	4	82
	Left Lateral Calyx	7	7	7	4	13	6	5	11	8	10	9	4	91
	Right Lateral Calyx	7	7	6	4	12	6	5	11	7	10	8	5	88
	Left Lobes	7	7	6	4	13	6	5	11	8	10	8	4	89
	Right Lobes	7	7	6	4	12	6	5	11	8	10	8	5	89
	Left Antennal Lobe	7	7	7	4	13	6	5	11	8	10	8	5	91
	Right Antennal Lobe	7	7	6	4	13	6	5	11	8	10	8	5	90
	Left Medulla	5	6	5	4	11	6	5	8	5	9	3	4	71
	Right Medulla	5	6	5	4	11	6	5	8	5	9	3	4	71
	Left Lobula	5	6	5	4	11	6	5	8	5	9	3	4	71
	Right Lobula	5	6	5	4	11	6	5	8	5	9	3	4	71
	Central Body	7	7	6	4	13	6	5	11	8	10	8	5	90
	Responsive	8	8	7	4	11	2	1	8	6	6	6	4	71
	Learners	6	6	3	1	1	1	0	3	2	0	2	3	28

e.m.) relative volumes for each of the left and right MB structural

Treatment	Age	n	Left Medial Calyx		Right Medial Calyx		Left Lateral Calyx		Right Lateral Calyx		Left Lobes		Right Lobes	
			Mean	S.E.M	Mean	S.E.M	Mean	S.E.M	Mean	S.E.M	Mean	S.E.M	Mean	S.E.M
Control	**3**	10	0.0049	0.0001	0.0049	0.0001	0.0055	0.0002	0.0054	0.0002	0.0057	0.0002	0.0056	0.0002
	12	11	0.0063	0.0004	0.0062	0.0003	0.0066	0.0003	0.0065	0.0003	0.0070	0.0004	0.0069	0.0005
Pre-eclosion	**3**	12	0.0049	0.0003	0.0048	0.0004	0.0054	0.0003	0.0054	0.0003	0.0056	0.0004	0.0058	0.0004
	12	11	0.0049	0.0002	0.0048	0.0002	0.0053	0.0002	0.0053	0.0002	0.0060	0.0004	0.0061	0.0004
Post-eclosion	**3**	13	0.0042	0.0002	0.0039	0.0002	0.0047	0.0002	0.0047	0.0003	0.0053	0.0003	0.0051	0.0003
	12	11	0.0046	0.0004	0.0047	0.0004	0.0048	0.0004	0.0050	0.0004	0.0056	0.0005	0.0056	0.0004
Continual	**3**	15	0.0045	0.0003	0.0045	0.0003	0.0049	0.0003	0.0048	0.0003	0.0055	0.0004	0.0057	0.0003
	12	9	0.0046	0.0003	0.0047	0.0002	0.0052	0.0002	0.0052	0.0002	0.0055	0.0003	0.0058	0.0002

Appendix 4.3 Statistical comparisons of MB component log relative volumes. Outputs from Linear Mixed Effects models in R (LMER) for each left and right MB component. Exposure treatments are comparisons to *control* workers ('intercept') with significant differences (alpha value of 0.05) highlighted in bold red and near significant (alpha value below 0.1) highlighted in bold black.

	Left lateral calyx				**Right Lateral Calyx**			
	Estimate	**Std. Error**	**t value**	**Pr(>|t|)**	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
(Intercept)	-1.92198	0.08431	-22.797	< 2e-16	-1.954796	0.092043	-21.238	< 2e-16
Pre-eclosion	-0.01531	0.03025	-0.506	0.6143	-0.002446	0.033216	-0.074	0.941474
Post-eclosion	-0.07453	0.02972	-2.508	**0.0141**	-0.071706	0.032559	-2.202	**0.030555**
Continual	-0.06262	0.02927	-2.139	**0.0354**	-0.056362	0.031492	-1.79	**0.07733**
age12	0.0564	0.03117	1.81	**0.074**	0.069288	0.034257	2.023	**0.046498**
size	-0.07666	0.01844	-4.157	**7.87E-05**	-0.072464	0.020154	-3.596	**0.000562**
Pre-eclosion 12-day	-0.09224	0.04278	-2.156	**0.034**	-0.097269	0.04704	-2.068	**0.041933**
Post-eclosion 12-day	-0.05796	0.04245	-1.365	0.1759	-0.038868	0.046662	-0.833	0.40737
Continual 12-day	-0.03017	0.04334	-0.696	0.4884	-0.033303	0.047157	-0.706	0.482126
	Left Medial Calyx				**Right Medial Calyx**			
	Estimate	**Std. Error**	**t value**	**Pr(>|t|)**	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
(Intercept)	-2.03232	0.11729	-17.328	<2e-16	-2.044129	0.112582	-18.157	<2e-16
Pre-eclosion	-0.01709	0.04253	-0.402	0.6929	-0.023156	0.043521	-0.532	0.6022
Post-eclosion	-0.0691	0.04163	-1.66	0.116	-0.101906	0.043572	-2.339	**0.0331**
Continual	-0.06131	0.0422	-1.453	0.1664	-0.052708	0.042915	-1.228	0.2385
age12	0.08574	0.04089	2.097	**0.0398**	0.082125	0.039265	2.092	**0.0403**
size	-0.06321	0.02573	-2.457	**0.0171**	-0.060876	0.024719	-2.463	**0.0169**
Pre-eclosion 12-day	-0.09821	0.05409	-1.816	**0.074**	-0.101028	0.051598	-1.958	**0.0545**
Post-eclosion 12-day	-0.06093	0.05457	-1.117	0.2682	-0.009961	0.051785	-0.192	0.8481
Continual 12-day	-0.05997	0.05696	-1.053	0.2962	-0.056115	0.052758	-1.064	0.2914
	Left mushroom body lobes				**Right mushroom body lobes**			

	Estimate	Std. Error	t value	Pr(>|t|)	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.80386	0.11279	-15.994	< 2e-16	-1.785747	0.106403	-16.783	< 2e-16
Pre-eclosion	-0.01251	0.04868	-0.257	0.801905	0.0035502	0.051321	0.069	0.9461
Post-eclosion	-0.03573	0.04783	-0.747	0.471485	-0.040101	0.050353	-0.796	0.4446
Continual	-0.02538	0.04805	-0.528	0.608378	-0.00089	0.050064	-0.018	0.9862
age12	0.05838	0.03674	1.589	0.116546	0.0599372	0.032878	1.823	**0.0725**
size	-0.10018	0.02437	-4.11	**0.000101**	-0.106033	0.022729	-4.665	**1.24E-05**
Pre-eclosion 12-day	-0.0729	0.05004	-1.457	0.149495	-0.079032	0.045117	-1.752	**0.0841**
Post-eclosion 12-day	-0.04121	0.04949	-0.833	0.407871	-0.024076	0.044262	-0.544	0.5882
Continual 12-day	-0.05776	0.05092	-1.134	0.26043	-0.050197	0.045146	-1.112	0.2699

Appendix 4.4 Statistical comparisons of MB component log relative volumes for only 12-day workers. Outputs from Linear Mixed Effects models in R (LMER) for each left and right MB components. Exposure treatments are comparisons to control workers

	Left Lateral Calyx				**Right Lateral Calyx**			
	Estimate	**Std. Error**	**t value**	**Pr(>|t|)**	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
(Intercept)	-1.70638	0.10261	-16.629	< 2e-16	-1.74978	0.10203	-17.15	< 2e-16
Pre-eclosion	-0.11234	0.02798	-4.015	**0.000279**	-0.1045	0.02936	-3.559	**0.027997**
Post-eclosion	-0.12595	0.02812	-4.479	**6.96E-05**	-0.1053	0.0293	-3.594	**0.023996**
continual	-0.08708	0.02954	-2.948	**0.005516**	-0.0851	0.03057	-2.784	**0.040158**
size	-0.11479	0.02412	-4.759	**2.96E-05**	-0.1048	0.02377	-4.409	**0.000102**
	Left Medial Calyx				**Right Medial Calyx**			
(Intercept)	-1.7031	0.14661	-11.617	1.96E-12	-1.97866	0.10664	-18.555	< 2e-16
Pre-eclosion	-0.1165	0.04381	-2.659	**0.03734**	-0.07133	0.0353	-2.021	**0.0839**
Post-eclosion	-0.121	0.04521	-2.675	**0.03475**	-0.10381	0.03539	-2.933	**0.02224**
continual	-0.1051	0.04731	-2.221	**0.05341**	-0.08004	0.03543	-2.259	**0.05838**
size	-0.1224	0.03472	-3.524	**0.00135**	-0.06711	0.02435	-2.756	**0.00783**
	Left Lobes Calyx				**Right Lobes Calyx**			
(Intercept)	-1.60984	0.13773	-11.688	8.22E-14	-1.6115	0.11954	-13.481	1.33E-15
Pre-eclosion	-0.08361	0.06726	-1.243	0.255557	-0.0736	0.07385	-0.997	0.351
Post-eclosion	-0.07097	0.06705	-1.058	0.326943	-0.0572	0.07372	-0.776	0.462
continual	-0.07702	0.06769	-1.138	0.293128	-0.0421	0.0741	-0.568	0.587
size	-0.13272	0.03068	-4.325	**0.000132**	-0.1336	0.02551	-5.237	**1.09E-05**

Appendix 4.5 Statistical comparisons of Antennal Lobe relative volumes for 3-day, age interaction and 12-day only. Outputs from Linear Mixed Effects models in R (LMER) for each left and right antennal lobe. Exposure treatments are comparisons to control workers ('intercept') with significant differences (alpha value of 0.05) highlighted in bold red and near significant (alpha value below 0.1) highlighted in bold black.

	Left Antennal Lobe				Right Antennal Lobe			
	Estimate	**Std. Error**	**t value**	**Pr(>|t|)**	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
(Intercept)	-2.25238	0.101941	-22.095	< 2e-16	-2.23093	0.105369	-21.172	< 2e-16
Pre-eclosion	0.020733	0.050424	0.411	0.68531	-0.00921	0.053712	-0.171	0.865714
Post-eclosion	-0.11825	0.049449	-2.391	**0.0271**	-0.10824	0.052731	-2.053	**0.054752**
Continual	-0.01083	0.049067	-0.221	0.82766	-0.03636	0.05237	-0.694	0.496535
age	0.004533	0.00355	1.277	0.20582	0.003341	0.003635	0.919	0.361187
size	-0.07975	0.021284	-3.747	**0.00038**	-0.08218	0.02197	-3.74	**0.000365**
Pre-eclosion 12-day	-0.00544	0.004931	-1.104	0.27331	-0.00201	0.005022	-0.4	0.690082
Post-eclosion 12-day	0.00467	0.004821	0.969	0.33603	0.005099	0.004895	1.042	0.301212
Continual 12-day	-0.00611	0.00492	-1.241	0.21877	-0.0046	0.004993	-0.922	0.359923

12 Day	Left Antennal Lobe				Right Antennal Lobe			
	Estimate	**Std. Error**	**t value**	**Pr(>|t|)**	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
(Intercept)	-2.20347	0.09229	-23.875	< 2e-16	-2.17777	0.0961	-22.661	< 2e-16
Pre-eclosion	-0.02117	0.03438	-0.616	0.569063	-0.02538	0.04015	-0.632	0.552
Post-eclosion	-0.08746	0.03372	-2.594	**0.058613**	-0.0729	0.03949	-1.846	0.12
Continual	-0.05583	0.03383	-1.65	0.171235	-0.069	0.03959	-1.743	0.137
size	-0.083	0.02079	-3.993	1.63E-04	-0.0887	0.02132	-4.161	8.09E-05

Appendix 5.1 Statistical comparisons of medulla, lobula and central body relative volumes for 3-day and age interaction. Outputs from Linear Mixed Effects models in R (LMER) for each left and right antennal lobe. Exposure treatments are comparisons to control workers ('intercept') with significant differences (alpha value of 0.05) highlighted in bold red and near significant (alpha value below 0.1) highlighted in bold black.

	Left Lobula				**Right Lobula**			
	Estimate	**Std. Error**	**t value**	**Pr(>|t|)**	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
(Intercept)	-2.2586223	0.106294	-21.249	<2e-16	-2.30022	0.100986	-22.778	<2e-16
Pre-eclosion	0.0108763	0.04845	0.224	0.824	0.019123	0.045289	0.422	0.675
Post-eclosion	0.0077257	0.049489	0.156	0.877	0.005565	0.046355	0.12	0.905
Continual	0.0692437	0.051674	1.34	0.188	0.061802	0.048505	1.274	0.209
age	0.0056551	0.00372	1.52	0.134	0.005484	0.003665	1.496	0.14
size	-0.033153	0.021746	-1.525	0.133	-0.02311	0.020725	-1.115	0.27
Pre-eclosion 12-day	-0.0009922	0.004858	-0.204	0.839	-0.00068	0.004796	-0.141	0.888
Post-eclosion 12-day	0.0006756	0.005074	0.133	0.895	0.000773	0.005	0.155	0.878
Continual 12-day	-0.0069821	0.005201	-1.343	0.185	-0.00577	0.005127	-1.126	0.265
	Left Medulla				**Right Medulla**			
	Estimate	**Std. Error**	**t value**	**Pr(>|t|)**	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
(Intercept)	-1.824369	0.123021	-14.83	<2e-16	-1.88609	0.118381	-15.932	<2e-16
Pre-eclosion	0.021842	0.055312	0.395	0.695	0.014527	0.052959	0.274	0.785
Post-eclosion	0.010907	0.056587	0.193	0.848	0.000482	0.054295	0.009	0.993
Continual	0.081163	0.059188	1.371	0.177	0.062775	0.056866	1.104	0.275
age12	0.004861	0.004424	1.099	0.276	0.004101	0.004454	0.921	0.361
size	-0.020616	0.025232	-0.817	0.418	-0.00593	0.024335	-0.244	0.809
Pre-eclosion 12-day	-0.004689	0.005786	-0.81	0.421	-0.00204	0.00584	-0.349	0.728
Post-eclosion 12-day	0.001757	0.006034	0.291	0.772	0.00382	0.006076	0.629	0.532
Continual 12-day	-0.008801	0.006188	-1.422	0.16	-0.00603	0.006236	-0.967	0.338
	Central Body							
	Estimate	**Std. Error**	**t value**	**Pr(>|t|)**				
(Intercept)	-2.6497726	0.192336	-13.777	< 2e-16				
Pre-eclosion	-0.0295062	0.096522	-0.306	0.76318				

Post-eclosion	-0.0124425	0.0947	-0.131	0.89692
Continual	-0.0101602	0.094013	-0.108	0.91516
age12	0.0007932	0.00671	0.118	0.90624
size	-0.1323609	0.040182	-3.294	**0.00157**
Pre-eclosion 12-day	0.0066758	0.009268	0.72	0.47374
Post-eclosion 12-day	-0.0024105	0.009038	-0.267	0.79049
Continual 12-day	0.0009818	0.009218	0.107	0.91549

Appendix 5.2 Statistical comparisons of medulla, lobula and central body relative volumes for 12-day cohort only. Outputs from Linear Mixed Effects models in R (LMER) for each left and right antennal lobe. Exposure treatments are comparisons to control workers ('intercept') with significant differences (alpha value of 0.05) highlighted in bold red and near significant (alpha value below 0.1) highlighted in bold black.

	Left Lobula				**Right Lobula**			
	Estimate	**Std. Error**	**t value**	**Pr(>|t|)**	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
(Intercept)	-2.13675	0.096144	-22.224	<2e-16	-2.18839	0.091477	-23.923	<2e-16
Pre-eclosion	-0.00311	0.033538	-0.093	0.9287	0.007936	0.028067	0.283	0.7859
Post-eclosion	0.006265	0.033772	0.186	0.8579	0.004908	0.028433	0.173	0.8676
Continual	0.014049	0.034614	0.406	0.696	0.015367	0.029251	0.525	0.6142
size	-0.05061	0.021365	-2.369	0.0209	-0.03862	0.020485	-1.885	0.0644

	Left Medulla	**Right Medulla**						
	Estimate	Std. Error	t value	Pr(>|t|)	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.75494	0.10872	-16.142	<2e-16	-1.81587	0.105049	-17.286	<2e-16
Pre-eclosion	-0.01764	0.03386	-0.521	0.618	-0.00504	0.028779	-0.175	0.866
Post-eclosion	0.01833	0.03427	0.535	0.607	0.02292	0.029454	0.778	0.458
Continual	0.01178	0.03524	0.334	0.746	0.014773	0.030336	0.487	0.639
size	-0.02753	0.02433	-1.132	0.262	-0.01446	0.023652	-0.611	0.544

	Central Body			
	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-2.56662	0.171232	-14.989	< 2e-16
Pre-eclosion	0.018225	0.07407	0.246	0.814562
Post-eclosion	-0.0284	0.072933	-0.389	0.712279
Continual	-0.00199	0.073101	-0.027	0.979291
size	-0.15025	0.037853	-3.969	0.000157

Appendix 6.1 Preliminary analysis of symmetry

For each individual for which we had total MB volume estimates I assessed the volume differentials (Δ) between the left and right MBs. Comparing whole MBs of 3 / 12-day workers showed the mean%Δ per worker to be smallest in *control* colonies of 1.24±0.003 / 2.19±0.005% (cov.=68.7 / 73.5%), and critically higher averages in exposed workers with *pre-eclosion* at 5.97±0.009 / 3.32±0.008% (cov.=49.1 / 70.8%), *post-eclosion* at 4.81±0.010 / 6.41±0.021% (cov.=63.2 / 90.2%) and *continual* at 3.36±0.012 / 5.35±0.021% (cov.=108.9 / 91.5%; Table S9). Specifically, compared to *control* there was a significantly higher Δ in the LCs in *post-eclosion* workers (t=2.07, p=0.045) and the Lo in *post-eclosion* and *continual* (t=2.69, p<0.01 & t=2.004, p=0.0506; Table S10), supporting that early-adult exposure does stress development.

Comparative analysis of volume differentials between the right and left MB hemispheres across treatments was performed with linear mixed-effects models (LMERs) in R version 3.0.1 (R Development Core Team 2014) using RStudio version 1.0.143, using the lme4 and lmerTest

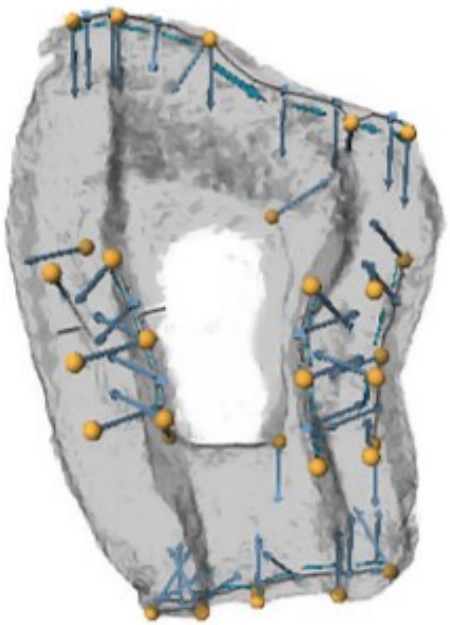
packages (Bates, Mächler et al. 2015, Kuznetsova A 2017). In addition to *treatment* as a fixed factor, we included *worker size* (inter-tegula width) to consider any disproportionate effect on small or large workers (Samuelson, Chen-Wishart et al. 2016).

Appendix 6.2 Preliminary morphometric analysis

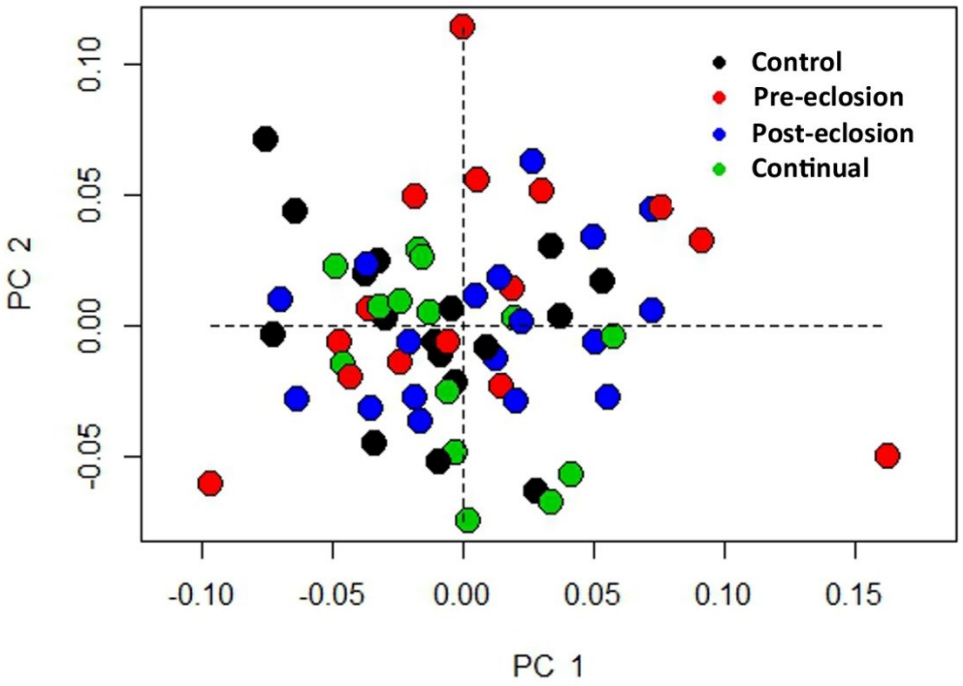
Landmark bases geometric morphometric analysis was carried out on the right lateral calyx of 64 bumblebee brains from across each pesticide treatment and control workers. 3D reconstructed samples of the right lateral calyces were exported from SPIERSView and saved as stl. files. Each sample was then imported into Stratovan Checkpoint for landmarks to be assigned. A series of corresponding points, identifiable across all samples were chosen; 31 Landmarks with 18 semi-landmarks placed between these to describe curves and placed on each 3D reconstruction (see Appendix 6.3 for landmark placement). Landmark coordinates were then exported for all samples for analysis in R version 3.0.1 (R Development Core Team 2014), RStudio version 1.0.143, using the Geomorph package (Adams, D. C., M. L. Collyer, and A. Kaliontzopoulou. 2018. Geomorph: Software for geometric morphometric analyses. R package version3.0.6.https://cran.rproject.org/package=geomorph.). Generalized Procrustes Analysis (GPA) was performed on the coordinates to exclude size and orientation. GPA optimally rotates, scales and translates each specimen to align the coordinates of corresponding points as closely as possible and produces aligned Procrustes coordinates that represent the shape of each specimen allowing best comparability (Gower 1975). PCA analysis was then used to compare Procrustes landmark coordinates across treatments (Appendix 6.4). Using the morphol.disparity function of geomorph, morphological disparity was estimated – the procrustes variance for each group, with pairwise comparisons

performed to identify differences between groups (Zelditch, Swiderski et al. 2012) (see Appendix 6.5 for pairwise comparisons).

Appendix 6.3 Lateral Calyx landmark and semi-landmark placements. Top down view of landmark placement on right lateral calyx sample. Yellow points denote full landmarks, lines indicate curves defined by semi-landmarks placed between full landmarks.



Appendix 6.4 PCA plot for Procrustes coordinates of right lateral calyx for each treatment.



Appendix 6.5 Morphological disparity between treatment groups for right lateral calyx.

Pairwise absolute differences between variances				
	Control	Pre-eclosion	Continual	Post-eclosion
Control	0.00000	0.00785	0.00098	0.00201
Pre-eclosion	0.00785	0.00000	0.00688	0.00584
Continual	0.00098	0.00688	0.00000	0.00103
Post-eclosion	0.00201	0.00584	0.00103	0.00000

P-Values				
	Control	Pre-eclosion	Continual	Post-eclosion
Control	1	0.01	0.79	0.38
Pre-eclosion	0.01	1	0.01	0.04
Continual	0.79	0.01	1	0.69
Post-eclosion	0.38	0.04	0.69	1

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