A molecular and cellular characterisation of the effects of neonicotinoid pesticides on the brain of the pollinator *Bombus terrestris*.



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by

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Dissemination of research

Prizes and Awards:

2019 Best Overall Oral presentation at the 29th Irish Environmental Researchers Colloquium-Environ 2019.

2019 Runner Up prize for best Oral presentation at the 8th Biology Research Day -Maynooth University.

Oral Presentations:

2019 Correlating neonicotinoid mode of action with bumblebee effects at the behavioural, cellular and molecular level. Guapo, F. and Carolan, J.C. **8th Biology Research Day -Maynooth University**.

2019 The mystery of the "Zombie bees": investigating the impact of Imidacloprid on pollinators. Guapo, F. and Carolan, J.C. **Biology Department Seminar Series Presentation**, Maynooth University.

2019 Molecular and behavioural characterization of neonicotinoid exposure on bumblebees reveals trends between chemical effect and their mode of action. Guapo, F. and Carolan, J.C. **29th Irish Environmental Researchers Colloquium-Environ 2019.**

2019 The road to becoming a scientist in ecotoxicology. Guapo, F. **Guest presentation for science in society elective (BI260), Maynooth University.**

2018 General assessment of the sublethal effects caused by the exposure to cholinergic pesticide on the important pollinator *Bombus terrestris*. Guapo, F. and Carolan, J.C. **7th Biology Research Day -Maynooth University**.

2018 Explaining the relationship between food production and animal pollination. What is the buzz about? Guapo, F. and Carolan, J.C. **Guest presentation for science in society elective (BI260), Maynooth University.**

2017 Structural Characterization of the Bumblebee Brain: an insight into the complexity of the insect's central nervous system. **Biology Department Seminar Series Presentation**, Maynooth University.

2017 A world without pollinators: A story about food production and pesticides. Guapo, F. and Carolan, J.C. **Guest presentation for science in society elective (BI260), Maynooth University.**

2017 Neuronal disruption after exposure to field relevant doses of clothianidin: a proteomic analysis of the neonicotinoid effects on the Bumblebee brain. Guapo, F. and Carolan, J.C. **Royal Entomological Society Insect Pollinator Special Interest Group Meeting National Museum of Scotland, Edinburgh.**

2017 Field relevant doses of neonicotinoid pesticide impair neuronal plasticity on the common pollinator *Bombus terrestris*. Guapo, F. and Carolan, J.C. **6th Biology Research Day -Maynooth University**.

2016 Label free mass spectrometry as a tool to assess the impact of sub lethal pesticide doses on the bee proteome. Guapo, F. and Carolan, J.C. **5th Biology Research Day**

2016 Toxicological impact of neonicotinoids on the insect's brain: A proteomic analysis of the Buff-tailed Bumblebee brain (*Bombus terrestris*). Guapo, F. and Carolan, J.C. **Irish Pollinator Research Network Conference**. Kildare, Ireland.

Poster Presentations

2018 Acute exposure to the neonicotinoid clothianidin at field relevant doses triggers differential responses in the neuronal cell proteome of the important pollinator *Bombus terrestris*. **28th Annual Meeting SETAC, 2018, Rome**. SETAC Europe 28th Annual Meeting.

2018 Maintenance, monitoring and mediation; functional analysis of the *B. terrestris* fat body. Larkin, D. E., Guapo, F. and Carolan, J. C. (2018). **7th Biology Research Day, Maynooth University.**

2016 Label free quantitative proteomics as a means of assessing pesticide delivery to bumblebees (Bombus terrestris). Guapo, F., Larkin, D. Carolan, J. C. **Computational and Molecular Biology Symposium 2016. Conway Institute, University College Dublin.**

Conference and Workshop Attendance

2019 **29th Irish Environmental Researchers Colloquium-Environ 2019.**

2019 **8th Biology Research Day** -Maynooth University

2019 **Irish Mass Spectrometry Society Annual Conference**. Dublin, Ireland.

2018 **7th Biology Research Day** -Maynooth University.

2018 **SETAC Europe 28th Annual Meeting**. Rome – Italy.

2017 **Royal Entomological Society Insect Pollinator Special Interest Group Meeting.** National Museum of Scotland, Edinburgh.

2017 **Hyphenated Analytical Techniques Workshop - Fundamentals, Applications and Challenges.** Department of Agriculture, Food and the Marine. Celbridge, Ireland.

2017 **6th Biology Research Day** -Maynooth University.

2017 **Irish Mass Spectrometry Society Annual Conference**. Dublin, Ireland

2017 **Biochemical Society Public Engagement Workshop**. Belfast, Northern Ireland.

2016 **5th Biology Research Day** -Maynooth University.

2016 **Biochemical Society Quantitative Proteomics and Data Analysis Workshop**. Chester, UK.

2016 **Irish Pollinator Research Network Conference**. Kildare, Ireland.

2016 **Irish Mass Spectrometry Society Annual Conference**. Dublin, Ireland.

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Event Organization

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I would like to dedicate this thesis to my grandmother Nilda, who passed away this year. She will always be loved and missed. And to all of those who cultivate a passion for science and want to help building a more rational, fair and democratic society.

Declaration

This thesis has not been submitted in whole or in part to this or any other university for any degree and is the original work of the author except where otherwise stated.

Signed Felipe Guapo de Melo **Date** 06/12/2019

Felipe Guapo de Melo

Abbreviations

µg	Microgram
µl	Microlitre
1D	One-dimensional
2D-DIGE	2-dimensional difference in gel electrophoresis
AA	Amino acid
Ach	Acetylcholine
AChE	Acetylcholinesterase
AL	Antennal lobe
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
AQUA	Absolute quantitation
ATP	Adenosine triphosphate
BLASTp	Basic local alignment tool for proteins
BP	Biological process
br	Basal ring
BR-C	Broad complex gene
CaE	Carboxylesterase
CAT	Catalase
cAMP	Cyclic adenosine monophosphate
CC	Cellular component
CLSM	Confocal laser scanning microscopy
cm	Centimetre
CNS	Central nervous system
D1	1 day old
D5	5 days old
D20	20 days old
DAPI	Diamidine-2-phenylindole
dH2O	Distilled water
DTT	Dithiothreitol
EcR	Ecdysome receptor
ER	Endoplasmic reticulum
FDR	False discovery rate
FTICR	Fourier-transform ion cyclotron resonance

g	g force
gr	Grams
GABA	Gamma aminobutyric acid
GI	Grey index
GO	Gene ontology
GPx	Glutathione peroxidase
GR	Glutathione reductase
GST	Glutathione S-transferase
HR/AM	High resolution accurate mass
IAA	Iodoacetamide
ICAT	Isotope-coded affinity tag
ICPL	Isotope-coded protein labels
iTRAQ	isobaric tag for relative and absolute quantitation
JAK/STAT	Janus kinase/ signal transducers and activators of transcription
JH	Juvenile hormone
JNK	Jun kinase
KCs	Kenyon cells
KCsN	Kenyon cell’s nucleus
KEGG	Kyoto encyclopaedia of genes and genomes
LC-MS/MS	Liquid chromatography mass spectrometry
LFQ	Label-free quantitation
LOEC	Lowest observed effect concentration
M	Molar
m/z	Mass/charge ratio
MAPK	Mitogen activated protein kinase
mAChr	muscarinic acetylcholine receptor
MF	Molecular function
mg	Microgram
min	Minute (s)
ml	Millilitre
MLA	Nortiterpenoid alkaloid methyllicaconitine
mm	Millimetre
mM	Millimolar
mRNA	Messenger RNA
MS	Mass spectrometry

MS1	Preliminary mass spectrum
MS2	Subsequent mass spectrum of peptide fragments
mTOR	Mechanistic target of rapamycin
nAChRs	Nicotinic acetylcholine receptors
nL	Nanolitre
OS	Oxidative stress
OXPHOS	Oxidative phosphorylation
PAGE	polyacrylamide gel electrophoresis
PCA	Principle component analysis
Pfam	Protein family
ppb	Parts per billion
ppm	Parts per million
PPI	Protein-protein interaction
proPO	Prophenoloxidase
rcf	Relative centrifugal force
RFC	Relative fold change
RH	Relative humidity
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPs	Ribosomal proteins
rRNA	Ribosomal RNA
s	Second (s)
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SILAC	Stable isotope labelling by amino acids in cell culture
snRNA	Small nuclear RNA
SOD	Superoxide dismutase
SSDA	Statistically significant differentially abundant
STRING	Search tool for the retrieval of interacting genes
TCA	Tricarboxylic acid cycle
TFA	Trifluoroacetic acid
TMT	Tandem mass tag
ToF	Time of flight
tRNA	Transfer RNA

UPR	Unfolded protein response
Usp	Ultraspiracle genes
Vg	Vitellogenin
Wnt	Wingless related integration site
YBP	Years before the present



“We all have a thirst for wonder. It's a deeply human quality. Therefore, you don't have to make stories up, you don't have to exaggerate. There's wonder and awe enough in the real world. Nature's a lot better at inventing wonders than we are.”

-Carl Sagan, Contact

Art by Pedro Busana at Faunart

Abstract

Bombus terrestris (L.) is one of the most important native and commercial pollinator species worldwide. Along with other pollinators their populations are in decline due to a multifactorial phenomenon that includes the extensive use of neonicotinoid insecticides. Thus, the characterisation and understanding of neonicotinoid effects on bees at the molecular level is essential to mitigate the risks of their use in the environment. This study initially characterised the brain proteomes of bumblebees in response to aging prior to assessing changes at the behavioural, cellular and molecular level as a response to neonicotinoid exposure. We demonstrated the highly catalytic nature of the developing bumblebee brain and how energy and carbohydrate metabolism increase in response to aging, while genetic information processes are downregulated. By considering differences in mode of action and mode of exposure to the neonicotinoids clothianidin and imidacloprid, the effects of acute and chronic oral exposure on bumblebee workers were determined. Neonicotinoids differentially impair energy metabolism and structural processes in the brain suggesting possible divergence of insecticide mode of action. Clothianidin and imidacloprid triggered different behavioural responses and toxicity in bees, with the former causing hyperactivity and the latter, temporal paralysis. Imidacloprid is less toxic to bumblebees and the brain physiology is differentially affected depending on chemical, dose or mode of exposure selected. The levels of the synapse associated protein synapsin increased in bumblebee brains for imidacloprid-exposed bees only, and functional annotation analysis of differential expressed proteins indicated impairment of intracellular transport, energy metabolism, translational activity, purines and pyrimidines metabolism, endocytic and exocytic activity and synaptic functioning as a whole. The pathways affected by neonicotinoid exposure vary depending on chemical and mode of exposure, which complicates the identification of biomarkers of neonicotinoid exposure in bumblebees. In addition, neonicotinoid metabolism in bees is poorly understood and these chemicals can accumulate in the bee body, which potentially contributes to long term toxicity. Overall the results presented in this thesis demonstrate individual and distinct ways by which neonicotinoids influence neuronal communication and provide novel insights into molecular aspects of bee health, through highlighting the pathways affected by aging and pesticide use on this important pollinator species.

Chapter I

General Introduction

1.1.Bee Evolution and Diversity

There are an estimated 25,000 species of bees in the world (Gould, 2015), members of the superfamily Apoidea, which in turn belong to the order Hymenoptera, that comprise 153,000 species of sawflies, ants, wasps and bees (Peters *et al.*, 2017).

Bee phylogenetics is principally inferred from molecular data as the preservation of fossils is rare (Cappellari *et al.*, 2013). However, it is clear that their evolution and diversification is closely linked to the appearance of the first angiosperm floral structures in the cretaceous period (Goulson, 2014). Prior to the origin of angiosperms, the majority of pollination was exclusively based on wind scattering mechanisms which was a very wasteful way to ensure plant reproduction. The evolution of flowers as a form of attracting insects, ensured a more efficient means of pollination which drove insects to become very efficient pollinators and one of the most ecologically important pollen feeding animals. The first plants to evolve flowers were water lilies and magnolias and it is probable that these plants were originally pollinated by beetles (Goulson, 2014). However, the revolution in insect animal pollination resulted in a rapid diversification of both groups, with an explosion and radiation of flower types and floral rewards for pollinators (Cappellari *et al.*, 2013). The development of long and tubular mouthparts with which to imbibe nectar, resulted in butterflies, some flies and bees to evolve into excellent pollinators through establishing coevolutionary relationships that have lasted for tens of millions of years (Goulson, 2014).

Bees are believed to have evolved from the wasp family Sphecidae that are known to capture prey and feed to their larvae, in small nests (Michez *et al.*, 2011; Goulson, 2014). The female wasp in this family builds nests, usually underground where they stock their prey to be used as food for their young. It is believed that at some point a species of Sphecidae wasp switched from stockpiling dead insects to pollen. It is difficult to know whether this was a gradual process, where larvae would be fed pollen only when their prey was scarce. However, the event of switching to feeding exclusively on pollen, is thought to represent the first step in the evolution of bees as we know them (Goulson, 2014).

The oldest known bee fossil is about 100 million years old (Poinar and Danforth, 2006). This is an individual preserved in amber that resembles the social stingless bees known to inhabit South America today. However, DNA analysis of different lineages of wasps and bees suggest that they diverged 124 and 111 million years before the present (YBP), shortly after the first angiosperm flowers evolved during the Cretaceous (Peters

et al., 2017). Since then, the group has diversified into several specialised pollination systems with angiosperms, which include seven contemporary bee families (Andrenidae, Apidae, Collectidae, Halictidae, Melittidae, Megachilidae and Stenotritidae) which are collectively clustered into the monophyletic group Anthophila (Figure 1.1) (Michez *et al*., 2011; Cappellari *et al.*, 2013; Peters *et al.*, 2017).

1.1.1. Sociality

One of the defining characteristics of bees is that different evolutionary groups display different levels of sociality, which involves a range of altruistic behaviours, specifically those where a group acts together to achieve a common goal, helping all individual parties (Andersson, 1984). The most organised form of sociality is termed eusociality, which is characterised by overlapping generations, cooperative care for the young and differential reproductive castes. A type of social structure found very clearly in honeybee and bumblebee species but that vary quite a bit among other evolutionary groups (Andersson, 1984).

The first bees are believed to have been solitary species (Michez *et al.*, 2011; Goulson, 2014; Peters *et al.*, 2017), which account for as many as 94 % of extant bee species (Kocher and Paxton, 2014). However, as their habits make them inconspicuous, they are rarely noticed in the wild. Solitary bees are also often small and dark coloured and have very variable life cycles, where females build their own nests in holes in the ground, trees or walls (Michener, 2007). Most of the known solitary bees inhabiting temperate regions of the world have only one generation per year and spend up to eleven months developing in their nests, where once provision is complete, females often abandon their brood or die shortly after offspring has reached adulthood (Michener, 2007; Goulson, 2014).

Primitively social bees maintain brood care or share a nest but there is no association with tasks used to maintaining their community. In addition, they may not be differentiated into reproductive castes (Kocher and Paxton, 2014).

Ultimately, eusociality is a rare and highly derived social system, although it has arisen at least four times in bee evolutionary history (Shell and Rehan, 2018), giving rise to a spectrum of eusociality where Euglossini, Ceratinini, Xylocopini and most sweat bees occupy the first level, being defined as solitary bees through a primitively eusocial origin, whereas corbiculate bees, which are those containing pollen baskets like Bombini are primitively eusocial and Meliponini and Apini represent the advanced eusocial bees (Figure 1.1) (Shell and Rehan, 2018). Eusociality has evolved only once in corbiculate

bees about 65 million YBP (Peters *et al.*, 2017). Later, at 30 to 40 million YBP the first bumblebee species (Bombini) arose in central Asia in a period of cooler temperatures, which may have encouraged bees to become larger and furrier (Goulson, 2014). These bees spread through Asia and Europe and seem to have migrated from Siberia to North America 20 million YBP, with only a few species reaching South America about 4 million YBP (Goulson, 2014).

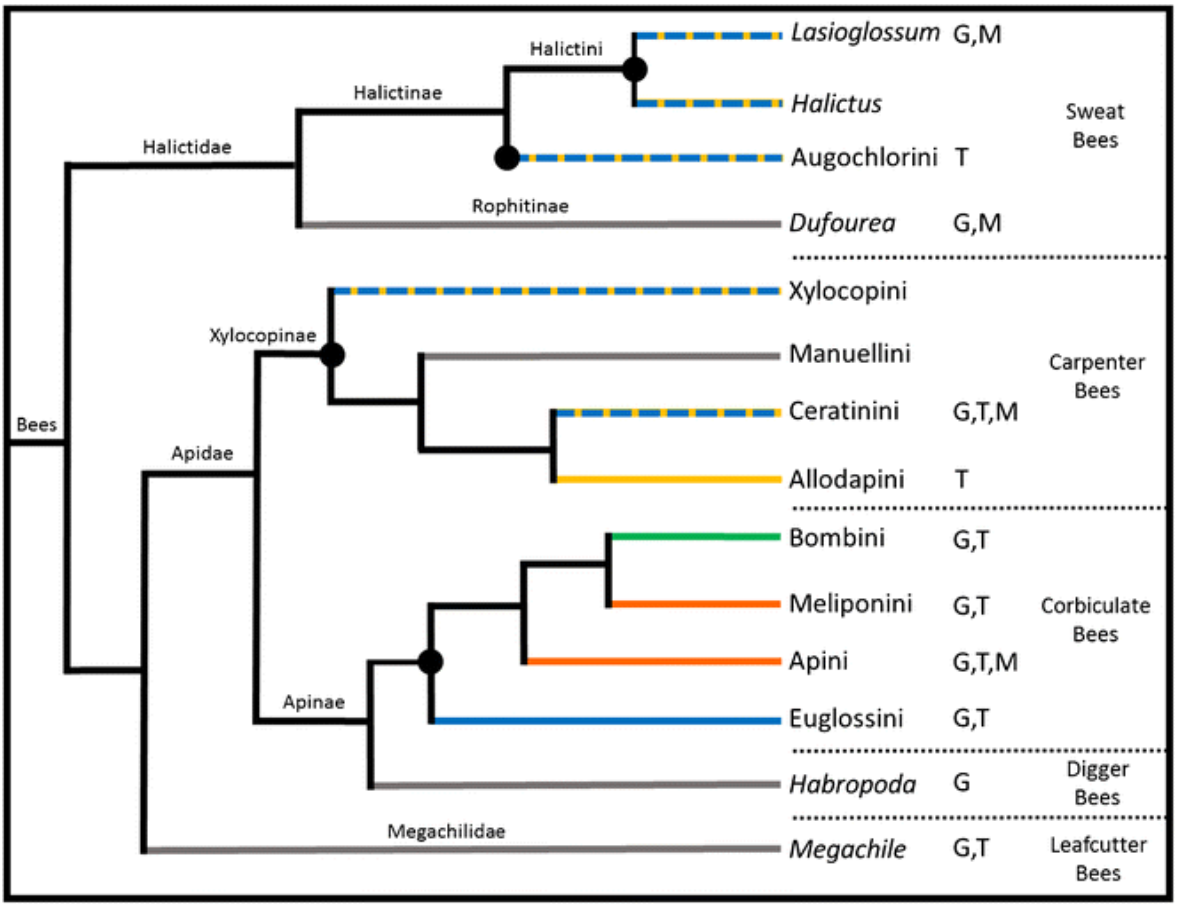


Figure 1.1 Phylogenetic relationships of major bee groups and social lineages. *Black nodes* indicate independent origins of sociality. *Branch colours* indicate lineages which are as follows: solitary (grey); solitary through to primitively eusocial *(blue)*; primitively eusocial (*green*); primitive through to advanced eusocial (*yellow*); advanced eusocial (*orange*) species. *Dashed branches with yellow* indicate lineages that contain facultatively social species. *Letters* indicate published resources for that lineage (*G* genome; *T* transcriptome; *M* methylome), adapted from Shell and Rehan, (2018).

1.1.2. Bumblebees

About two hundred and fifty of the known species of bees are bumblebees (*Bombus*) (Cameron *et al.*, 2007) of which there are two main types; the true and the cuckoo bumblebees (obligate social parasites of true bumblebees) (Williams, 1998). Although the majority of the bumblebee species in the world belong to the true bumblebees division, there are 45 species of cuckoo bumblebees, belonging to the subgenus *Psithyrus* (Pedersen, 2002; Kozmus *et al.*, 2011).

The true bumblebees are eusocial and form colonies comprising hundreds of individuals. They have a division of labour (egg-layer vs. foragers) among co-operating adult females of two generations, comprising mothers (the queen) and daughters (workers), living in a single nest. The two main female castes within eusocial colonies are workers, whose main responsibilities are foraging, brood care, nest building, defence and the queens, who are almost solely responsible for egg laying. Colonies usually contain only one mated queen and can have several non-reproductive workers (Michener, 2007).

Bumblebees are large and resilient bees with workers having a body length between 7-27 millimetres; their body, as with most insects is composed of three distinct segments, the head, thorax and abdomen (Michener, 2007). The head contains the mouthparts, antennae, eyes, different glands and the brain. The thorax contains the salivary glands, oesophagus, a ventral nerve chord and flight associated muscles. Their abdomen contains the stinging apparatus along with digestive organs, fat cells, ventral nerve chord and ganglia, the dorsal vessel through which haemolymph is pumped into the haemocoel and a generally undeveloped reproductive system in workers and a mature ovary in queens (Williams, 1998).

The whole body is covered in a thick layer of hair which may be of different colours and colour patterns. Banding, colouration, and structural differences occur between bumblebee castes and species and can be used to distinguish them morphologically (Figure 1.2). Queens are usually distinguished by size as they are larger than both their worker and male counterparts and males can be differentiated from workers by the absence of a stinger, a longer antennae and sometimes larger size (Williams, 1998; Alford, 2009).

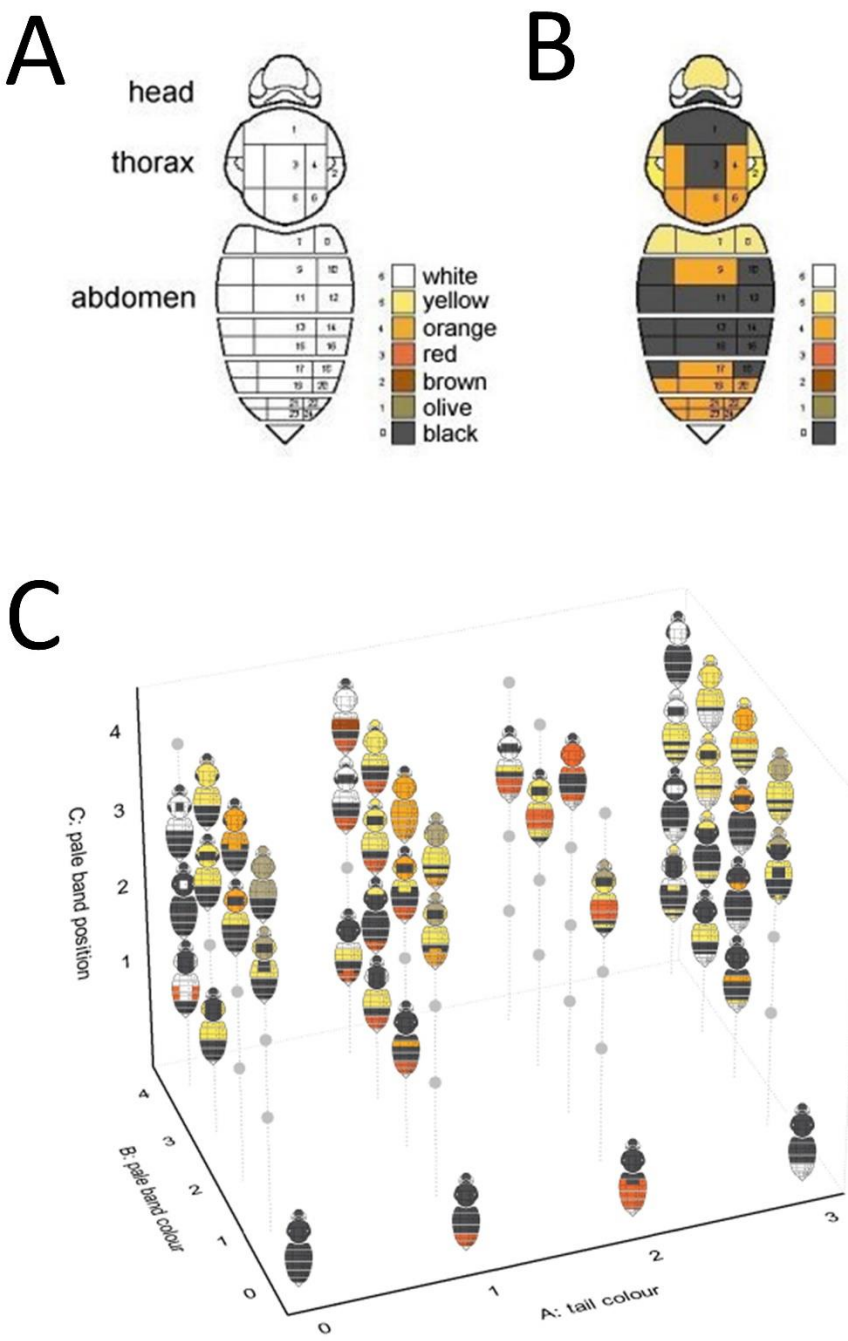


Figure 1.2 (A) Division of the worker dorsum into 23 colour pattern elements and (B) example of different colouration found in bumblebees around the world. (C) Three-dimensional colour pattern space defined for bumblebees with division of four main colour groups (0, 1, 2 and 3) and respective derivatives, adapted from Williams, (2007).

1.1.3. Global distribution and habitat

Bumblebees have a worldwide distribution, with the exception of sub-Saharan Africa and Oceania, and reach their highest diversity in temperate, alpine, and arctic zones (Goulson, 2014). In Africa, Asia and Europe bumblebees are particularly species rich in the Southern Tertiary Mountains which span from Tibet and the Himalayas westwards

towards the Alps and Pyrenees; and in the north-temperate steppe zone running eastwards from the Baltic to inner Mongolia. There are currently 63-68 species described in Europe, 68 in North Asia, 124 in East Asia and 27 and 73 in south and west Asia respectively (Williams and Jepsen, 2016). Although a smaller diversity of bumblebees is found in the Americas, they remain quite species rich around the western mountains which extend from the Rocky Mountains in North America (47 species) southwards to the Andes in South America where 26 species are recognised (Williams, 1998; Williams and Jepsen, 2016).

Bumblebees are most commonly associated with cool, open, flower rich habitats with characteristic shingle, sand dune, saltmarsh or old meadow vegetation (Williams, 1988), from which they obtain a continuous supply of nutrients in the form of pollen and nectar throughout the colony life cycle (Carvell, 2002). Their habitats often experience one annual period of adverse weather which delimits their annual cycles (most often a cold winter). Thus, the variety and quantity of bee species in a geographical area is determined by the diversity of plant species available, as individual bee species may have diversified feeding and nesting requirements and are sometimes niche habitat specialists (Williams, 1998; Fitzpatrick *et al.*, 2006).

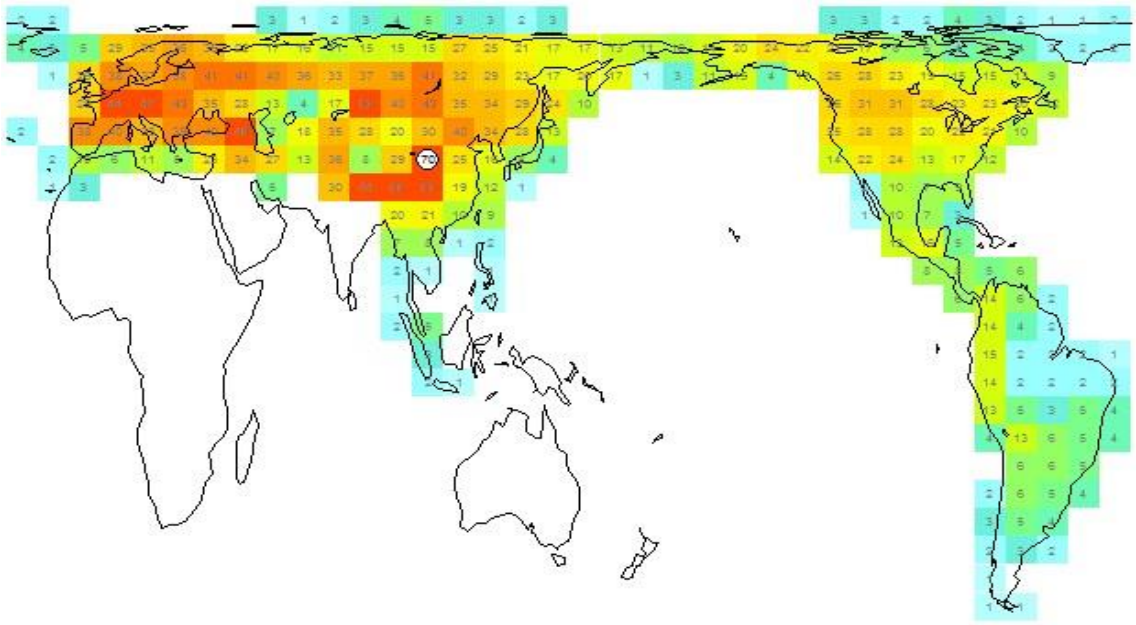


Figure 1.3 Worldwide distribution of bumblebee diversity as counts of species richness. The number of species richness is recorded within each large equal area (611,000 km^2) grid cell. An equal area colour scale is used to emphasise the difference among regions with red signalling highest species richness in an area. The white spot marked by the number 70 represents a global hotspot in Sichuan/Gansu. Bumblebees are particularly species rich in Asia and Europe, adapted from Williams (1998) and Natural History Museum webpage (https://www.nhm.ac.uk/research-curation/research/projects/bombus/introduction.html) .

Bumblebees are principally dependent on three main habitat resources to complete their colony cycle: nesting, forage, and overwintering sites. Overwintering takes place at about 2 or 15 cm underground in shaded areas usually near trees and in banks without dense vegetation and in sandy well drained soils. Selected sites are typically north facing slopes which are believed to be chosen to prevent diapausing queens from emerging too early on a warm or sunny day in the winter (Liczner and Colla, 2019) and many queens can be found hibernating in the same hibernation chamber or location.

In relation to nesting bumblebees exhibit generalist patterns with wild nests being found most commonly in forests, agricultural landscapes, grasslands and urban areas with nest density and colony survival being determined by the availability of forage resources (Liczner and Colla, 2019).

1.1.3.1. *Bombus terrestris*

Bombus terrestris (Linnaeus 1758) the buff-tailed bumblebee, is one of Europe's most common bee species and important natural pollinators. *B. terrestris* can be identified by a single yellow band at the top of the thorax, a single yellow band at the top of the abdomen and white stripes at the bottom of the abdomen (tail). Workers and queens can be differentiated not only by size, but the queen usually exhibits a tail with darker hairs (buff coloured) (Alford, 2009; Goulson, 2014) (Figure 1.4).

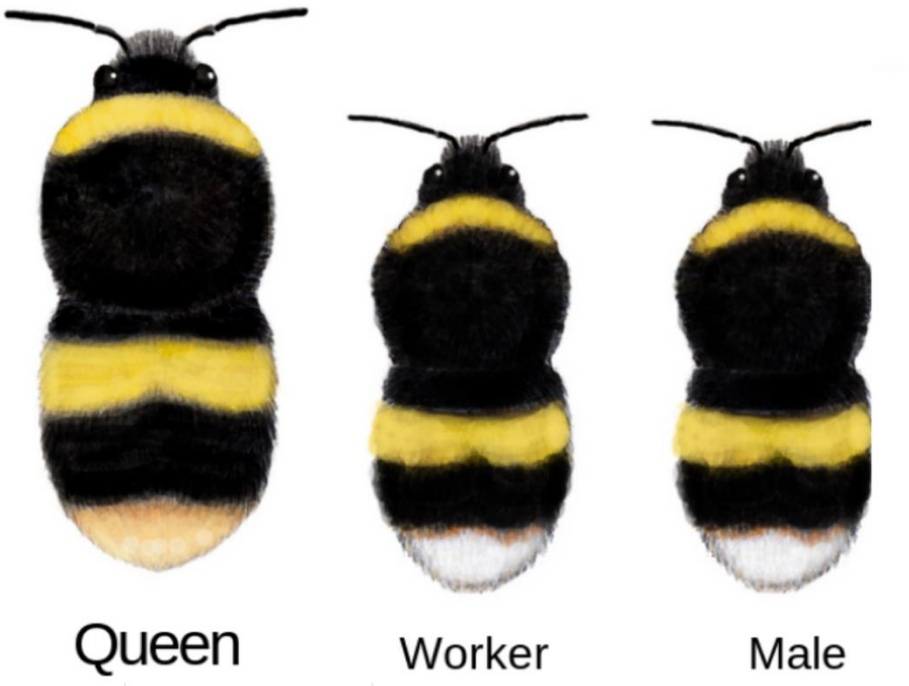


Figure 1.4 Caste differentiation among *B. terrestris.* Source: White-tailed bumblebees - Bumblebee Conservation Trust.

B. terrestris colonies are located below ground and comprise 100 - 300 workers which are produced and controlled by a single queen (Alford, 1975, 2009). The annual life cycle begins when queens emerge in spring after several months of hibernation and establish a nest for the purpose of laying eggs (Figure 1.5). Eggs hatch into larvae after four days and require a further 10-14 days during which they are dependent on the queen and workers for feeding, to pupate and metamorphose into an adult. Caste differentiation among females is apparent within the colony with a single queen solely undertaking the central reproductive duties whilst sterile female workers perform other tasks essential for colony success such as foraging and feeding larvae (Alford, 2009). *B. terrestris* colonies switch from the production of diploid workers to the production of haploid males and gynes (virgin queens) later in the season, representing a colony switch from the growth and expansion phase to the sexual maturation phase of colony development. This switch appears to be endogenously controlled by the queen, but the exact mechanism by which this event occurs remains unknown (Holland, 2013; Holland and Bourke, 2015). Once the production of sexual bees commences, the colonies enter the final stages of development and begin to die. Mature gynes and males leave the colonies to mate and never return. Mated gynes go into diapause during the colder months while males and the remaining colony perish (Alford, 2009).



Figure 1.5 Bumblebee colony lifecycle. Queens emerge in spring after several months of hibernation and establish a nest for the purpose of laying eggs. Eggs hatch into larvae and take several days before metamorphosing into an adult. Colonies go through an intensive period of growth with the production of diploid workers that care for the colony and the brood. Later in the summer colonies switch to the production of haploid males and gynes (virgin queens). Mature gynes and males then leave the colonies to mate and never return. Mated gynes go into hibernation during the colder months while males and the remaining colony perish. Image used with the permission of Pedro Busana (Faunart Illustration), adapted from Colgan et al., (2019).

1.1.3.2. Contribution to pollination

Pollination is the physical act of transferring pollen from the anther of one plant to the stigma of the same or different plant of the same species. This process is necessary and beneficial for both insect and plant and ensures plant reproduction and maintenance of genetic diversity, while providing the necessary nutrients required by a bee in the form of pollen and nectar (Goulson *et al.*, 2008; Fontaine *et al.*, 2011). Although managed and wild bees are known to contribute to the vast majority of pollination of a wide range of crops (Figure1.6 and 1.7), many other insects and animals also participate including flies,

beetles, moths, butterflies, wasps, ants, birds, bats, among others (Rader *et al.*, 2016, 2019).

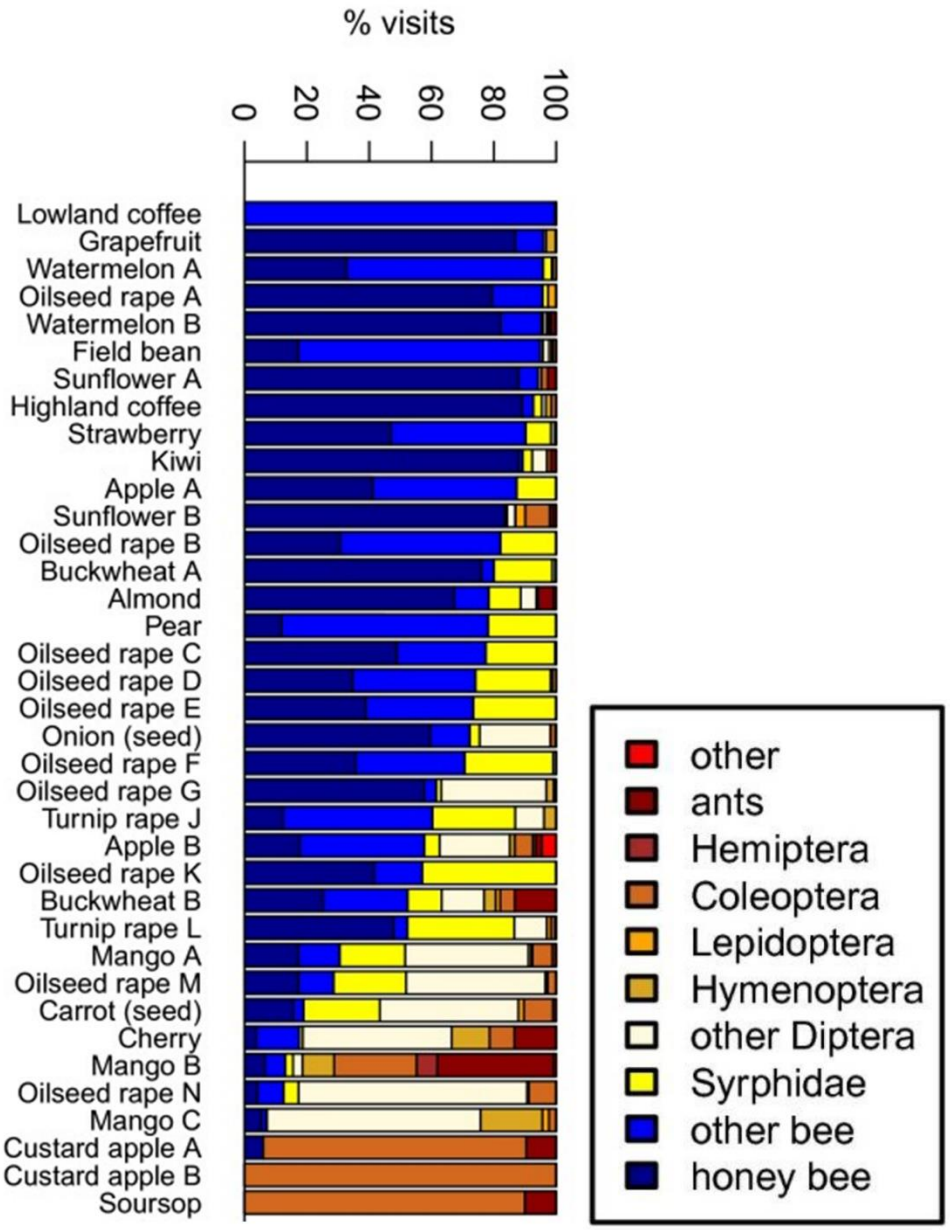


Figure 1.6 Percentage and diversity of pollinators per crop culture providing crop pollination services (visits) adapted from Rader *et al.* (2016)**.** Other bees and honeybees account for the vast majority of pollination of a wide range of commercial crops.

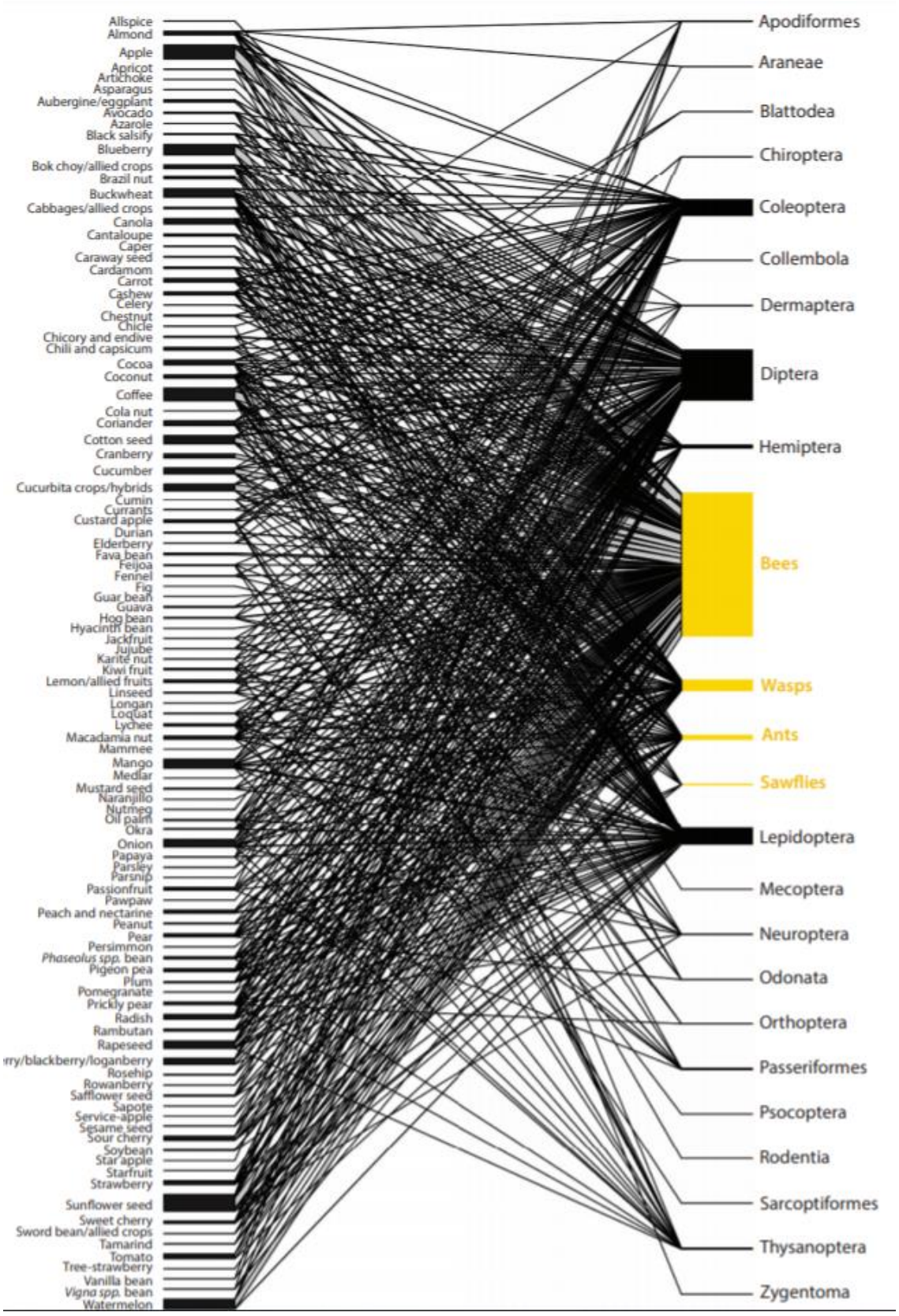


Figure 1.7 A crop–flower visitor network of 105 commercial crops (left) and main orders of pollinators (right). The width of the bars is based on the number of taxa found to visit that crop group in the literature reviewed; the wider is the bar, the more taxa visited that crop. The yellow bars and labels represent the groups that make up Hymenoptera, most of which are bees which visit 77% of the 105 crops assessed. Diptera was the second most frequent flower-visiting order (72% of crops), followed by Lepidoptera (54%) and Coleoptera (51%), adapted from Rader *et al.*, (2019).

Over 70% of the food crops consumed by humans benefit from pollination (Klein *et al.,* 2007), with the value of ecosystem services provided by pollinators to global agriculture being estimated at around €153 billion (Gallai *et al.,* 2009) to €213 billion per annum (Lautenbach *et al.,* 2012; Potts *et al.,* 2016). Rader *et al.* (2016) investigated the number of visits of different insect groups across 37 different crops which was then updated to a total of 105 crops of commercial interest that benefit from animal pollination (Rader *et al.,* 2019). In their study they highlight the interesting relationship between different pollinators and plant groups demonstrating that pollinators show preference for certain plants and that the diversity of insects is what guarantees efficient pollination and the sustainability of economic agricultural practices.

Bees can be flower specialists or generalists which determines their pollination preference (Michener, 2007). The length of their proboscis, determines whether the tongue can reach the nectar in the flower, thus gaining the reward from its pollination activity. Access to floral rewards is the ultimate factor determining if a bee species will become a stable pollinator of the respective plant (Michener, 2007). *B. terrestris* relies on its robust size and long tongues, giving them the ability to access a huge variety of flowers. Furthermore, this bee species is able to employ buzz-pollination, a phenomenon where bees use their powerful wing muscles to vibrate the flower directly which increases the efficiency of pollen transfer in numerous economically important food crops such as tomatoes, eggplants and a variety of berries (Suzuki *et al*., 2007; Cameron *et al.,* 2011; Cordoba and Cocucci, 2011). This efficacy has led to the domestication and commercial use of bumblebees in order to meet growing demands for food production (Graystock *et al.*, 2016).

1.2 Bumblebee declines-cause and effect

So far, only three bumblebee species are thought to have become extinct globally: *Bombus rubriventris*, *Bombus melanopoda* and *Bombus franklini*. However, about 34.7% of species are in decline globally (Arbetman *et al.*, 2017) and many more are listed as endangered or at risk and are being assessed for red list status by the IUCN (Goulson, 2014; Williams and Jepsen, 2016). Recent recorded reductions in bumblebee populations in Europe and North America have led to environmental and economic concerns of global decline as it increases the pressure on food production and ecosystem maintenance systems (Goulson *et al.,* 2008; Williams and Osborne, 2009; Szabo *et al.,* 2012; Giannini *et al.,* 2015). Several stressors have been implicated as potentially contributing to losses of global bee populations including pesticides, parasites, pathogens,

climate change and subsequently impaired nutrition, all of which can act both individually and in combination to negatively impact bee health (Goulson *et al.*, 2015; Smart *et al.*, 2016). In order to effectively protect and conserve both wild and commercial bumblebee populations it is essential to understand through which physiological mechanisms these stressors interact provoking negative effects on these important pollinators.

1.2.1 Environmental stressors and reduced genetic diversity

Environmental stressors that negatively impact global bee populations include habitat fragmentation (Goulson *et al.*, 2008), exposure to agrochemicals, disease, climate change and decreased resource diversity (Biesmeijer *et al.*, 2006; Goulson *et al.*, 2015; Simon-Delso *et al.*, 2015). Changes in land use due to increased crop monoculture and urbanisation can reduce floral and habitat resources for bees, creating a poorer diet (Rortais *et al.*, 2017) and also resulting in the isolation of colonies that become at risk of inbreeding and having reduced genetic diversity. In addition, climate change can dramatically disrupt the normal phenological activity of bees at both colony and individual level (Stone and Willmer, 1989; Simon G Potts *et al.*, 2010) and change or limit the climatic distribution of bee species increasing competition for space and resources (Williams, 2007).

1.2.1.1 Pathogens and parasites

Bumblebees are, in their role as pollinators, frequently exposed to and infected by a broad range of parasites, including protozoans, fungi, bacteria, and viruses. These pathogens can often evolve a specialised virulence equilibrium with their hosts (Goulson *et al.*, 2015; Rabajante *et al.*, 2015). However, if this equilibrium is broken by any disturbances, the impact of pathogens as stressors on a population may be increased (Meeus *et al.*, 2018). It is commonly accepted that parasites negatively impact bee health and drive bee declines both individually and in combination with other stressors.

Although numerous mechanisms are implicated in the spread of pathogens and parasites within bee populations, pathogen spill over from commercial colonies into wild populations has been recently and repeatedly demonstrated as a principal mechanism of parasite infection increases (Meeus *et al.*, 2011; Whitehorn *et al.*, 2013). The social nature and high population densities of most managed bee species (honeybees and bumblebees) facilitates the harbouring and spread of pathogens within colonies (Meeus *et al.*, 2011; Whitehorn *et al.*, 2013). In addition, the movement of colonies across large geographical

regions for crop pollination services, increases their potential to spread pathogens to wild bees including either novel or invasive pathogens (Cameron *et al.*, 2016).

Among the most prevalent pathogens infecting bumblebees are the protozoan trypanosome *Crithidia bombi* and the microsporidian *Nosema bombi* (McIvor and Malone, 1995; Imhoof and Schmid-Hempel, 1999). Both pathogens infect the bumblebee Malpighian tubules, digestive tract, haemolymph, fat and nerve tissues and the ventriculus and can cause substantial reductions in fitness and survival at the individual and colony levels by impacting foraging efficiency, the survival of over wintering queens, colony founding success, colony size and overall colony reproductive output (Durrer and Schmid-Hempel, 1994; McIvor and Malone, 1995; Fries *et al.*, 2001; Brown *et al.*, 2003; Otti and Schmid-Hempel, 2008).

1.2.1.2 Pesticides and their use in the modern agricultural industry

Pesticides encompass a large variety of compounds such as insecticides, fungicides, herbicides, rodenticides, molluscicides, nematicides, plant growth regulators and others (Aktar *et al*., 2009). Classes of pesticides that are considered highly toxic to insect pests (i.e. direct contact toxicity days after application to crops) include carbamates, organophosphates, synthetic pyrethroids, chlorinated cylcodienes and chloronicotines (Claudianos *et al.*, 2006). The introduction and use of synthetic insecticides coupled with the subsequent development and introduction of herbicides and fungicides has contributed dramatically to pest control and agricultural output (Aktar *et al*., 2009). However, it is becoming increasingly apparent that beneficial insects, including pollinators are also being negatively impacted (Hopwood *et al*., 2016; Mommaerts *et al.*, 2010; Blacquière *et al.*, 2012; Sanchez-Bayo and Goka, 2014; Tavares *et al.*, 2015; Sánchez-Bayo *et al.*, 2016; Zaluski *et al.*, 2017).

In recent years, the use of insecticides that act on the insect (including bumblebee) cholinergic system such as carbamates and organophosphates has increased considerably with one particular pesticide class, the neonicotinoids being widely used over the past two decades (Goulson, 2013). These systemic insecticides upon application, spread throughout the plant tissues making them toxic to any insect that feeds on it. This protects the plant from direct damage inflicted by herbivorous (mainly sap feeding) insects and indirectly by reducing insect vectored viruses (Simon-Delso *et al.*, 2015). The exposure of bees to neonicotinoids occurs primarily through the ingestion of residues in the pollen and nectar of contaminated plants which may present at parts per billion (ppb) to part per

million (ppm) levels (Desneux *et al.*, 2007; Blacquière *et al.*, 2012; van der Sluijs *et al.*, 2013; Sánchez-Bayo *et al.*, 2016; Buszewski *et al.*, 2019). Once ingested the neonicotinoid acts on the insect nicotinic acetylcholine receptor and has the ability to disrupt neuronal cholinergic signal transduction systems (Elbert *et al.*, 2008; Palmer *et al.*, 2013). Neonicotinoid exposure can have major negative impacts on bees at the individual through to the colony levels. Individual effects include the modulation of immunocompetence, which increases susceptibility to other stressors such as pathogens (Di Prisco *et al.*, 2013), deleterious effects on foraging and motor activities (Williamson, Baker and Wright, 2013; Williamson, Willis and Wright, 2014; Manjon *et al.*, 2018) and direct effects on learning and memory (Stanley *et al.*, 2015). Colony level effects have the potential to alter the production of the next generation of bees and to date neonicotinoid effects have been reported on colony establishment, growth and development (Whitehorn *et al.*, 2012), brood and sexual caste production (Woodcock *et al.*, 2017) and the susceptibility of colonies to colony collapse disorder (Goulson *et al.*, 2008; Potts *et al.*, 2010; Lu *et al.*, 2014; Stanley *et al.*, 2016).

Studies that have investigated the molecular effects of neonicotinoids on invertebrates are increasing in number. For instance the neonicotinoid imidacloprid affects ribosome biogenesis, lipid and phospholipid metabolic processes and genes with crucial role in regulation of focal junction, development and axonogenesis in mussels (Dondero *et al.*, 2010). Expression level changes in immune (Aufauvre *et al.*, 2014) ribosomal, oxidative phosphorylation and metabolism genes were also reported post neonicotinoid exposure in honeybees (Shi *et al.*, 2017). And alterations in pentose phosphate metabolism and sucrose metabolism genes have been described exclusively in the brain of honeybees after exposure to the neonicotinoids clothianidin, imidacloprid and thiamethoxam (Christen *et al.*, 2018). However, fewer studies involving neonicotinoid effects on bumblebees are available and those that have been published report that imidacloprid and clothianidin can affect processes such as mitochondrial function and alternative splicing (Moffat *et al.*, 2015a; Colgan *et al.*, 2019). Therefore, major shortcomings in our understanding on how these chemicals alter the bumblebee on the molecular and cellular level still exist. To address these knowledge gaps, research was conducted as part of this thesis with the aim of determining the physiological mechanisms of neonicotinoid interactions with the brains of these important pollinators. This will be achieved through the interpretation of alterations of protein abundance levels in the main central nervous system organ, the brain, and much of the research presented in the following chapters will describe the use of mass spectrometry-based proteomics as a means of providing greater understanding of

the bumblebee neurological system and of determining the changes in the bee brain, triggered by neonicotinoid exposure. It is anticipated that this new level of insight into neonicotinoid effects on the bumblebee neuronal system will provide a better understanding of the risks associated with widespread neonicotinoid use and enable the development of more effective protection and conservation strategies for both wild and commercial bumblebee populations.

1.3 Mass spectrometry based proteomics

Prior to the application of mass spectrometry, protein analysis was mostly dependent on the purification of individual proteins and complexes followed by activity and biochemical characterisation of purified material. Apart from being quite laborious, these experiments relied on the extent to which the proteins could be purified (Demmers, 2015) and provided information that was limited to the "one gene-one protein- one function paradigm that restricted previous generations of molecular biologists (Bensimon *et al*., 2012).

The development of electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI) for the analysis of intact proteins in the 1980s, and their coupling with an online liquid chromatography system in the 1990s, first conducted by the Hunt group from the University of Virginia, enabled large scale analysis of biological systems, through the identification of several constituent proteins and peptides simultaneously (Mann, 2016). Later on, associating the electrospray nano liquid chromatography mass spectrometry (LC-MS/MS) platform, with efficient database searching algorithms using MS/MS spectra, made possible by the advancements of genomics, led to the dawn of the MS-based proteomics age. Permitting accurate and rapid measurement of the mass-to-charge ratio (m/z) of thousands of molecules which has seen constant improvement in sensitivity and resolution over the past 25 years (Walther and Mann, 2010; Mann, 2016). The large scale analysis of proteins by mass spectrometry has now become an indispensable tool for the global characterisation of biological systems in many biomedical and research laboratories. Through the use of different equipment and continuous improvements in bioinformatic infrastructure, large quantities of data can now be processed and analysed, obtaining biological information by ordering proteins and their associated functions in linear pathways, according to the molecular biology paradigm. In addition, this high throughput approach has also shed light on how translated products can determine the phenotype from a systems biology

perspective, taking into consideration contextual relationships and crosstalk between linear pathways (Bensimon *et al.*, 2012; Mann, 2016).

A number of different proteomic strategies now exist to characterise the molecular landscape of cells and tissues, including the incorporation of heavy stable isotopes or label free comparison techniques and their respective and powerful statistical assessment tools (Demmers, 2015). The applications for these techniques can be dependent on the type of mass spectrometer available, which nowadays include the time of flight (ToF), the quadrupole, the Ion Trap, the FTICR and the Orbitrap mass spectrometers and their use, has the potential to influence the performance of the quantitative analysis in determined systems (Demmers, 2015).

ToF analysis works by identifying the size of the particle based on the velocity of an ion. The quadrupole mass spectrometer generates an electric field and obtains information on the movement of the ions through this field. The ion trap is a type of quadrupole that uses oscillating static direct current and radio frequency electric fields to trap ions. The FTICR traps ions in a magnetic field and their movement is translated into m/z ratios. The Orbitrap generates a high-resolution mass spectrum using Fourier transformation after trapping the ions in an orbital motion around a spindle. Some of the current equipment are hybrids that can employ one of more of the instruments mentioned for the analysis of peptides and proteins (Demmers, 2015).

Among the quantitative mass spectrometry techniques, 2-dimensional difference in gel electrophoresis (2D-DIGE) has been superseded by metabolic and chemical labelling of proteins and label free quantitative approaches. The metabolic and chemical labelling consists of the use of isotopically labelled tags which can be distinguished in the mass spectrometer as they differ in mass, while not altering the protein and peptide properties such as the retention times on a chromatography column (Demmers, 2015), thereby allowing for simultaneous analysis of tagged proteins in a single mass spectrum or LC-MS run. Metabolic or chemical labelling techniques aim for a relative quantitative measurement and include ^{15}N labelling, ^{13}C labelling, stable isotope labelling using amino acids in culture (SILAC), isotope-coded affinity tag (ICAT), isotope-coded protein labels (ICPL), tandem mass tags (TMT) and isobaric tag for relative and absolute quantitation (iTRAQ), dimethyl labelling and ^{18}O labelling (Angel *et al.*, 2012; Demmers, 2015).

Other techniques, however, aim for absolute quantitation (AQUA) of peptides and proteins which can be achieved by spiking the samples with a known concentration of a synthesised peptide or protein containing heavy isotopes. As in most labelling techniques, systematic quantitation errors can be incorporated into the sample depending on the stage

at which the label is incorporated. A series of factors have the capacity to influence this error incorporation, with chemical labelling being more susceptible than metabolic labelling. However, metabolic labelling is slower and depends on metabolic active cells which restricts its use considerably (Angel *et al.*, 2012).

1.3.1 Label free quantitation.

The development of label free quantitation (LFQ) methods was a consequence of the development of faster and more sensitive mass spectrometers and systems with highly reproducible and stable ionisation capabilities. This approach is not dependant on expensive labelling kits and can make use of two major approaches for quantitation: spectral counting and intensity based quantitation, which are based on the assumption that peptides that are more abundant will be detected and fragmented more often and as such, the patterns of peptide amino acid fragmentation (MS/MS counts) or relative peptide intensities provide information on protein abundance (Demmers, 2015).

Intensity based quantitation, involves quantifying the total amount of a peptide in a specific retention time window, for which the area under the curve in the chromatogram is accurately determined. Issues with different physicochemical properties of peptides that influence the identification and improvements in quantitation have been achieved by incorporating mathematical corrections and normalisation steps that ensure control over the amount of proteins loaded onto the LC-MS system and thus efficient quantitation. A method that combines peptide counting, spectral counting and ion intensities into a normalised spectral index has also contributed to the reduction in variance of complex mass spectrometry data quantitation in proteomic analysis (Griffin *et al.*, 2010; Demmers, 2015).

The employment of equipment and techniques such as those described above make it now possible to detect and quantify a rich diversity of hundreds to thousands of protein isoforms often spanning several orders of magnitude in biological samples (Angel *et al.*, 2012). Detection usually follows a series of steps post sample preparation involving the initial fractionation of the peptide mixture, that is immediately ionised either by ESI or MALDI and then analysed by MS. The power of this analysis lies in the measurement accuracy of the MS system and on the ability to perform tandem MS (MS/MS) measurements that provide additional information for the peptide amino acid sequence. Typical LC MS/MS involves the acquisition of a preliminary mass spectrum (MS1) of the precursor peptide, dissociation of isolated precursor ion of interest into smaller

fragments either by collision-induced dissociation or electron capture or transfer dissociation, and subsequent mass analysis of these fragments (MS2)(Angel *et al.*, 2012).

A typical proteomics pipeline involves the characterisation of complex samples of proteins using a high resolution accurate mass (HR/AM) spectrometer to gather mass spectrometry data, the identification and quantification of peptides and proteins followed by the analysis of these proteins to determine the profile for the specific tissue/fraction being analysed or the statistically significant changes in protein abundances between samples. For the purposes of this thesis the workflow outlined in Figure 1.8 was adopted using the Thermo Fisher Q-Exactive Orbitrap HR/AM spectrometer following the procedures and approaches outlined in Cox *et al.*, (2014). Proteins were identified, quantified and normalised using the MaxQuant software consisting of the Andromeda search engine (Cox *et al.*, 2011) and the predicted proteome derived from the genome sequencing initiative for the buff tailed bumblebee *B. terrestris* (Ben M Sadd *et al.*, 2015).

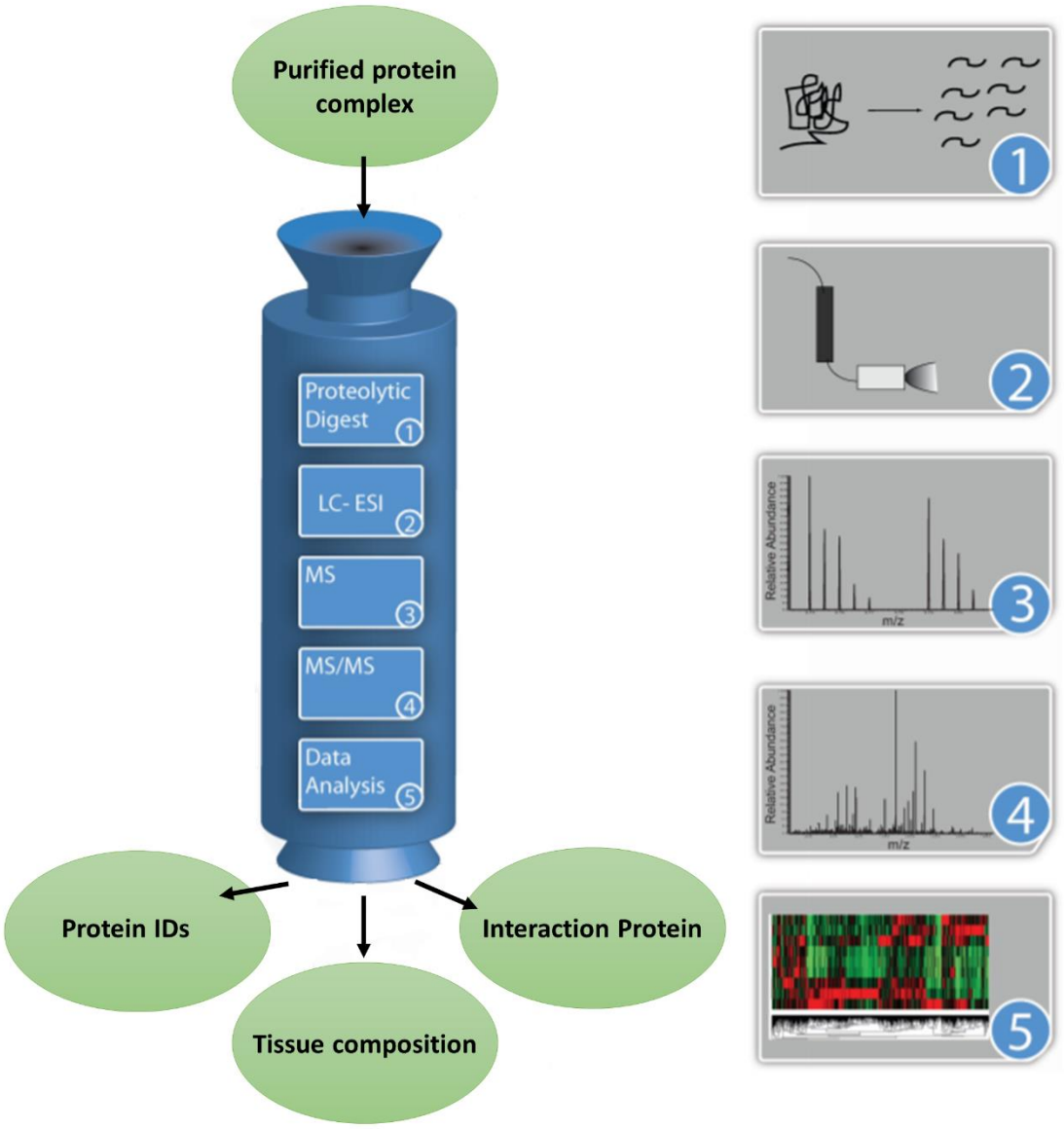


Figure 1.8 A typical proteomics workflow. Different samples are processed and fed into the proteomics pipeline, yielding different results depending on the application. The pipeline consists of several steps, listed in the different panels: proteolytic digest (1), the separation and ionisation of peptides by liquid chromatography (LC) coupled to electrospray ionisation (2), their analysis by MS (3), fragmentation of selected peptides and analysis of the resulting MS/MS spectra (4) and data analysis, including identification and quantification of proteins from several detected peptides, as well as downstream bioinformatic analysis depending on the specific application (5). In the workflow described here, mass spectrometry is performed with a linear ion trap–Orbitrap instrument (LTQ-Orbitrap) and computational proteomics is done within the MaxQuant framework. LC-MS/ MS, liquid chromatography coupled online to mass spectrometry; MS, mass spectrometry; MS/MS, tandem mass spectrometry; Protein ID, protein identification; m/z, mass- to-charge ratio. Adapted from (Walther and Mann, 2010).

1.4 Overview of thesis objectives

The principal aim of this thesis was to obtain fundamental biological information on the brain of the important bumblebee species *B. terrestris* through the characterisation of its proteome, taking into account the spatial, temporal and chemically dynamic complexity of this organ. MS-based proteomics enables the high throughput and comprehensive characterisation and quantification of proteins in a biological sample that

is closer to the phenotype than either the static genome or the overly fluctuating transcriptome (Walther and Mann, 2010).

While the proteome is highly complementary to the genome, it differs from cell-to-cell and time-to-time and is highly representative of the biological phenotype. It explores the large interconnection network of functionally and physically interacting proteins in a way that far surpasses traditional individual protein centric approaches such as antibody or epitope tagged protein detection, through western blot or microscopy (Walther and Mann, 2010). Therefore, MS-based LFQ proteomics is a core technique that allows for the unbiased and extensive identification and characterisation of the complex systems governing the organisation and functioning of cells and tissues under changing conditions.

In this thesis MS-based LFQ proteomics was used to characterise the brain proteome of *B. terrestris* under adult developmental stages and insecticide exposure challenges.

We begin by producing a comprehensive profile of the adult bumblebee brain proteome and determining the principal biological processes that account for typical brain function and organisation to serve as a reference for subsequent experiments. This was followed by a characterisation of the brain proteome across different ages to determine how age and in-colony experience influence the molecular landscape, responsible for the acquisition of complex behaviours and learning, which are necessary for the division of labour among bumblebee workers (Chapter II). This proteomic approach resulted in new insights into the potential molecular basis of complex behaviours, behavioural plasticity and even the ecology of bumblebees.

The characterisation of the bumblebee brain proteome enabled investigative experiments to determine the effects of environmental stressors on the expression levels of key proteins involved in brain homeostasis. These experiments focused on assessing the impact of the neonicotinoid insecticides clothianidin and imidacloprid on the brain of *B.s terrestris* and were designed to specifically test the hypothesis that sublethal doses of neonicotinoid would alter the brain proteomes of exposed bees. We also aimed to differentiate the chemical mode of action (Chapter III) and chemical mode of exposure (Chapter IV) for clothianidin and imidacloprid based on behavioural observations and differences in brain protein expression levels. It was anticipated that intrinsic differences in mode of action and exposure effect between the different chemicals and the consequences of exposure on pathways upstream and downstream to those expected would be determined. The datasets produced from characterisation of clothianidin and imidacloprid exposure (both chronic and acute) bumblebee brains, were subsequently

screened to determine whether potential biomarkers of neonicotinoid (either individual or general) exposure could be identified (Chapter V). Finally LC/MS was conducted to investigate the distribution, metabolism and accumulation of insecticide residues in the three main insect body sections (heads, thorax and abdomens) (Chapter VI) which assisted the interpretation of results from preceding chapters specifically in terms of amount of pesticide that is delivered to and remaining in the target tissue.

We anticipate that the new insights produced in this thesis improves the evidence base regarding neonicotinoid effects on bees and makes a valuable contribution to the development of more effective protection and conservation strategies for both wild and commercial bumblebee populations, in addition to the development and assessment of safer chemicals.

Chapter II

Proteomic characterisation of the adult bumblebee (*Bombus terrestris L.*) brain through age dependent development.

2.1. Introduction

2.1.1. Insect nervous system development

The process of differentiation in the nervous system of holometabolous insects is driven by a repetitive cycle of neuroblast proliferation and quiescence followed by elimination. Initially, proliferation of the stem cell neuroblasts gives rise to a neural groove and ridge that develops into the primitive nervous system that arises ventrally to the germ band. Although neuroblast division is necessary for self-renewal, they are also involved in the production of ganglion mother cells that differentiate into neurons or glial cells. It is estimated that 30 neuroblasts are initially present in *Drosophila* (Tan *et al.*, 2011), these are scattered cells that are delaminated from the neuroectodermal layer. The expression of proneural genes allows for the differentiation of ectodermal cells into neuroblasts instead of epidermal cells, and the subsequent expression of neurogenic genes guarantees that no neighbouring cells can differentiate into neuroblasts (Klowden, 2013b).

During late embryogenesis, the majority of neuroblasts are eliminated by apoptosis. One cell remains in each potential segmental ganglion and after becoming quiescent these cells proliferate during larval development. The pupal stage brings a new process of neuroblast elimination (Klowden, 2013b). This is regulated by the action of ecdysteroids, which are the major steroid hormones coordinating developmental transitions in insects (Uryu *et al.*, 2015). A heterodimeric nuclear hormone receptor complex of proteins, encoded by the ecdysome receptor (EcR) and ultraspiracle (usp) genes, regulate the expression of ecdysone-responsive elements. The ecdysone-response *Broad-Complex* (BR-C) gene acts as a key regulator of this cascade and encodes several zinc finger containing different isoforms (Z1-Z4) (Mugat *et al.*, 2000). During nervous system development an initial set of the Z3 isoforms of the BR-C ecdysteroid signalling pathway expressing primary neurons are responsible for establishing the overall configuration of the larval nervous system. These are later replaced by secondary neurons expressing the Z4 and Z1 BR-C isoforms during the pupal phase of neurogenesis (Younossi-Hartenstein *et al.*, 1996; Truman, 1996; Klowden, 2013b).

Neurons innervating larval structures are destined to disappear in the adult and they die soon after the larval-pupal moult. Other larval neurons are then remodelled and used for other functions. During the pupal stage the larval dendrites are pruned which is then followed by an outgrowth of the adult dendrites. The proliferative clusters of neuroblasts surrounded by glial cells are formed during this stage. The clusters localised in the anterior region give rise to the protocerebrum, deutocerebrum and tritocerebrum of the

brain and neurons are then reconfigured to make new connections and serve new functions in the adult brain. Clusters in other segments produce the suboesophageal and the abdominal ganglia. The growing axons and dendrites are sensitive to external guidance cues, and they extend throughout the embryo, being initially attracted to the midline and then expanding until they have branches through the whole organism (Stollewerk and Simpson, 2005; Klowden, 2013a).

The death of neurons during the larval-pupal moult is derived from hormonal oscillation that exists during the last larval instar where two peaks in ecdysteroid hormone abundance occur. The first peak occurs in the absence of juvenile hormone (JH) and makes the neurons more sensitive to the second and larger peak that causes neuronal degeneration of larval connections, initiating the differentiation of adult neuronal processes (Truman, 1996; Klowden, 2013). Understanding the underlying mechanisms of nervous system differentiation in insects is crucial for the correct interpretation and analysis of factors influencing bumblebee adult brain plasticity, in response to the environment. The following sections will present our current understanding of the insect nervous system morphology and functioning, and the relevance to the understanding of neurological functions in the bumblebee brain.

2.1.2. Adult insect nervous system function and morphology

The insect nervous system is composed of a peripheral and central part. The peripheral nervous system includes all the nerves that radiate from the central nervous system. These include the nerves that innervate muscles, stretch receptors, innervate the reproductive system and spiracles, and the various sensory receptors. Another component of the peripheral nervous system, is termed the visceral nervous system, which is responsible for innervating the gut, the heart, and the endocrine glands and forms a network of peripheral ganglia that is associated with digestive processes (Grueber *et al.*, 2005; Klowden, 2013a).

The central nervous system (CNS) of an insect comprises a bilaterally symmetric brain and a nerve cord, that extends through the thorax and abdomen, lying ventrally to the gut (Figure 2.1). It is formed of ganglia that are present in each segment of the body. The head contains two types, the supraesophageal and suboesophageal ganglia. The suboesophageal ganglion or posterior brain is made up of three fused segments and is responsible for controlling the mouthparts, it also has a dorsal lobe for connecting the anterior brain to the rest of the CNS. When reference is made to the insect brain, the supraesophageal part (or anterior brain) is generally being considered, the structure of

which is very variable and can vary even within species, which is mostly observed in eusocial insects (Avarguès-Weber *et al.*, 2012; Klowden, 2013b).

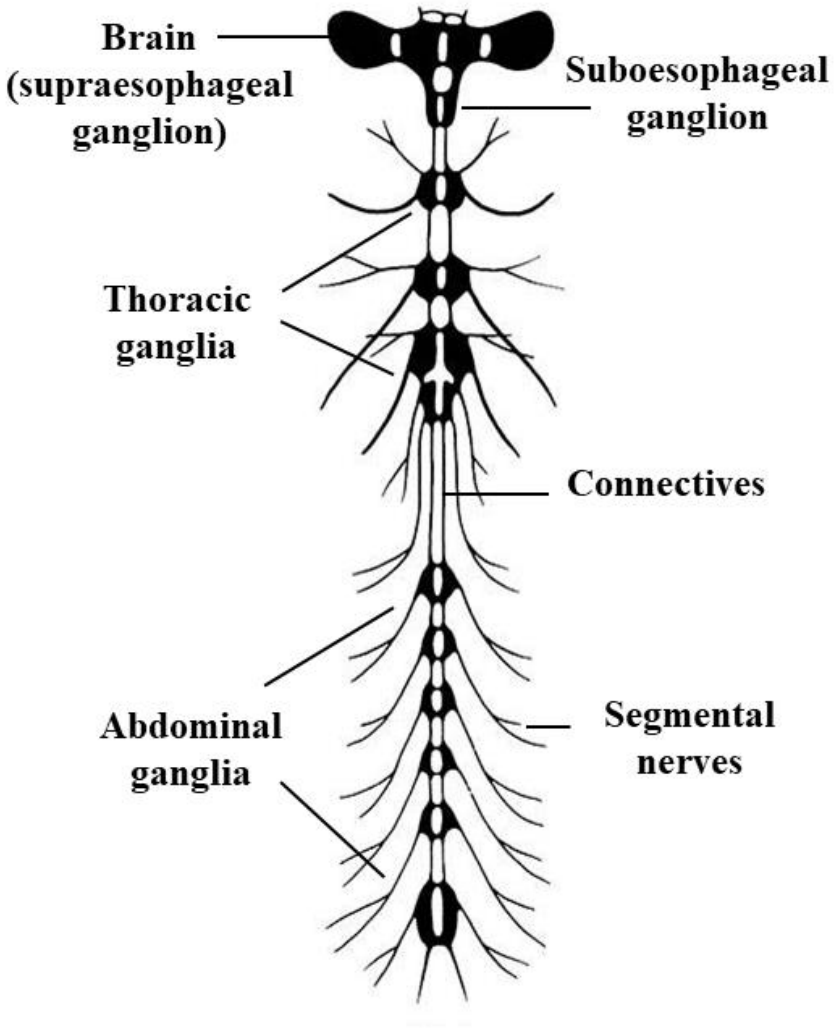


Figure 2.1 The generalised central nervous system of insects highlighting the arrangements of ganglia in the ventral nerve cord (Image adapted from Klowden, 2008).

It is estimated that the number of nervous cells in the insect brain vary from approximately 200,000 in the fruit fly *Drosophila melanogaster* to more than a million in the cockroach *Periplaneta americana* (Fahrbach, 2006). The brain is divided in three easily recognizable compartments, the protocerebrum which comprises the mushroom bodies, the central complex, the pars intercerebralis and the lateral accessory lobes, receiving input from the compound eyes and the ocelli, as well as having neurosecretory neurons, and regions important for sensory integration, learning and memory. The deutocerebrum receives input from antennal organs and the tritocerebrum which is

connected to the visceral nervous system and the ventral nerve cord, receives sensory input from the mouthparts (Homberg *et al.*, 1989; Klowden, 2013a).

The ventral nerve cord consists of a chain of ganglia which are formed by the soma of motor neurons, interneurons, and neurosecretory cells. Fibre bundles formed of the axons belonging to motoneurons, interneurons and incoming sensory afferents, connect the ganglia throughout the whole nerve cord to the brain. The insect nervous system is thus decentralised, meaning that the brain is not responsible for everything that happens in an insect. The body ganglia have localised control over the insect limbs, while the thoracic ganglia controls wings and legs, and is also responsible for obtaining environmental information such as wind strength and sounds, sending information back to the brain. Movement and mating are controlled by the nerve cord, and the brain is the main organ for information processing, although it is suggested that the body ganglia may also participate in information processing to some degree (Avarguès-Weber *et al.*, 2012).

2.1.2.1. The bee brain

The brain of bumblebee workers is a small structure, measuring approximately 5 mm in length and comprises an estimated one million neurons (for reference, humans have 100 billion neurons) (Fahrbach, 2006). Historically, brain complexity was associated with the capacity to carry out complex activities. Therefore, the brain of insects was not perceived to be able to process complex phenomena, such as learning and memory. This founded the idea that the insect brain must be “hard wired”, a concept that states that all the connections in the insect brain are fixed, allowing for a rapid but immutable response, in the same way as our reflexes. Pheromone-induced behaviour is a prime example of a hard wired type of response (Avarguès-Weber *et al.*, 2012).

The rapid neuroplastic responses on the structure of neuronal compartments and cells, that results from experience, would then be restricted to more complex organisms. Some small brained insects seem indeed to be hard wired, but that is not the case for bees. Apart from seeing ultraviolet and polarized light and to exhibit communication with symbolic dances, bees also appear to have neuroplasticity in individual neurons. This contributes greatly to the view that individual bees are extremely important for the decision making in the hives, and that they are able to store memories of past events and to assess the behaviour of other bees, based on their own past experience (Avarguès-Weber *et al.*, 2012).

Bee brains are much larger than those of other insects such as the fruit fly which have brains that are 50 times smaller than bees, with one tenth of the total number of neurons. The increased number of neurons account for a more elaborate structural division in the brain, including a developed visual system and mushroom bodies, which allows for the integration of multiple senses (Fahrbach and Velarde, 2009).

2.1.2.1.1. The mushroom bodies

In bees, the mushroom bodies occupy more than 20% of the bee brain, having about 340,000 neurons in the honeybee brain. In fruit flies, it accounts to only 2% of the brain having approximately 2,500 small neurons. In structure they can be defined as a pair of neuropils or group of neurons clustered together, that are subdivided into three regions: the calices, that serve as the input areas; the peduncle, which is the transfer region, and the alpha and beta lobes, that are the output regions (Figure 2.2, MB) (Fahrbach, 2006).

The greater the size of the mushroom bodies, the more compartments there are, which directly correlates with increased potential to produce new interconnections, and therefore new cognitive abilities. Such cognitive abilities are derived from the activities of the mushroom bodies neuronal cells, known as Kenyon cells (KCs). At least three subpopulations of KCs have been identified in the honeybee brain, all of which vary in dendritic and axonal morphologies, and in the neurotransmitters used. These cells are known to undergo structural alterations during the life of an individual, priming the neuronal connections and brain capacity for information processing (Strausfeld, 2002; Fahrbach, 2006) .

The mushroom bodies and its unique KCs form a system of neurons allowing for elaborate interconnections in the brain. They receive input from sensory neuropils, including the antennal lobe, and are essential for processing olfactory, mechanosensory and gustatory information. The mushroom bodies are also crucial for the processing and storage of information associated with pattern recognition learning, memory and sensory inputs and behaviour, and have also been implicated in visual coordination, locomotor activity and spatial orientation (Fahrbach, 2006; Klowden, 2013a). Therefore, mushroom bodies seem to act like a combination of the human hippocampus and the cortex, changing sensory information, into value-based information. This integration develops a signal that leads to memory over time, increasing the ability that bees have to navigate in different situations (Avarguès-Weber *et al.*, 2012).

Studies involving experiments that have inhibited the development of these structures, using genetic manipulation, have shown that individuals with deformed mushroom bodies suffer from loss of short- and long-term memory (De Belle and Heisenberg, 1996; Mizunami *et al.*, 1998; Raabe *et al.*, 2004). These findings suggest a strong correlation between mushroom body size and memory, as well as size and behavioural complexity, especially in hymenopterans. It is thought that the reason for this is the remarkable plasticity displayed by the Kenyon cells, in that they will readily and actively rebuild their neural fibres, acting as a sort of neural substrate on which new memories can grow and thus innervating new neuronal processes (Campusano *et al.*, 2007; Szyszka, 2008; Klowden, 2013a).

2.1.2.1.2. The central complex

The central complex is also referred to as the protocerebral bridge, the centre body and the noduli (Figure 2.2, CB). The function of the central complex is not yet clear and may vary, depending on the insect species. Studies have demonstrated that the central complex is not connected to the mushroom bodies, but is connected to the ocelli, which may suggest a strong correlation to the pathway responsible for polarised vision. In locusts it is connected to the ventral cord suggesting an additional role in movement (Klowden, 2013a).

Insects that can perform asymmetrical leg movements (such as weaving silk threads) are known to have highly developed central complexes. There is also a possibility that it plays a vital role in flying behaviour, since changes in the activity of neurons, at the region of the central complex, are seen at the start and end of flight, in locust grasshoppers (Song *et al.*, 2017).

Experiments that supressed the development of the central complex in *Drosophila*, generated individuals that were unable to fly and displayed uncoordinated movements. It seems that the central complex is not necessary for initiation of leg movement, but for its control, as the flies with undeveloped central complexes had difficulty orientating themselves and could not flight straight. It seems therefore, that the central complex can be implicated in visual and olfactory pattern recognition, flight control and limb coordination (Klowden, 2013a; Honkanen *et al.*, 2019)

2.1.2.1.3. The optic lobes

The optic lobes correspond to the portion of the protocerebrum that extends to the compound eyes, it is formed by three groups of neuropils (the lamina, medulla and the

lobula) (Figure 2.2, Lo, Me, La). They process the sensory information originating from the compound eyes. The lamina is the outermost neuropil and receives the direct inputs from the photoreceptors of the compound eyes, which then, transmits the information through connections to the medulla and then to the innermost neuropil, the lobula. The visual information is then sent to the calices in the mushroom bodies, where it is processed (Klowden, 2013a).

2.1.2.1.4. The antennal lobes

The antennal lobes are part of the deutocerebrum, which innervates sensory receptors and the muscles of the antennae (Figure 2.2, AL). They are divided into a series of glomeruli, which are dense synaptic regions surrounded by glial cells. The number of glomeruli varies according to the insect species, but there are areas with hundreds of thousands of receptor synapses, being formed between sensory neurons, interneurons and other neurons that project into the mushroom bodies, in the protocerebrum. Antennal receptors become activated by pheromone blends or food odours (Hansson and Anton, 2000; Klowden, 2013a).

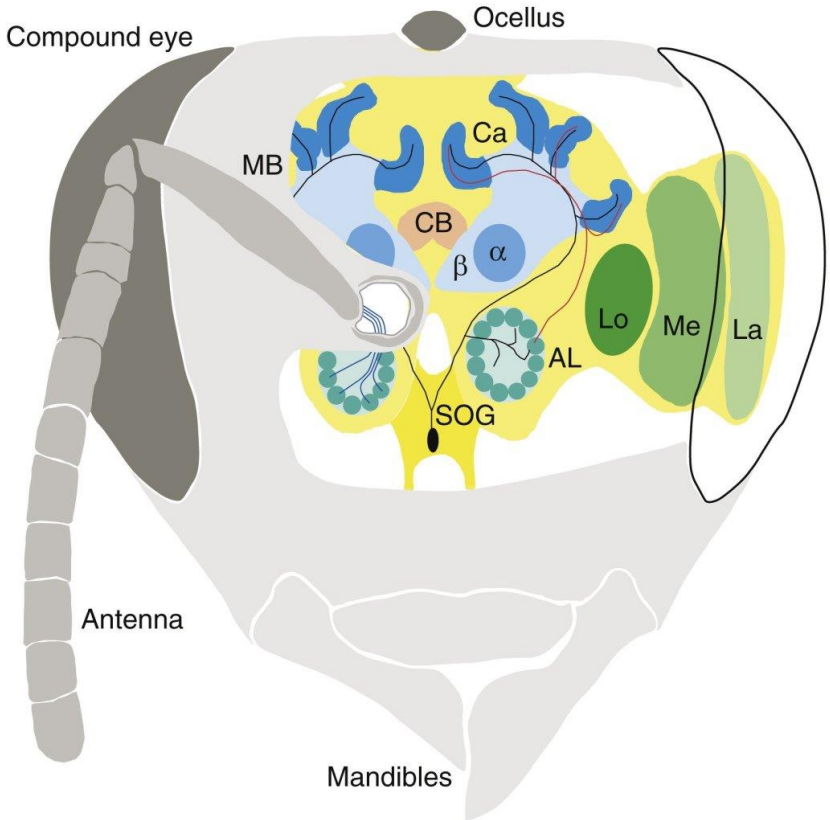


Figure 2.2 Bee head schematics showing the main neuropil structures in the brain. (MB = mushroom body, Ca = calyx, CB= central body, α and β lobes integrating the pedunculus. Lo = lobula, Me = Medula, La = Lamela. AL =antennal lobe, SOG = suboesophageal ganglion) Image from Chittka and Niven, (2009).

2.1.2.2. Neurons

The nerve cells, or neurons, are the principal components of the nervous system. They can integrate information, undergo excitation, and transmit information by electrical and short-range chemical signalling. Chemical signalling occurs through synapses from one neuron to the next, forming complex neural networks. Neurons develop from proneural cells during embryogenesis as neuroblasts, that delaminate from neuroectoderm, dividing asymmetrically to produce ganglionic mother cells, which then divide to form glial support cells and neurons (Oland and Tolbert, 2003; Klowden, 2013a).

The basic structure of a neuron consists of a cell body called soma or perikaryon and the projections that form axons and dendrites. Protein synthesis occurs exclusively in the soma. Therefore, synthesised metabolites have to be transported to the neuronal projections. The fact that protein synthesis occurs near the nucleus, means that the soma of neurons is rich in Golgi complexes and rough endoplasmic reticulum and these organelles are typically absent from the axons (Mendenhall and Murphey, 1974; Li and Strausfeld, 1997; Klowden, 2013a).

Axons originate from the soma at the axon hillock and are responsible for carrying information away from the cell. They are rich with smooth endoplasmic reticulum and neurosecretory vesicles. Dendrites, on the other hand, are the tree like branches that extend from the soma and are specialised in receiving information. These occur through the activation of specific receptors for the various neurotransmitters acting in the synaptic cleft, or in the case of sensory receptors, to environmental stimuli such as light, vibration and air or water borne molecules (Cuntz *et al.*, 2008; Klowden, 2013a).

Most insect neurons are monopolar, bearing a single axon from the soma, but neurons involved with peripheral receptors may be bipolar, and specialised neurons often referred to as internal stretch receptors, are mostly multipolar, being responsible for monitoring internal organ functions (French and Torkkeli, 2012; Klowden, 2013a) (Figure 2.3). According to their function, they may be divided into sensory or motor neurons, interneurons and neurosecretory neurons (Figure 2.4). Sensory neurons carry the information from sensory receptors, while motor neurons, regulate muscle contraction. The connection between the two is usually mediated by interneurons, which make up the majority of nerve cells in the central nervous system. Neuron axons, dendrites and synapses aggregate to form neuropils, while the cell bodies of motor neurons and interneurons are situated peripherally. The soma of interneurons and motor neurons make

up the ganglia of the insect central nervous system, while the cell body of sensory neurons are usually located near the environment sensing organ itself (Klowden, 2013a).

The KCs are specific types of neurons that have small somas (4-7 μm) and send characteristic dendritic arborizations to form the calyces, and bifurcating axons to the pedunculus, forming parallel fibre bundles called α and β lobes (Figure 2.2, α and β) (Schurmann, 1987). They are divided into several subpopulations according to their peripheral or central location in the calyces, but the functional implications of these differences are still unknown.

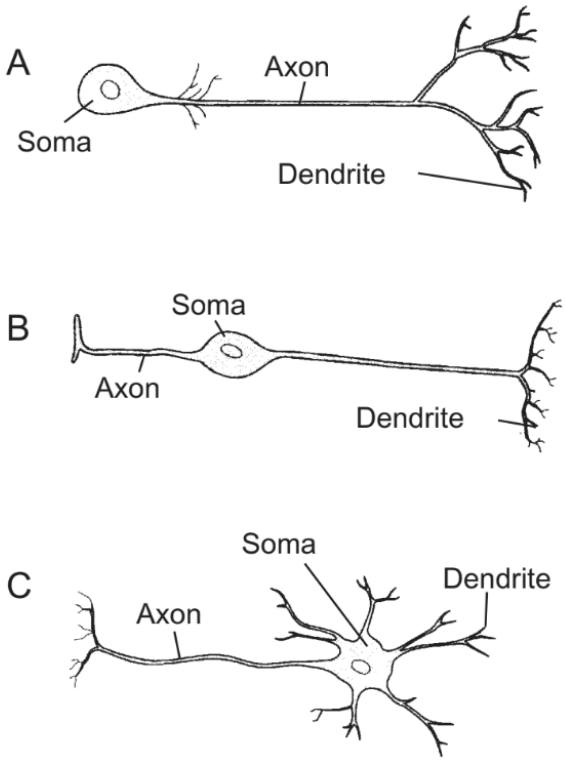


Figure 2.3 Schematic representation of insect neurons, (A) monopolar, (B) bipolar and (C) multipolar. Image from Klowden, (2013a).

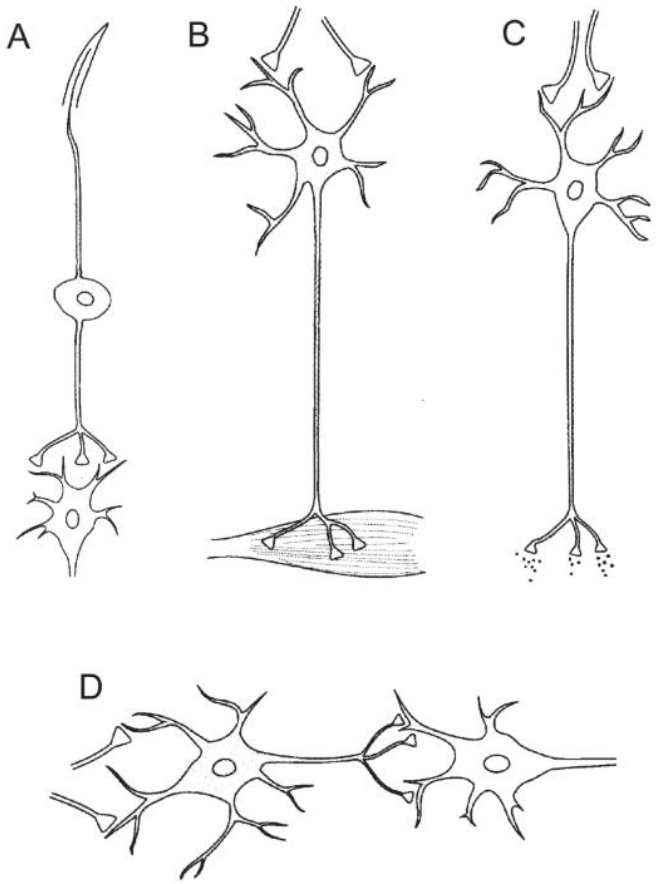


Figure 2.4 Schematic representation of types of neurons (A) sensory, (B) motor, (C) neurosecretory, (D) interneuron. Image from Klowden, (2013a).

2.1.2.3. Glial cells

In contrast to vertebrates, glial cells do not account for the majority of cells in the nervous system of insects (Klowden, 2013a). It is estimated that they constitute about 10-25% of the insect brain, but this does not mean they are less important. Insects have an open circulatory system, thus glial cells provide not only support and guidance for axonal growth, but a system of barriers that maintain the chemical and ionic differences, that are responsible for the electrical potential required for neuron functioning. They contribute to the blood brain barrier, preventing certain molecules from entering the central nervous system, balancing the levels of potassium and sodium between the potassium rich haemolymph and the neuronal cells, and keeping neurotransmitters from crossing back into the haemolymph (Carlson and Saint Marie, 1990; Carlson *et al.*, 2000; Klowden, 2013a).

Glial cells are mainly classified according to their location. A layer of perineurial glia (perineurium) is responsible for secreting an extracellular neural lamella that surrounds the entire nervous system, creating the blood-brain barrier system in insects. They are collectively referred as the nerve sheath. Pereneurial glial cells regulate

glycogen levels within themselves, suggesting that they may be responsible for transferring metabolites to the neurons. Beneath this layer of perineurial cells, there are larger and less numerous flattened cells, called sub perineurial glia, that appear to regulate the permeability of the blood brain barrier (Carlson *et al.*, 2000; Jackson and Haydon, 2008) . Cortex glial cells are found beneath the subperineurial cells, providing outgrowth instructions, and maintaining the neuronal cell body. Surrounding the soma of neurons are the neuropil glia, which consist of different subtypes of glia cells with flattened cell bodies. One major subtype of this group is the astrocyte, that surrounds synapses and may modulate its signalling, having the potential to influence nervous system transmission (Oland and Tolbert, 2011; Klowden, 2013a).

2.1.2.4. Neuronal stimulation and the main neurotransmitters acting on the insect central nervous system

Every cell in an organism engages in ion transport across their membranes, which in turn, generates an electrical potential difference between the inside (negative), and the outside of the cell (positive). The action potential is created by protein pumps that take in potassium, while pumping out sodium, magnesium and calcium. In neurons, the difference in electrical potential or polarization, can reach about -70mV, in the absence of nervous transmission (Klowden, 2013a). When receptors at the dendrites of neurons are stimulated by light, mechanical or chemical signals, the neurons undergo a depolarization proportional to the amount of stimulation, known as receptor potential. The generation of the receptor potential triggers the all-or-nothing depolarization along the axon, known as the action potential, which is responsible for activating synapses, through the conversion of electrical energy to chemical energy, releasing neurotransmitters and thus stimulating neighbouring neurons (Klowden, 2013a).

There are several types of neurotransmitters, neuromodulators and neuropeptides regulating synaptic transmission. Some are excitatory, while others have inhibitory effects. Acetylcholine (Ach) is the most common excitatory neurotransmitter in insects, it is released by interneurons in the neuropil regions, and by sensory neurons mediating fast excitatory cholinergic neurotransmission via Ach induced activation of pentameric nicotinic receptors (Sattelle *et al.*, 2005; Klowden, 2013a). Glutamate is another type of excitatory neurotransmitter, which is critical in the neuromuscular junction of insects and all invertebrates, its agonist action causes striated muscle contraction, and passes on sensory information to the central ganglia (Bravarenko *et al.*, 2003). The Gamma Aminobutyric acid (GABA) is a neurotransmitter that inhibits the postsynaptic membrane

both in the neuropil and at the neuromuscular junction. Thus, GABA acts as a neuromodulator being able to either affect the release of neurotransmitters at the presynaptic membrane or control the intensity of the response at the postsynaptic membrane. Several biogenic amines like histamine, dopamine, serotonin, tyramine and octopamine can also function as neuromodulators and neurotransmitters. Octopamine regulates almost every physiological process in invertebrates, acting in the central nervous system, desensitizing sensory inputs, and regulating the animal's response patterns. It appears to induce the release of fatty acids from the fat body, initiate stress responses and control the activity of flight muscles. It also increases the sensitivity of sensory receptors through the modification of membrane resistance, adaptation of receptors to stimuli and enhancement of postsynaptic responsiveness (Blenau and Baumann, 2001). Dopamine acts by controlling heartbeat rate and nerve evoked contraction of the foregut muscle. These two and other amine neurotransmitters, also act in a variety of osmoregulation processes, and can control the levels of glucose in the haemolymph (Fieber, 2019).

Serotonin plays an important role in learning, affecting a number of membrane currents such as the K^+, whose reduction by PKA- dependent phosphorylation leads to depolarization and an increase in membrane excitability. This affects short term learning and increases the release of glutamate in the synapses, and new protein synthesis, which is believed to be involved in long term learning (Byrne and Hawkins, 2015). Nitric oxide also acts as a neurotransmitter in insects and may be involved in processes such as learning and memory formation in invertebrates in a way that induces neuronal plasticity, rather than, or in addition to, the effects that glutamate has in vertebrates (Osborne, 1996; Klowden, 2013a).

The purines ATP and adenosine, plus the pyrimidines also act as neurotransmitters and neuromodulators. ATP and its metabolites are present in presynaptic vesicles and are necessary for active transport to concentrate neurotransmitters into vesicles, thus they induce modulatory and often excitatory effects, through the activation of calcium channels. Purinergic receptors can also activate adenylyl cyclase-linked G proteins modulating olfaction, gustation, axon regeneration and inhibition of sexual maturation in arthropods, echinoderms and annelids (Burnstock and Verkhratsky, 2009).

There are also more than 70 families of neuropeptide transmitters acting and regulating the action of neurotransmitters in the nervous systems, including proctolin, tachykinins and the FMRFamide, that act in the synaptic cleft. These will not be further discussed here but a review of some families is available in Fieber (2019).

Neurotransmitters are degraded at the synaptic cleft by enzymes after they have been bound, allowing some materials to be recycled and preventing neurons from being continually activated by a stimulus that is no longer present, thus opening the receptor to stimulation by a second release of neurotransmitters. Carbamate and organophosphate insecticides act by inhibiting the enzyme acetylcholinesterase, which degrade the neurotransmitter Ach and neonicotinoid insecticides, are known to bind irreversibly to the nicotinic acetylcholine receptor, thus interfering with nervous system signalling within the insect (Klowden, 2013a; Williamson and Wright, 2013; Buszewski *et al.*, 2019).

2.1.3. Brain investment and maturation under colony level selection

Neuroblasts in proliferative clusters surrounded by glial cells from the anterior region of the nervous system form the protocerebrum, deutocerebrum and tritocerebrum structures of the adult brain as we know it, during the pupal developmental stage (Klowden, 2013b). Although it is known that growing axons and dendrites are sensitive to external guidance cues and form new connections post emergence, very little is known about the cellular changes in response to organismal aging and external experience.

Studies with small insect species have demonstrated that adults can undergo significant neurogenesis. It is estimated that a cricket can add as many as 20 new KCs per day to each calyx of the mushroom bodies, resulting in the addition of hundreds of new neuronal cells to the brain during the life of the insect (Cayre and Strambi, 1994). This process is critical to optimise the learning abilities of the animal and its adaptation to its environment (Cayre *et al.*, 2007). Neurogenesis is principally regulated by developmental hormones, with juvenile hormones (JH) promoting neurogenesis and ecdysteroids reducing the number of KCs added to mushroom bodies. Although this phenomenon seems to be absent in certain insect taxa, including fruit flies and honeybees, a second type of neuronal plasticity is responsible for changing the shape of the brain of adult bees (Fahrbach and Velarde, 2009).

Changes in the volume of the neuropils in the mushroom bodies are caused by strengthening and thickening of axonal and dendritic arborization. Such growth increases the number of synapses in these regions and several studies in social Hymenoptera document that the final volume of the mushroom body neuropil is experience dependant, meaning that worker honeybees, which are responsible for foraging, have bigger neuropils than nestmates that perform hive tasks (Ismail and Fahrbach, 2006; Smith *et*

al., 2010). Changes in brain volume have also been described in relation to division of labour and position in the social hierarchy in two species of social wasps (O'Donnell *et al.*, 2015, 2018). It is important to understand these structural changes in the brain as studies have demonstrated that the bigger the neuropil, the greater their information processing capacity (Krofczik *et al.*, 2008).

In honeybees, it has been proposed that an initial period of mushroom body growth occurs following adult emergence, but that the continuity of such growth is dependent on foraging experience later on (Fahrbach *et al.*, 1998). The model for neuronal plasticity of adult honeybee brains proposes that KCs respond to various internal and external factors with dendritic growth. The internal factors also correspond to fluctuations in the levels of the expression of juvenile hormone, which peaks at around the second or third day post emergence, followed by a peak of ecdysteroids around day 3 or 4. These hormones known to act in neurogenesis, activate a series of nuclear receptors that trigger dendritic growth in the mushroom body neuropils, independently of foraging, for about 15 days post emergence. After that, the juvenile hormone becomes extremely upregulated, switching behaviour and leading bees to forage. Once foraging, increased muscarinic signalling dictates gene expression and increases dendritic growth (Fahrbach and Velarde, 2009; Dobrin *et al.*, 2011).

Honeybees have age dependant division of tasks with newly emerged bees staying in the hives, where they are responsible for a series of maintenance activities, starting from nurse responsibilities, where they care for the brood and the queen. The transition to other activities such as food storage, comb construction, removal of dead bees and guarding the colony, occurs later in the life of the worker bee, until they finally switch to foraging at around day 21 or 24 of life (Fahrbach and Robinson, 1995). Behavioural transitions within the hive are not absolute and can be altered depending on the necessity of the colony, but the shift from hive activities to foraging is usually more dramatic. The exact timing of these transitions is not fixed and is influenced by weather, nutritional status and most importantly, colony age structure. This behavioural maturation is principally associated with fluctuations in expression level of hormones, as described above, and with physical changes to different brain regions as evidenced by Fahrbach and Robinson (1995) who demonstrated changes in the volumes of the antennal lobes, between one day old bees and nurse bees, and on neuropil volume of the mushroom bodies, between recently emerged bees and older workers and foragers. Therefore, such changes in brain structure can be associated with learning and division of labour.

However, bumblebees have a different structural colony organization, with division of labour not being so defined by age. Although workers carry out activities that are similar to honeybees, such as nursing brood and building and maintaining the colony structure, the initiation of foraging, contrastingly, is correlated with body size more than age (Riveros and Gronenberg, 2009). Therefore, questions exist regarding how the adult bumblebee brain develops and matures, and how the incidence of neuroplasticity is related to behaviour development and social organization.

Jones *et al.* (2013) demonstrated that environmental experience, during the first week of development of newly emerged workers of *B. impatiens*, significantly affected the size of the mushroom bodies and antennal lobes, in the same manner as suggested for honeybees. Neuropil size and volume were also increased significantly during the first 21 days of adult life. These changes were observed in the optic and antennal lobes and in the mushroom bodies, with relative volume increases of 14.8% in the optic lobes and 16% in the antennal lobes, after only two days post emergence. The mushroom bodies were also significantly larger after only 2 days but showed consistent growth over the first 7 days of adult life, having a relative volume increase of 20.3% after 3 weeks.

These results are congruent with the findings for honeybees, but the age-related neuroplasticity of bumblebees may contribute to a more flexible developmental transition in behavioural tasks, due to a rapidly maturing brain, which allows workers to respond to changing colony requirements via alterations in neuronal circuitry.

To investigate and visualize the changes occurring in biochemical pathways controlling neural plasticity in the adult bumblebee brain, we used label free quantitative proteomics to characterise the total brain proteome of bumblebee workers at day 1, 5 and 20 post emergence.

2.2. Chapter aims and hypothesis

Compelling evidence suggests that experienced bees make the best choices related to foraging, taking less time than inexperienced bees to extract nectar and pollen; and they handle complicated flowers with ease (Heinrich, 1979). Such differences in performance and problem solving are associated with learning and memory behaviour. We have seen how the structure of the brain changes with age and experience, and we now set out to investigate the molecular changes in the brain resulting from these processes of maturation. The principal aims of this chapter were to:

1. Objectively characterise the general brain proteome of an adult bumblebee worker.

2. Investigate the changes in brain proteome associated with bee aging and in-colony experience, and to infer if such changes can be associated with behavioural plasticity or cognitive capacity in bumblebees.

We hypothesise that the molecular profile of bumblebee brains differs greatly in relation to age, and that such differences are crucial to help explain different degrees of social organization.

2.3. Experimental outline and methods

2.3.1. Colony maintenance *Bombus terrestris audax*

Commercial colonies (two week young research colonies) of *B. terrestris audax* were obtained from Biobest (supplied by Agralan UK) and maintained at standard environmental conditions of 25°C ± 1°C and 60% ± 5% relative humidity with continuous darkness or red light and fed sugar solution (Biobest) *ad libitum* and 2 g of pollen (Biobest) every day. Colonies where the founding queen had died (in transit) or where males had started to emerge from pupal cells, were not used for any molecular experiments. Colonies were acclimatised at laboratorial conditions for at least 48h before beginning the experiments.

2.3.2. Worker acclimatization for brain proteome characterization experiment

Workers were removed from four different colonies, colour-tagged using a water-based paint to allow for tracking within colonies, before being transferred to a plastic box (w 16,5cm; h 8,5cm; d 12cm) (max 10 bees per box (2 or 3 from each colony)). Autoclaved fine sand was used to cover the bottom of the boxes and pollen and 50% sucrose (w/v) were provided *ad libitum.* Bees, which each represent one biological replicate, were acclimatised, and kept in these boxes before brain dissection and protein extraction took place.

2.3.3. Age controlled worker tagging

One healthy commercial colony with a few workers and a laying queen was chosen to be used in the age comparison experiment. The colony was monitored day after day

with three check-ups being done daily to identify recently emerged bees (callows). Once identified, callows were colour tagged by application of a small drop of a water-based paint on the bee's thorax. Each monitoring day was assigned a different colour to allow for tracking of time in relation to individual emergence. After being tagged, bees were returned to their same colony, allowing for normal social interaction throughout the duration of the experiment. Three groups were defined in this study to be used in the experiment: recently emerged bees (<1 day old) were assigned as the D1 experimental group; 5-day old bees were assigned to the D5 experimental group and the third with 20-day old workers was assigned to D20. No clear size difference was observed between any of the bees chosen for this experiment. Bees were anesthetised in a CO_2 chamber and brains were dissected immediately at the time-points described above. Each bee brain representing one biological replicate.

2.3.4. Sample preparation for mass spectrometry-based proteomics

Five brains for each experimental group (D1, D5, D20) and 6 brains of non-age controlled workers kept in microcolonies for a maximum of 7 days were dissected and transferred into 200 µl of 6 M urea, 2 M thiourea buffer, supplemented with a protease inhibitor cocktail (Roche) and immediately snap frozen using liquid nitrogen (biological replicates). Samples were stored at -20 ºC until the dissections were completed. After thawing on ice, brains were homogenised and centrifuged for 5 min at 9,000 x g to pellet any cellular debris.

The supernatant was then removed and quantified using the Qubit™ protein quantification system (Invitrogen), following the manufacturer's instructions. Samples were chosen accordingly to the most similar protein quantities and protein profiles obtained on an 1D SDS page gel. Eighty-five micrograms of each sample were precipitated using the 2D Clean Up Kit (GE Healthcare), following the manufacturer's instructions. The resulting protein pellet was resuspended in 50 µl of 6 M urea, 2 M thiourea, 0.1 M Tris-HCl, pH 8.0 buffer, and re quantified using the Qubit™ system. 105 µl 50 mM ammonium bicarbonate was added to 20 µl of each sample. Proteins were reduced with 0.5 M of dithiothreitol (DTT) at 56ºC for 20 min and alkylated with 0.55 M iodoacetamide (IAA) at room temperature for 15 min, in the dark. One microliter of a 1% w/v solution of Protease Max Surfactant Trypsin Enhancer (Promega) and 1 µg Sequencing Grade Trypsin (Promega, 0.5 µg/µl stock) was added to give a protein:trypsin

ratio of 40:1. The protein/trypsin mixture was incubated at 37°C overnight. Digestion was terminated by adding 1 µl of 100% trifluoroacetic acid (Sigma Aldrich) and incubation at room temperature for 5 min. Samples were centrifuged at 13,000 x g for 10 min and the supernatant was removed and purified for mass spectrometry using C18 Spin Columns (Pierce), following the manufacturer's instructions. The eluted peptides were dried using a Speedy Vac concentrator (Thermo Scientific Savant DNA120) and resuspended in 2% v/v acetonitrile and 0.05% v/v trifluoroacetic acid (TFA). Samples were sonicated for 2 min to aid peptide resuspension followed by centrifugation at 13,000 x g for 5 min. The supernatant was removed and a volume equivalent to 1 µg was loaded onto a QExactive (ThermoFisher Scientific) high-resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system.

2.3.5. Proteomic analysis

Protein profiles were determined in three biological replicates for each group, using label-free, mass spectrometry-based proteomic profiling. Peptides were separated on a 2% to 40% acetonitrile gradient on a 500 mm length EasySpray PepMap column, using a 120 minutes' reverse-phase gradient at a flow rate of 250 nL min^{-1}. All data were acquired with the mass spectrometer operating in automatic data dependent switching mode. A full MS scan at 140,000 resolution and range of 300–1700 m/z was followed by an MS/MS scan, resolution 17,500 and range of 200–2000 m/z, selecting the 15 most intense ions prior to MS/MS.

2.3.6. Protein identification and abundance level quantitation

Protein identification and LFQ normalisation of MS/MS data was performed using Max-Quant v1.6.5.0 (http://www.maxquant.org) following the general procedures and settings outlined in (Hubner *et al.,* 2010). The Andromeda search algorithm (Cox *et al.,* 2011) incorporated in the MaxQuant software was used to correlate MS/MS data against the NCBI reference sequence database for *B. terrestris* (UniProt Consortium, 2015)(entries, downloaded May 2017) with duplicates and redundant sequences removed and a contaminant sequence set provided by Max-Quant. Prior to the proteomic searches the *Bombus* sequence database was modified so that each protein annotation included the homologous Uniprot ID for *Apis mellifera* and *Drosophila melanogaster*. The following search parameters were used: first search peptide tolerance of 20 ppm, second search peptide tolerance 4.5 ppm with cysteine carbamidomethylation as a fixed modification and N-acetylation of protein and oxidation of methionine as variable modification and a

maximum of two missed cleavage sites allowed. False Discovery Rates (FDR) were set to 1% for both peptides and proteins and the FDR was estimated following searches against a target decoy database. LFQ intensities were calculated using the MaxLFQ algorithm (Cox *et al.*, 2014) razor and unique peptides with a minimum ratio count of two peptides across samples. Peptides with a minimum length of seven amino acids were considered for identification and proteins were only considered identified when more than one unique peptide for each protein was observed.

2.3.7. Proteomic characterisation of the total brain proteome of workers of *Bombus terrestris*

Perseus v.1.5.5.3 (http://www.biochem.mpg.de/5111810/perseus), was used for data analysis, processing and visualisation. Normalised LFQ intensity values were used as the quantitative measurement of protein abundance for subsequent analysis. The data matrix originating from two other mass spectrometry runs with adult worker brains was first filtered for the removal of contaminants and peptides only identified by site and LFQ intensity values were $\log_2$ transformed.

Proteins not found consistently in all replicates of at least one group were omitted from the analysis. As three different datasets were used to characterise a standard brain proteome, a list of identified proteins in each dataset was copied and merged, and only proteins identified by ≥2 peptides were kept. Following removal of replicates a list of all individual proteins identified in the brain proteome was constructed.

The unique Uniprot ID for all identified proteins was extracted from the identifier string and used to query the BlastKoala platform using the taxonomy ID 30195 (Brite and Kegg mapper), for *B. terrestris,* and the STRING v 11 and the GOrilla databases, against the reference organism *Drosophila melanogaster*. Medium network specificity values were applied on the analysis and GO term enrichments, obtained for biological process, molecular function and cellular compartments, were analysed to characterise the total brain proteome.

2.3.8. Proteomic data analysis and characterisation of aging and maturation in the brains of *Bombus terrestris*

Perseus v.1.5.5.3 (http://www.biochem.mpg.de/5111810/perseus), was used for data analysis, processing and visualisation. Normalised LFQ intensity values were used as the quantitative measurement of protein abundance for subsequent analysis. The data

matrix was first filtered for the removal of contaminants and peptides only identified by site. LFQ intensity values were log_2 transformed and each sample was assigned to its corresponding group (D1, D5 and D20).

Proteins not found in three out of three replicates in at least one group were omitted from the analysis. A data imputation step was conducted to replace missing values, with values that simulate signals of low abundant proteins chosen randomly from a distribution specified by a downshift of 2.1 times the mean standard deviation (SD) of all measured values, and a width of 0.1 times this SD. Principal component analysis was performed on all replicates. Individual pairwise Student t-test comparison was also carried out between D1 and the other two timepoints to identify statistically significant differently abundant proteins (SSDA) (-log p value $\geq$ 1.3).

Volcano plots were generated in Perseus by plotting the negative log p-values on the y- axis and log_2 fold-difference values on the x-axis for each pair-wise comparison to visualise changes in the total brain proteome through aging. SSDA proteins with abundance changes greater than 1.5 relative fold change (RFC) were highlighted. A numeric Venn diagram analyses comparing each developmental group identified exclusively expressed proteins which were analysed individually.

The Uniprot ID for all SSDA proteins was extracted from the identifier string and used to query the BlastKoala and STRING v 11 databases, using the same parametres described above. GO term enrichments were ranked based on number of genes identified in the dataset in relation to the total number of background genes for that gene ontology (% coverage). This provided a complete characterisation of the brain proteome throughout aging.

2.4.Results

2.4.1. Characterisation of the adult bee brain proteome

The characterisation of the brain proteome was conducted through the bioinformatic grouping of all proteins identified in the brain of *B. terrestris* after 3-hour long mass spectrometry runs per sample. Proteins not found consistently (3 out of 3) in the non-aged controlled biological replicates and in 3 out of 3 within each of the age-controlled biological replicates for each treatment group were omitted from the analysis. All identified proteins were collated, and replicates were removed resulting in the final identification of 2,574 unique proteins that represent the general brain proteome of an adult worker bumblebee.

The BlastKoala analyses resulted in the annotation of over 76.1% of entries uploaded against the *B. terrestris* taxonomy ID 30195. The majority of the proteome was annotated to 4 main functional categories including genetic information processing (20.3%), protein families: genetic information processing (18.2%), environmental information processing (9.2%) and carbohydrate metabolism (9.2%). The remaining terms were divided into other categories, including cellular process (7.4%), protein families: metabolism (5.4%), organismal systems (4.8%), protein families: signalling and cellular process (4.5%), energy metabolism (4.4%), lipid metabolism (3.2%) and human diseases (2.6%) and 10.8% accounting for the remaining of the categories (Figure 2.5).

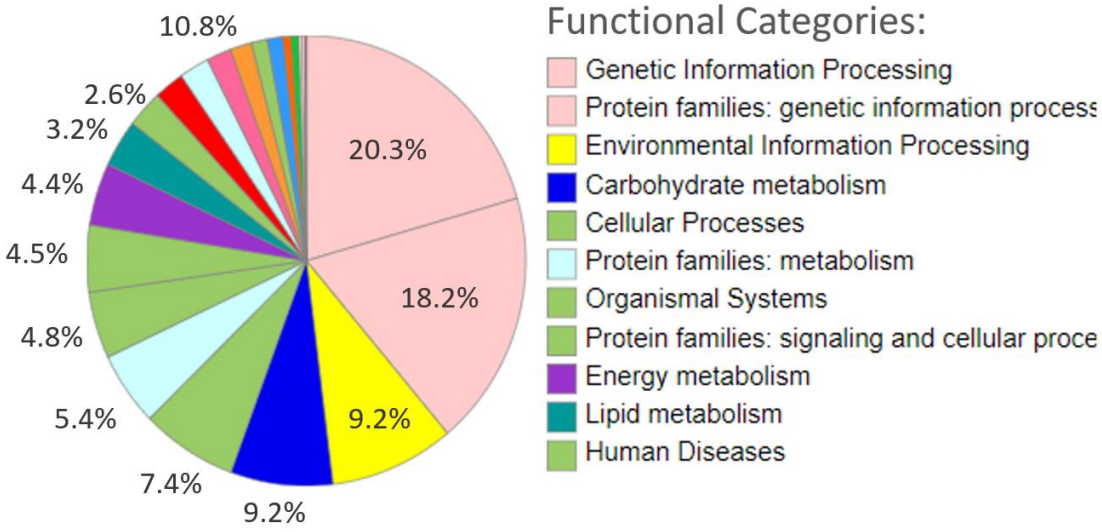


Figure 2.5 BlastKoala pathway analysis of the brain proteome. A global overview of biological processes associated with the bumblebee brain proteome. Among the biological processes were those related to normal cell housekeeping including numerous metabolism-associated processes, genetic information processing, organismal systems and environmental information processing. Out of 2,574 entries, 76.1% of the proteins received at least one annotation.

Out of the 2,500 uploaded proteins representing the standard bumblebee brain proteome, BlastKoala KEGG annotation analysis identified six hundred and fifty-two proteins with enzymatic activity, fifty-three with kinase activity, 65 phosphatase and 76 had peptidase activity, all of which represented the 3 main enzymatic classes in the brain proteome (Table 2.1).

Only 6 transcription factors were annotated in BlastKoala, 19 proteins were members of the transcription machinery and 118 proteins were associated with messenger RNA biogenesis and the spliceosome (92).

Within our brain proteome, a number of proteins were associated with translation and protein stability, including 92 members of ribosomes, with 30 being related to ribosome biogenesis and 46 with translation factors. Seventy-one proteins were annotated as chaperones and folding catalysts, and 76 were members of the ubiquitin system while 44 were constituents or related to the proteasome.

Three hundred proteins were associated with membrane trafficking, 82 being transporters, 8 associated with the secretory system, 79 were cytoskeleton proteins and 218 were associated with the exosome (Table 2.1). In regard to other DNA processes, 128 proteins were annotated as chromosome associated proteins and 26 with DNA repair and recombination processes. In addition, 109 proteins were associated with mitochondrial biogenesis, 47 to GTP binding proteins and 21 to Ion channels. The complete list of KEGG modules enriched by BlastKoala and represented in figure 2.5 is available in Table 2.1 and in Appendix 2 (Table 2.7.1).

Table 2.1 Brite KEGG Mapper Reconstruction result of the standard bumblebee brain worker proteome.

KEGG Brite	Pathway description	Genes and Proteins
Protein families: metabolism		
ko01000	Enzymes	652
ko01001	Protein kinases	53
ko01009	Protein phosphatases and associated proteins	65
ko01002	Peptidases and inhibitors	80
ko01003	Glycosyltransferases	11
ko01004	Lipid biosynthesis proteins	18
ko01006	Prenyltransferases	8
ko01007	Amino acid related enzymes	29
ko00199	Cytochrome P450	2
Protein families: genetic information processing		
ko03000	Transcription factors	6
ko03021	Transcription machinery	19
ko03019	Messenger RNA biogenesis	118
ko03041	Spliceosome	92
ko03011	Ribosome	92
ko03009	Ribosome biogenesis	30
ko03016	Transfer RNA biogenesis	41
ko03012	Translation factors	46
ko03110	Chaperones and folding catalysts	71
ko04131	Membrane trafficking	300
ko04121	Ubiquitin system	76
ko03051	Proteasome	44
ko03032	DNA replication proteins	14
ko03036	Chromosome and associated proteins	128
ko03400	DNA repair and recombination proteins	26
ko03029	Mitochondrial biogenesis	111
Protein families: signalling and cellular processes		
ko02000	Transporters	82
ko02044	Secretion system	8
ko04812	Cytoskeleton proteins	80
ko04147	Exosome	215
ko02048	Prokaryotic defense system	2
ko04030	G protein-coupled receptors	5
ko04040	Ion channels	21
ko04031	GTP-binding proteins	47
ko04052	Cytokines and growth factors	1
ko04515	Cell adhesion molecules	4
ko04090	CD molecules	18
ko00535	Proteoglycans	4
ko00536	Glycosaminoglycan binding proteins	9
ko00537	Glycosylphosphatidylinositol	10
ko04091	Lectins	6

Characterisation of the bumblebee brain proteome using BlastKoala also enabled the visualisation of important cellular processes in the context of neuronal functioning, such as the synaptic vesicle cycle (Figure 2.6 A) and other housekeeping processes such as glycolysis (Figure 2.7 B), oxidative phosphorylation (Figure 2.7 A) and endocytosis (Figure 2.6 B), that were almost completely represented in the identified proteome and several signal transduction pathways, associated with environmental information

processing, that are also heavily represented in the brain proteome (Table 2.7.1 Signal transduction, Appendix 2).

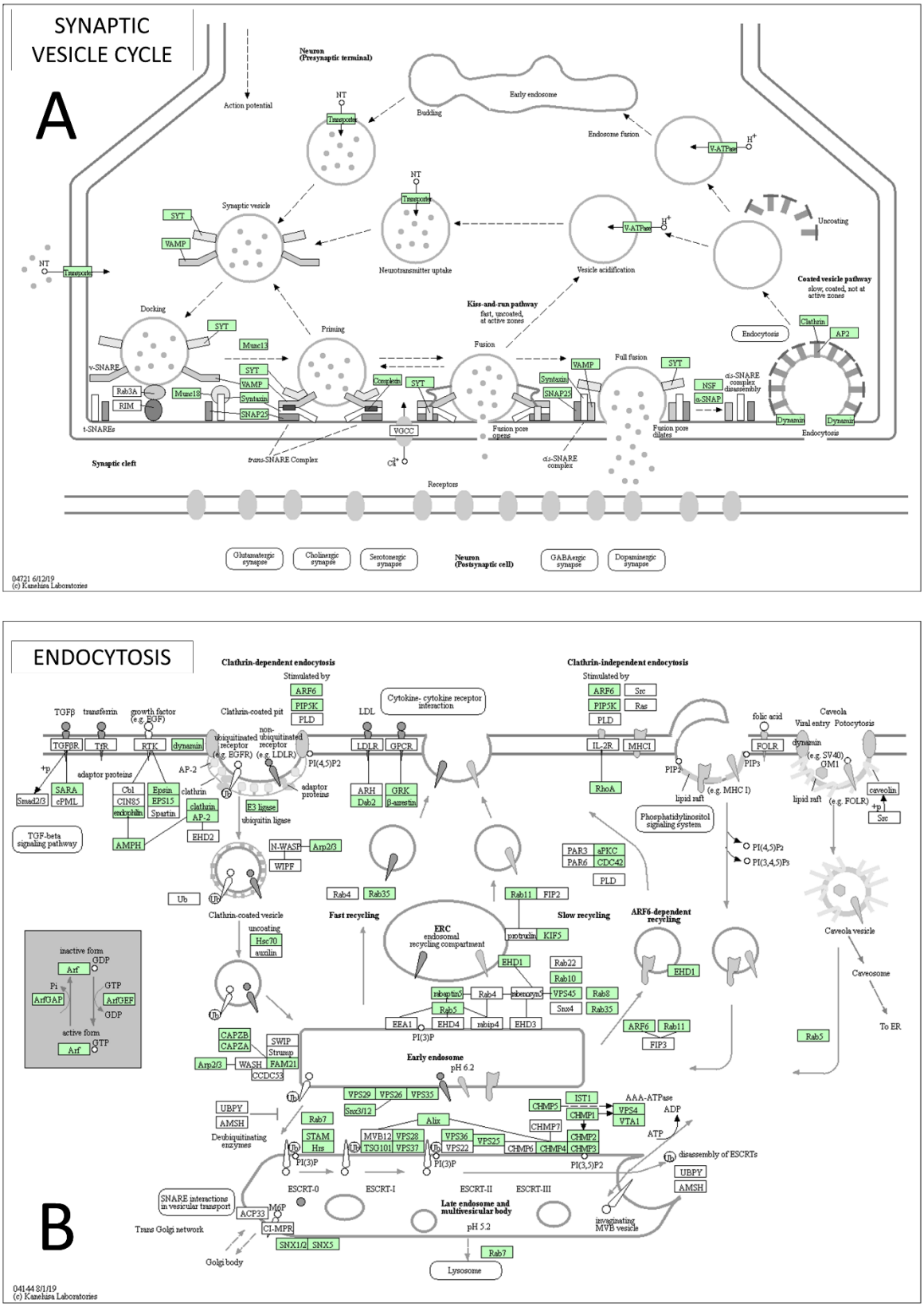
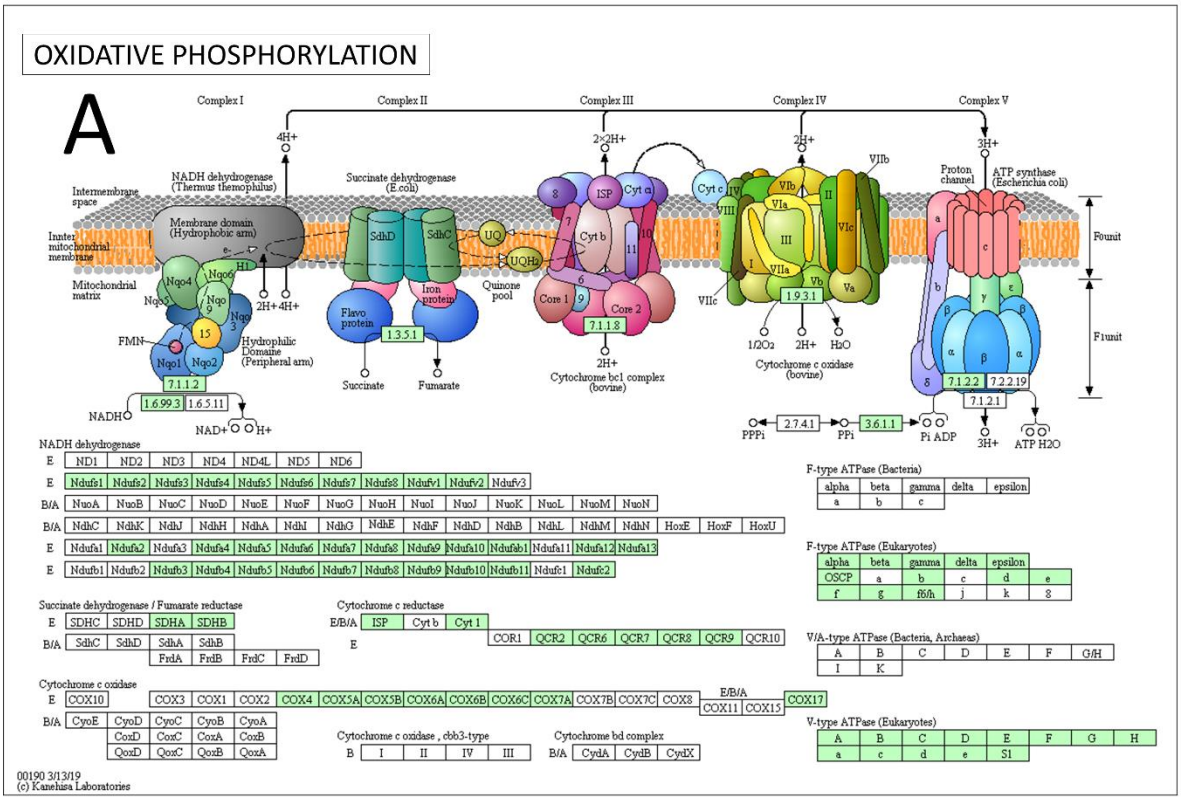


Figure 2.6 BlastKoala analysis overview of the pathways of interest enriched within the brain proteome. Within each pathway diagram, green elements indicate members of the pathway identified in our dataset. (A) Synaptic vesicle cycle, (B) Endocytosis.



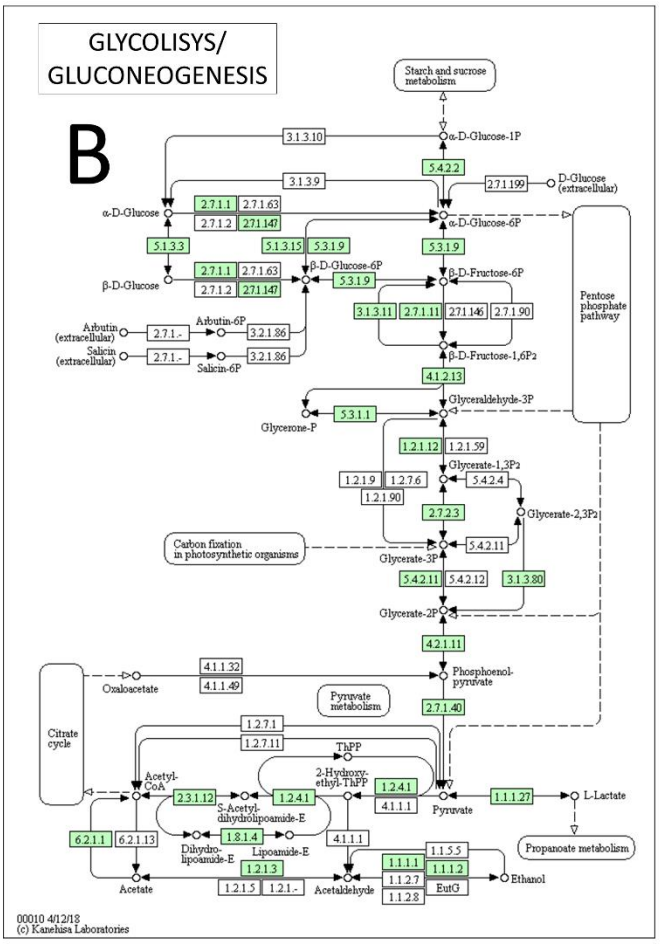


Figure 2.7 BlastKoala analysis overview of primary metabolic pathways enriched within the brain proteome. Within each pathway diagram, green elements indicate members of the pathway identified in our dataset. (A) Oxidative phosphorylation, (B) Glycolysis/Gluconeogenesis.

In order to obtain further information about the brain proteome, we carried out gene ontology enrichment analysis using the GOrilla platform. The molecular function enrichment results show two main branches of annotations, one for binding and one for catalytic activity, with the latter, having a smaller and more significant p-value, as demonstrated by colour intensity of each enriched term (Figure 2.8). Other significant components include oxidoreductase activity, specifically related to the electron transfer process and NADH and NADPH activity (Figure 2.8). To a lesser degree, nucleotide binding and actin, are enriched molecular function terms in the brain proteome.

The examination of biological processes resulted in a much more populated diagram, with well-defined branches involving processes particularly associated with metabolism and transport (Figure 2.9). The most enriched terms were organic substance transport, vesicle mediated transport, and other forms of intracellular transport and protein localization. In addition, the generation of precursor metabolites and energy, through oxidation reduction processes, electron transport chain and mitochondrial electron transport NADH to ubiquinone, were enriched. Other enriched metabolism terms were the nucleobase containing small molecule metabolic process, the carboxylic acid metabolic process and organic acid metabolic process (Figure 2.9).

Cellular component enriched terms were defined by a threshold p value of 10^{-5} (Figure 2.10) and among the main terms enriched, were the mitochondrial respiratory chain complex, and components of vesicles, which were constantly highlighted in the clear branching of 6 parental terms that include; cell part, organelle part, synapse, membrane- enclosed lumen, organelle and synapse part (Figure 2.10).

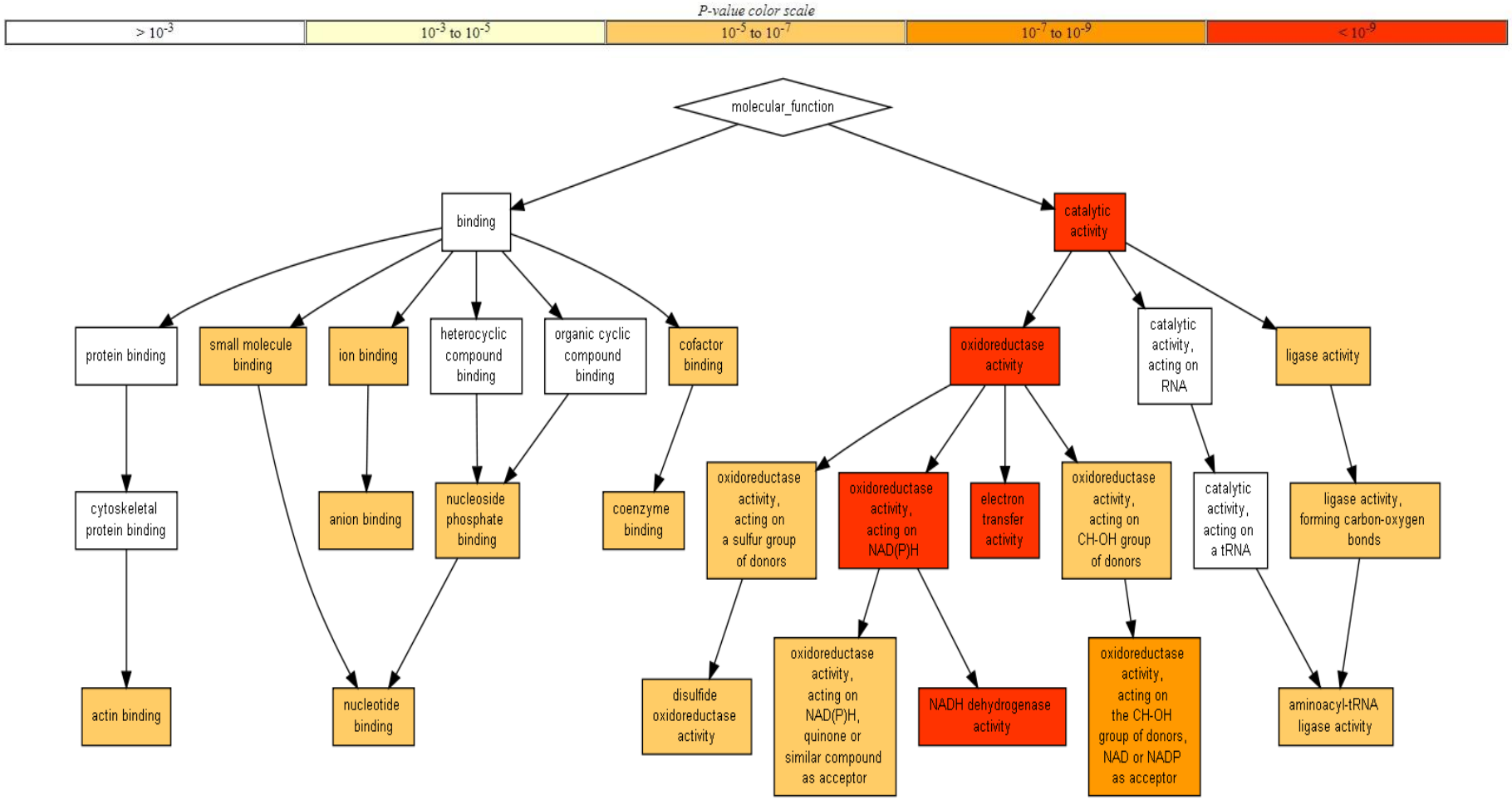


Figure 2.8 Hierarchy of enriched gene ontology molecular function terms within *B. terrestris* brain proteome compared to a background whole *Bombus* proteome. Flow diagram of the overall organisation of terms in the brain proteome. The p- value scale corresponds to significance with white corresponding to lower significance ($p > 10^{-3}$) and red representing high significant ($p<10^{-9}$). P value threshold 10^{-5}.

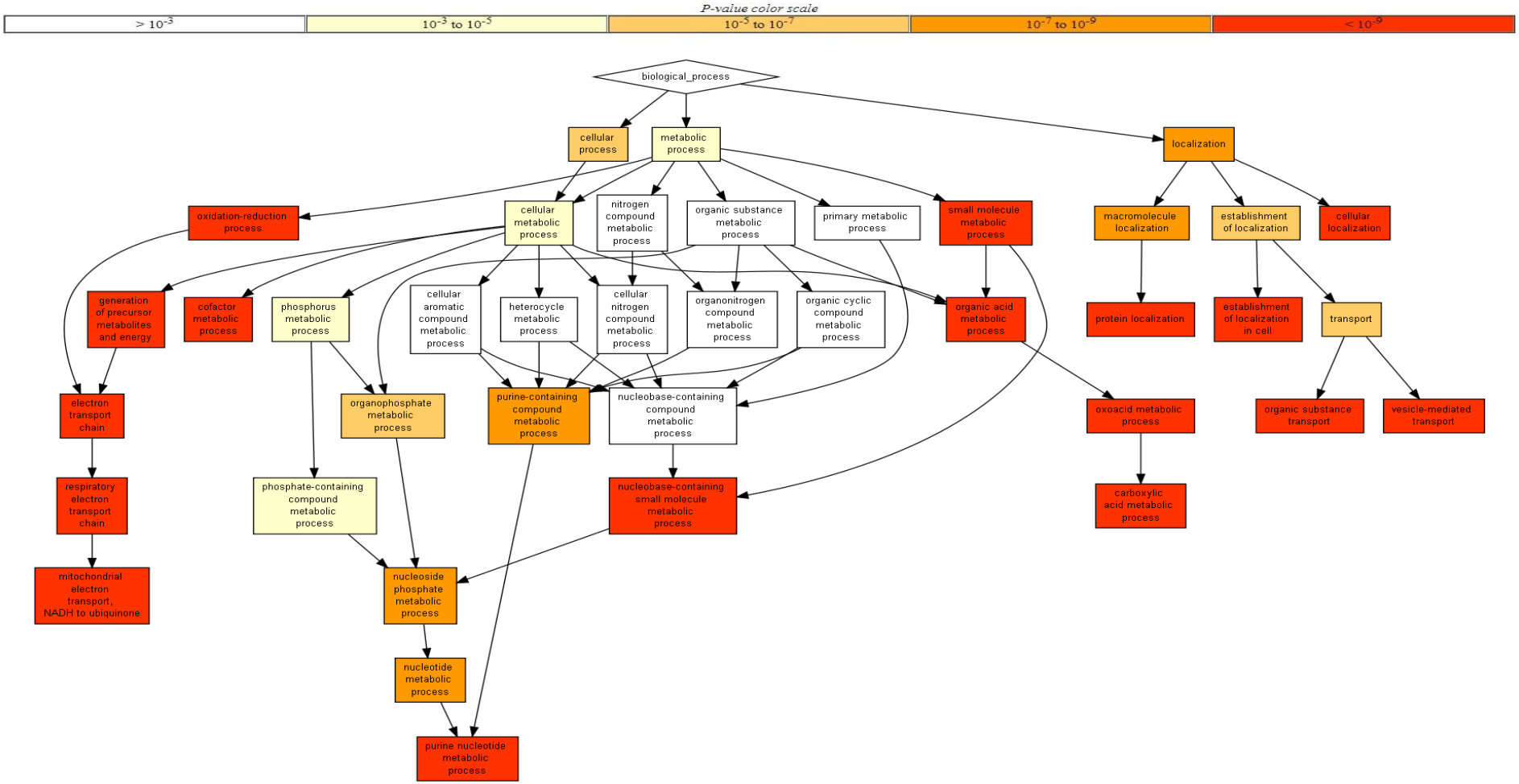


Figure 2.9 Hierarchy of enriched gene ontology biological process terms within the *B. terrestris* brain proteome compared to a background whole *Bombus* proteome. Flow diagram of the overall organisation of terms in the. The p- value scale corresponds to significance with white corresponding to lower significance ($p > 10^{-3}$) and red representing high significant $p<10^{-9}$). P value threshold 10^{-9}.

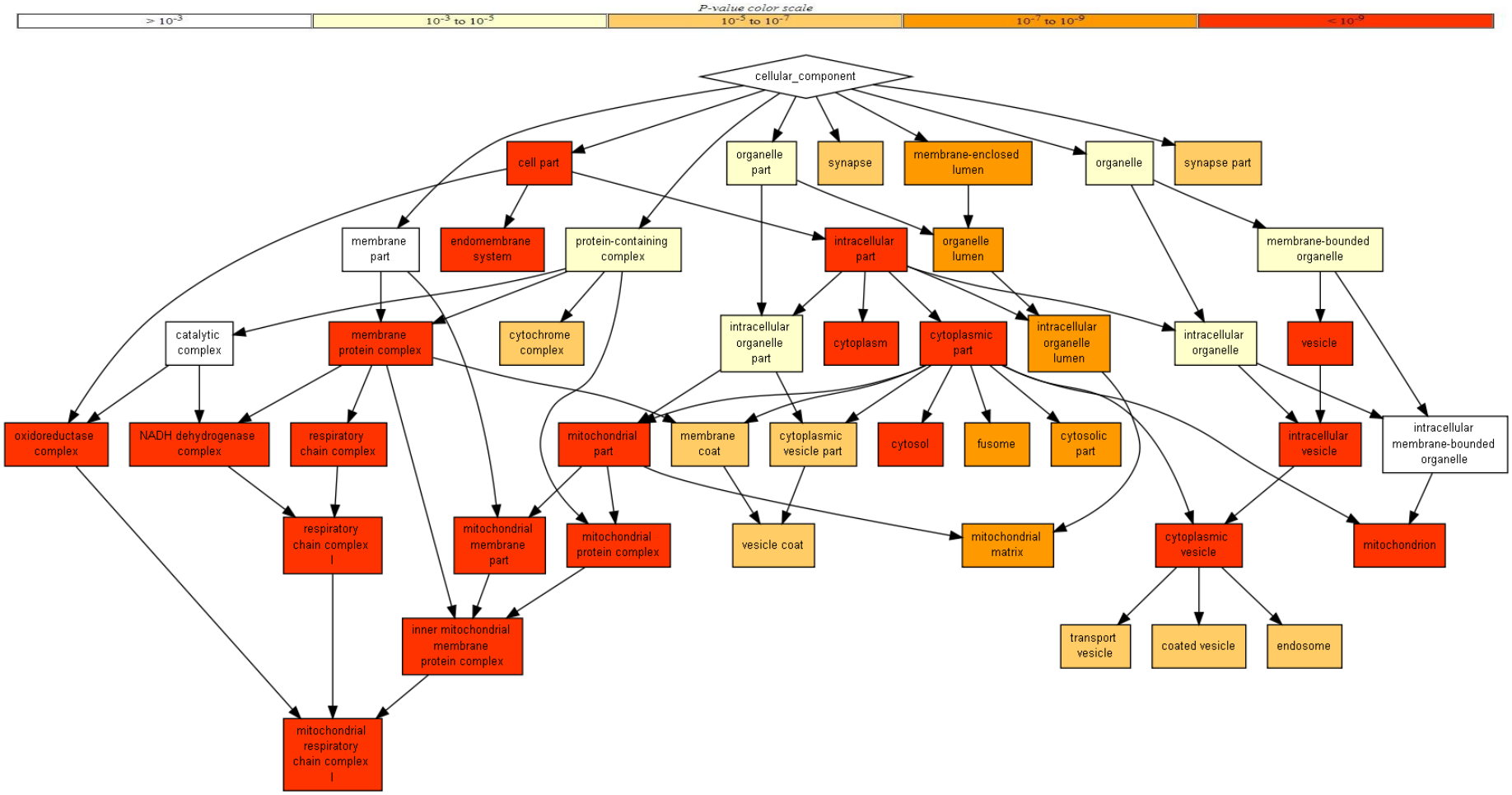


Figure 2.10 Hierarchy of enriched gene ontology cellular process terms within the *B. terrestris* brain proteome compared to a background whole *Bombus* proteome. Flow diagram of the overall organisation of terms in the. The p- value scale corresponds to significance with white corresponding to lower significance ($p > 10^{-3}$) and red representing high significant ($p<10^{-9}$). P value threshold 10^{-5}

2.4.2. Effects of age and brain maturation on the brain proteome of *Bombus terrestris*

To observe and characterise the brain proteome during development and aging, brains of worker bees were dissected on emergence day, 5 days post emergence and twenty days post emergence. These proteomes were compared using a principal component analysis, that clearly differentiated each replicate within its own group, suggesting major changes in the proteome during adult brain maturation and development. Component 1 and 2 accounted for 57.1% and 10.6% of total variability in the dataset, respectively. The biggest differences were seen when the group of newly emerged bees (green) was compared with the other two groups of mature bees (blue D20 and orange D5) (Figure 2.11).

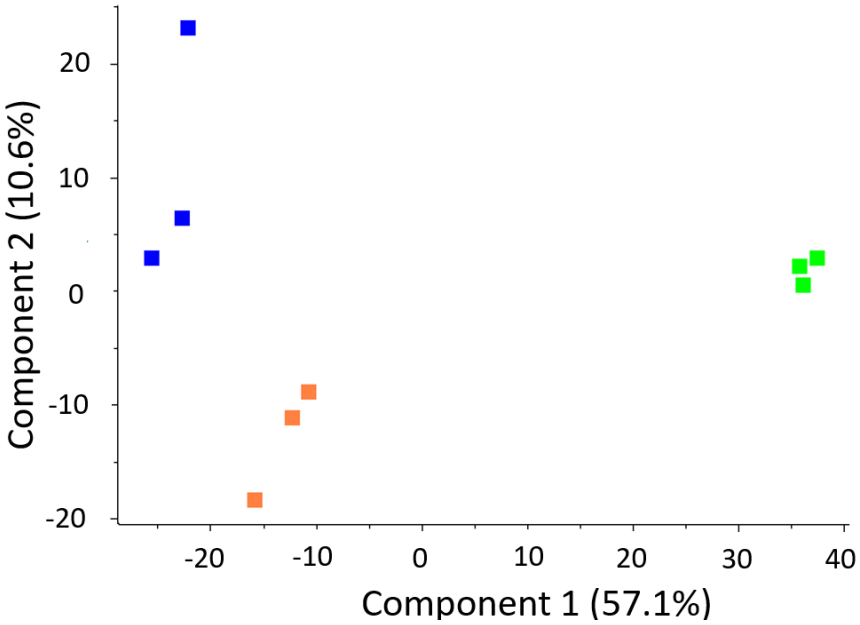


Figure 2.11 Principal component analysis utilizing the Benjamin-Hochberg FDR cut off 0.05 in all identified proteins per age group. Green = Recently emerged bees (D1), Orange = 5 days old bees (D5), Blue = 20 days old bees (D20). Clear separation between age groups were obtained with biggest differences seen in the brains of recently emerged workers of *B. terrestris*.

Two sample Student's t-tests comparing the proteome of workers at day 1 against the proteome at day 5 and day 20, resulted in the identification of 509 and 743 statistically significant differently abundant proteins (SSDA) at day 5 and day 20 respectively ($p \leq 0.01$) and fold change differences greater than 1.5. Out of the comparison between day 5 and day 1, 148 proteins had their abundances increased, while 361 proteins had

abundances decreased in response to aging and in-colony experience (Figure 2.12). The main biochemical pathways changing during maturation were carbohydrate, energy and lipid metabolism and environmental information processing, which increased in abundance, while pathways related to genetic information processing, environmental information processing, cellular process and glycan biosynthesis and metabolism, decreased in abundance (Figure 2.13).

The majority of these processes had similar expression profiles in the proteome of 20-day old workers when compared to recently emerged bees (D1). However, at this time-point a greater number of SSDA proteins were identified with 259 having increased abundance in response to aging and in-colony experience, while 484 had decreased abundance (Figure 2.12). The top five KEGG enriched terms, again, included carbohydrate, lipid and energy metabolism and protein families of genetic information and environmental information processing. Unlike the previous comparison, carbohydrate metabolism is represented to a smaller degree, while energy metabolism was increased. The top 5 downregulated processes were genetic information processing, protein families: genetic information process, environmental processing, human diseases and protein families: metabolism (Figure 2.13).

Of the ten top proteins with increased and decreased abundance identified by pairwise Student's t-tests comparison of the proteomes of D5 and D20 against D1 (Table 2.2), 7 out of the top 10 proteins with increased abundance were increased both at day 5 and day 20 in comparison to D1. These were vitellogenin, the uncharacterized proteins LOC100649693, LOC100645788 and LOC100646603, the protein inositol monophasphatase 2, the Glycine-rich cell wall structural protein and the cytochrome P450 6a2, whereas within the top 10 proteins with decreased abundance, only 3 proteins were shared (the probable chitinase 10 protein, the calsyntenin-1 and the thyroid receptor-interacting protein 6).

Another similarity found by these analyses was that among the SSDA proteins in both comparisons, a greater number of elements was found to be of decreased abundance in comparison with the D1 brain proteome. The complete list of SSDA proteins can be visualised in Appendix 2, Tables 2.7.2 and 2.7.3.

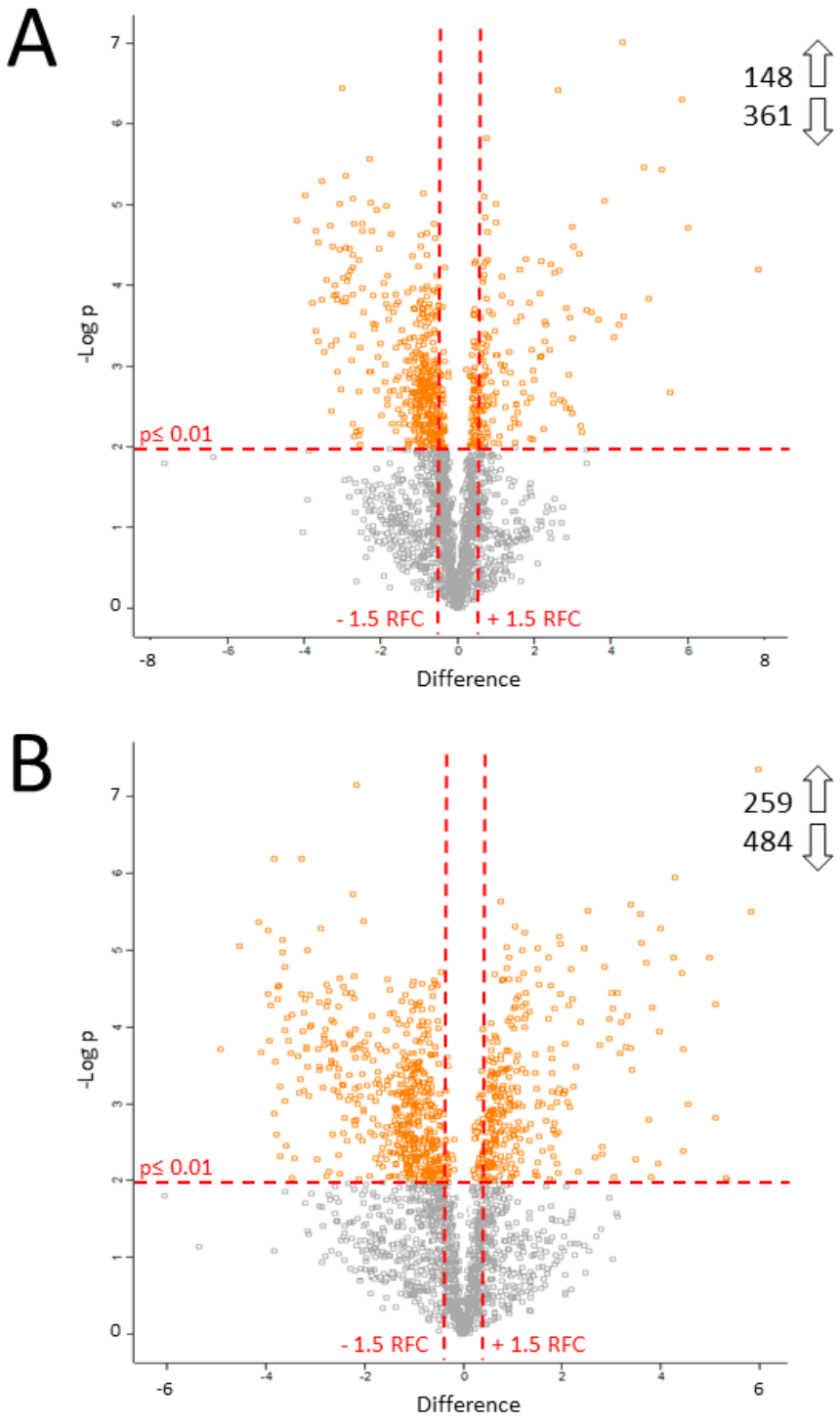


Figure 2.12 Volcano plots derived from pairwise Student's t-tests showing patterns of expression of individual proteins in the total brain proteome and SSDA proteins expression ($p \leq 0.01$, red horizontal dotted lines and relative fold changes (RFC) greater than 1.5, vertical dotted lines). (A) D1 vs D5 proteomes, 509 identified SSDA proteins (B) D1 vs D20 proteomes, 743 identified SSDA proteins. Arrows show number of increased and decreased abundant SSDA proteins. -log p: -log 10 of the p-value, Difference: $\log_2$ fold difference.

Table 2.2 The top 10 proteins with increased and decreased abundance identified from a pairwise Student's t-test comparing LFQ intensities of all proteins identified in each age group proteome. Asterisk marks proteins found in both comparisons.

	Top 10 Increased abundance proteins			Top 10 Decreased abundance proteins		
	Fold change	**Protein name**	**Protein Id**	**Fold change**	**Protein name**	**Protein Id**
D5	230.9	Vitellogenin *	XP_012163499.1	18.0	Probable chitinase 10 *	XP_020721052.1
	64.0	Uncharacterized protein LOC100643149	XP_003393266.1	15.8	Calsyntenin-1 *	XP_003400538.1
	58.3	Uncharacterized protein LOC100649693 *	XP_012165470.1	13.6	Beta-ureidopropionase	XP_003403150.1
	46.7	Inositol monophosphatase 2 *	XP_003403245.2	13.0	Uncharacterized protein LOC100645018	XP_003398832.1
	39.8	Uncharacterized protein LOC100645788*	XP_003393207.1	12.8	Uncharacterized protein LOC100647299	XP_003397075.2
	31.6	Glycine-rich cell wall structural protein *	XP_020721219.1	12.4	Laminin subunit gamma-1 isoform X1	XP_003396946.1
	28.9	Uncharacterized protein LOC100646603 *	XP_012172148.1	12.2	Septin-1	XP_012170301.1
	20.2	Cytochrome P450 6a2 *	XP_003400131.1	11.7	Thyroid receptor-interacting protein 6 *	XP_003400696.1
	19.7	Endothelin-converting enzyme 1	XP_012170197.2	11.5	Down syndrome cell adhesion molecule-like protein Dscam2	XP_020718321.1
	18.5	Uncharacterized protein LOC100645163	XP_003394861.1	11.2	Alanine--tRNA ligase, cytoplasmic isoform X1	XP_012167104.1
D20	63.2	Uncharacterized protein LOC100649693	XP_012165470.1	30.2	L-selectin	XP_003403165.1
	56.7	Uncharacterized protein LOC100645788	XP_003393207.1	23.2	Arylsulfatase J-like	XP_012174370.1
	40.3	Vitellogenin	XP_012163499.1	17.8	Iron/zinc purple acid phosphatase-like protein	XP_003399144.1
	34.6	1,5-anhydro-D-fructose reductase	XP_012172366.1	17.2	Beta-hexosaminidase subunit beta-like	XP_003397815.1
	34.6	Glycine-rich cell wall structural protein	XP_020721219.1	15.5	Zinc finger CCCH domain-containing protein 13	XP_020719123.1
	31.7	Uncharacterized protein LOC100646603	XP_012172148.1	15.4	Calsyntenin-1	XP_003400538.1
	23.6	Protein lethal (2) essential for life	XP_003393098.1	15.4	Putative serine protease K12H4.7	XP_012165494.1
	22.1	Cytochrome P450 6a2	XP_003400131.1	14.9	Probable chitinase 10	XP_020721052.1
	21.8	Inositol monophosphatase 2	XP_003403245.2	14.3	Thyroid receptor-interacting protein 6	XP_003400696.1
	21.8	Uncharacterized protein LOC100649369	XP_003393941.1	14.2	Alpha-N-acetylglucosaminidase	XP_003397173.1

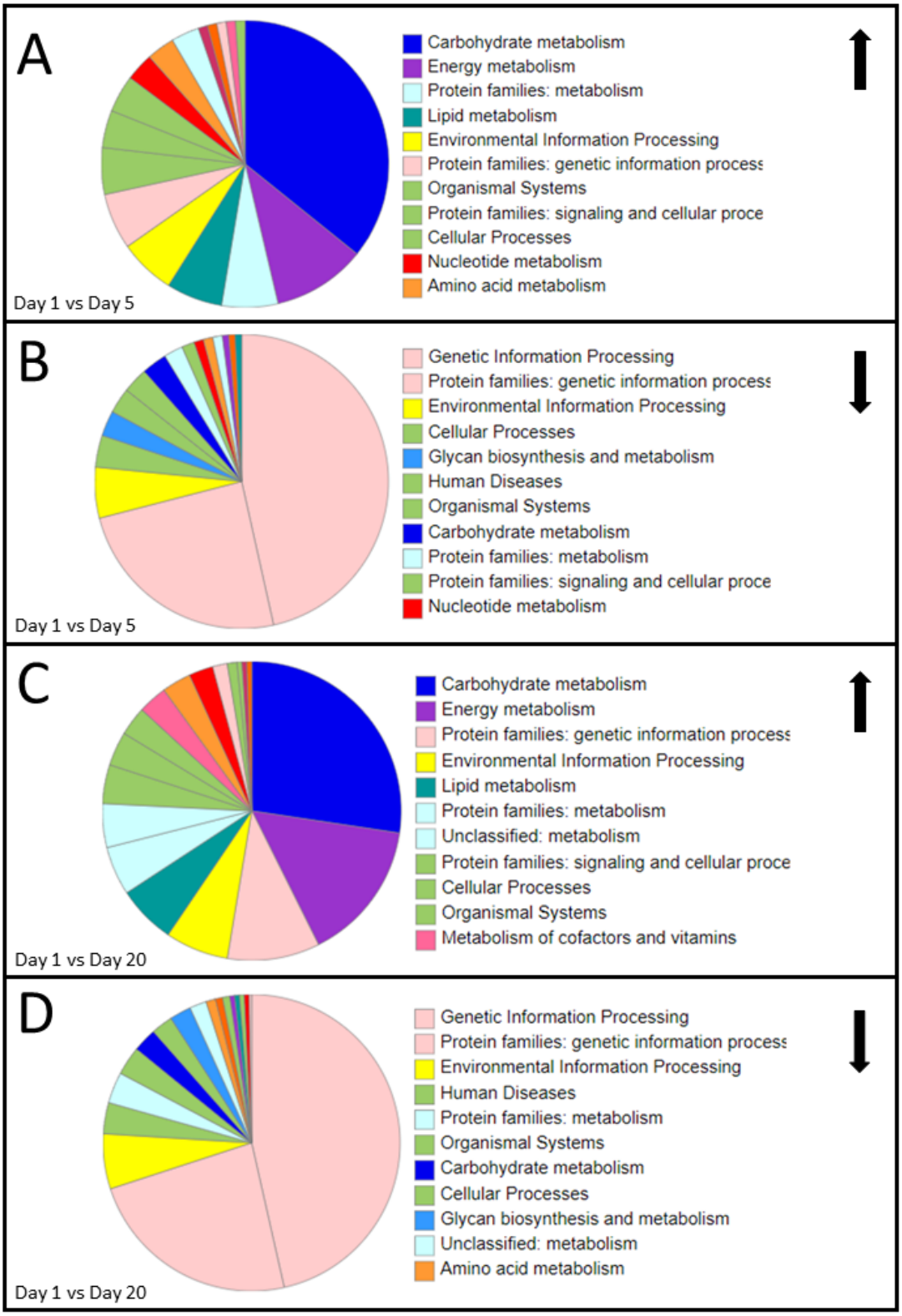


Figure 2.13 BlastKOALA pathway analysis of SSDA proteins demonstrating the diversity of biological processes affected by aging and in colony experience. (A = Increased pathways in D5 in comparison with D1, B = decreased pathways in D5 in comparison with D1, C= Increased pathways in D20 in comparison with D1, D = decreased pathways in D20 in comparison with D1. The main increased processes in aging are carbohydrate metabolism and energy metabolism, while the main decreased processes are genetic information processing and protein family's genetic information processing.

A different approach was then taken to elucidate more specific differences and similarities between the three-age brain proteome development groups. A multiple sample ANOVA test with a permutation-based FDR (0.05) on all identified proteins in at least three out of three replicates in each group (2,100), identified 1,551 proteins with statistically significant changes in abundance among all age groups (SSDA2). These proteins LFQ expression levels were Z-scored and visualized through hierarchical clustering (Figure 2.14).

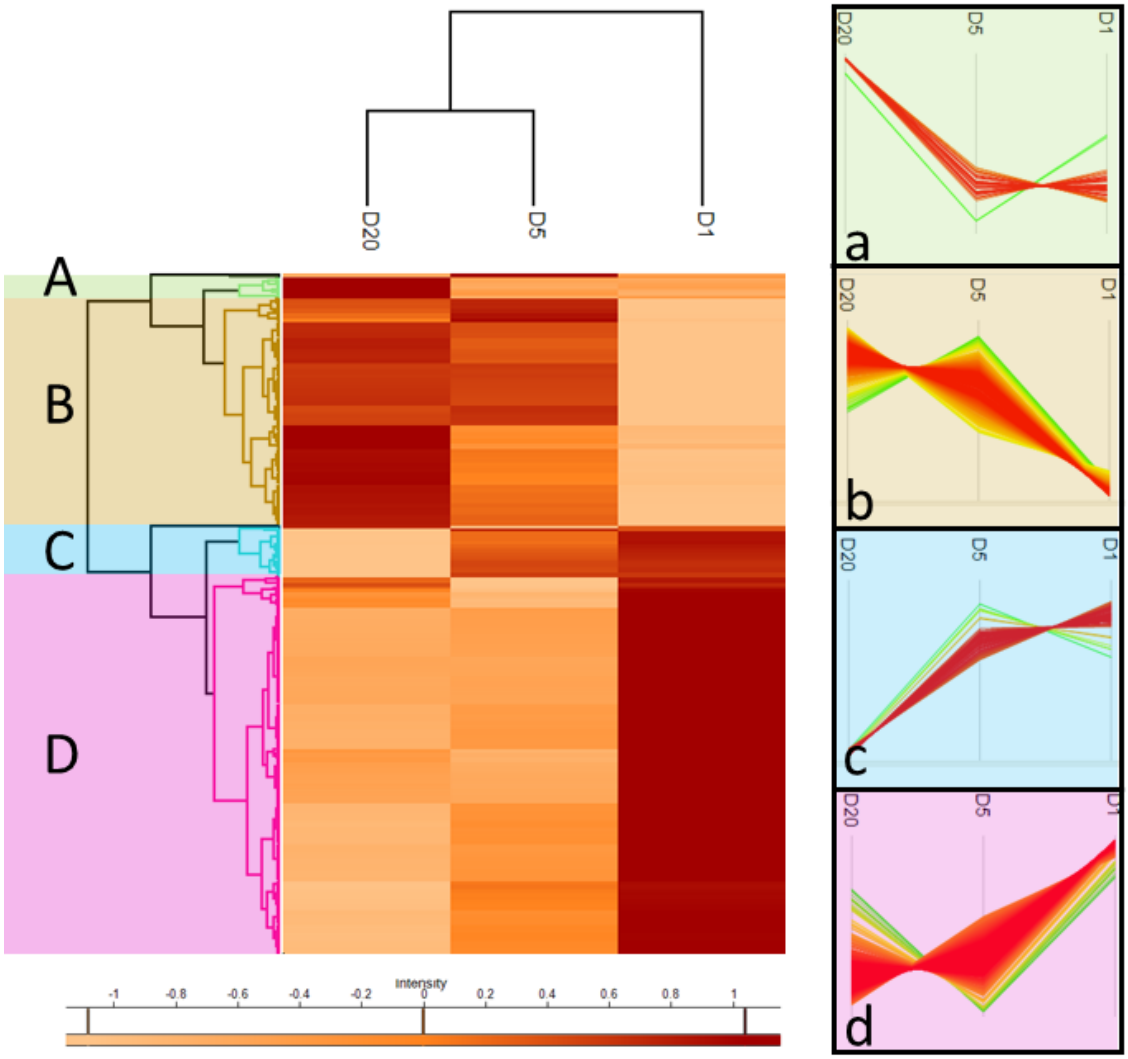


Figure 2.14 Hierarchical clustering of the mean intensity values for 1,551 SSDA proteins among the different samples comparing the brain proteome over aging and in colony experience. Heat map colours represent the relative expression of proteins in samples (Left). Cluster expression trends of identified proteins are represented by the coloured lines (Right). These proteins have been divided into groups based on age specific expression profiles (Clusters A, containing 37 proteins with increased abundance at D20, cluster B with 396 proteins with decreased abundance at D1, cluster C with 85 proteins with decreased abundance at D20 and cluster D with 658 proteins with increased abundance at D1). Relative abundances (z-scored normalised) of individual proteins can be read from the intensity spectrum and visualized in a,b,c and d respective to each cluster.

Hierarchical clustering identified 4 main clusters of protein expression between the replicates of each age group. Cluster A contained 37 proteins, representing the group of proteins with increased abundance at day 20 in comparison to days 5 and 1. Cluster B contained 396 proteins with increased abundance in matured brains (Day 5 and Day 20), in comparison to day 1. Cluster C with 85 proteins, was representative of a young brain containing proteins highly expressed at day 5 and on the day of emergence, and cluster D was characteristic of a newly emerged brain, containing 658 proteins highly expressed at day 1 (Figure 2.12).

Furthermore, 65 proteins were found to be exclusively expressed in the brains of newly emerged workers and 5 proteins were exclusively expressed in the brains of 20-day old workers (Table 2.3). No single protein was found to be exclusively expressed in 5-day old bumblebee brains. These results corroborate the PCA results where the brains of D5 and D20 bees are more similar to each other, with the D1 brain being more distinct.

The list of D20 exclusively expressed proteins was added to the proteins present in cluster A prior to biological functional analysis using the platform STRING. This step was not repeated for D1 exclusive proteins, as these represented a bigger group, so they were analysed on their own.

Table 2.3 Exclusive proteins identified in all 3 replicates of 20-day old worker bee brains.

Fasta headers	Protein IDs
XP_003393267.1	HIG1 domain family member 1C
XP_003398495.1	Deoxyribose-phosphate aldolase
XP_012168855.1	72 kDa inositol polyphosphate 5-phosphatase
XP_012170000.1	Protein lethal (2) essential for life
XP_020722624.1	Aldose reductase

2.4.2.1. Describing the newly emerged brain

The brain proteome of a newly emerged bee is populated mainly by genetic information processing pathways, as this is the main downregulated enrichment obtained from KEGG analysis in D5 and D20 (Figure 2.11, downregulated pathways). In addition, evidence that the brain is not yet completely formed post emergence is obtained from the STRING enrichment terms associated with the exclusively expressed proteins at D1 (Table 2.4 exclusives and cluster D). These comprise a series of processes involved in the assembling of the brain structures, including terms such as the central nervous system morphogenesis, optic development, maintenance of epithelial integrity, axon point

recognition and substrate adhesion dependant cell spreading, as well as the presence of proteins belonging to the Wnt signalling pathway (Table 2.4). Therefore, this is a stage in which the brain is still undergoing severe restructuring and development, and is highly dependent on transcription, translation and the synthesis of organelles such as the endoplasmic reticulum, Golgi complex and the proteasome, distributing and regulating the components necessary to ensure axon extension, and the building of neurological connections and processes.

2.4.2.2. Describing the young mature brain

A swift increase in metabolism characterises the young mature brain of worker bumblebees (Figure 2.11). These include carbohydrate, lipid and energy metabolism (Figure 2.11 A and C), whereas genetic information processing is among the main biochemical pathways being downregulated as the brain ages (Figure 2.11 B and D). An increase in neurotransmitter metabolism, glycolytic energy production as well as oxidation-reduction processes are characteristic of a maturing brain, which then also shows increased mitochondrial translation (Clusters B and C figure 2.12, table 2.4).

Proteins associated with the endoplasmic reticulum are also present to a lesser degree in relation to the newly emerged brain (Cluster D, figure 2.12 and table 2.4). The brain at five days post emergence is thus metabolic active and responsive to a series of different neurotransmitters and has increased energy production.

2.4.2.3. Changes triggered by age and in colony experience: the mature brain proteome

The maturation development process results in a highly synaptically active brain, one with increased energy production mechanisms being used to feed into neurotransmitter metabolism, oxidation reduction processes, lyase activity and other signalling pathways as evidenced by clusters A and B, figure 2.12 and table 2.4.

The mature brain also shows a significant increase in the levels of vitellogenin, several uncharacterized proteins LOC100649693, LOC100645788 and LOC100646603, and of the protein inositol monophasphatase 2, the Glycine-rich cell wall structural protein and the cytochrome P450 6a2, and a considerable downregulation of terms associated with neuronal morphogenesis. The complete list of GO enrichment terms obtained from the list of proteins from cluster 2 shows a variety of biological terms related with the metabolism of several insect acting neurotransmitters like acetylcholine, GABA,

glutamate, dopamine and several terms associated with energy metabolism (Appendix 2, table 2.7.5). These results suggest that the mature brain is fully responsive to the environment and its main activities are related to the maintenance of cell functioning, to the detriment of axon extension processes and nervous system morphogenesis.

Table 2.4 Top 10 Functional enrichment results obtained using the platform STRING based on the list of cluster constituent proteins identified from the hierarchical clustering.

	Column A			Column B		
	GO	Definition	% coverage	GO	Definition	% coverage
Cluster A	GO:0030016	Myofibril	5.6			
	GO:0016829	Lyase activity	3.1			
Cluster B	GO:0009448	Gamma-aminobutyric acid metabolic process	100.0	GO:0004449	Isocitrate dehydrogenase (NAD+) activity	100.0
	GO:0045213	Neurotransmitter receptor metabolic process	100.0	GO:0002046	Opsin binding	100.0
	GO:0002032	Desensitization of G protein-coupled receptor signaling pathway by arrestin	100.0	GO:0004069	L-aspartate:2-oxoglutarate aminotransferase activity	100.0
	GO:0006116	NADH oxidation	100.0	GO:0004427	Inorganic diphosphatase activity	100.0
	GO:0061615	Glycolytic process through fructose-6-phosphate	100.0	GO:0008603	cAMP-dependent protein kinase regulator activity	100.0
Cluster C	GO:0015929	Hexosaminidase activity	60.0	GO:0015030	Cajal body	15.4
	GO:0016231	Beta-N-acetylglucosaminidase activity	50.0	GO:0005761	Mitochondrial ribosome	14.8
	GO:0030620	U2 snRNA binding	40.0	GO:0032543	Mitochondrial translation	13.8
	GO:0005762	Mitochondrial large ribosomal subunit	16.0	GO:0005686	U2 snRNP	10.5
	GO:0005763	Mitochondrial small ribosomal subunit	15.6	GO:0008536	Ran GTPase binding	9.5
Cluster D	GO:0048081	Positive regulation of cuticle pigmentation	100.0	GO:0019749	Cytoskeleton-dependent cytoplasmic transport, nurse cell to oocyte	100.0
	GO:0070861	Regulation of protein exit from endoplasmic reticulum	100.0	GO:0031036	Myosin II filament assembly	100.0
	GO:0000461	Endonucleolytic cleavage to generate mature 3'-end of SSU-rRNA from (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	100.0	GO:0051016	Barbed-end actin filament capping	100.0
	GO:0003331	Positive regulation of extracellular matrix constituent secretion	100.0	GO:0061031	Endodermal digestive tract morphogenesis	100.0
	GO:0006422	Aspartyl-tRNA aminoacylation	100.0	GO:0070863	Positive regulation of protein exit from endoplasmic reticulum	100.0
Exclusives D1	GO:0050072	m7G(5')pppN diphosphatase activity	66.7	GO:0021551	Central nervous system morphogenesis	20.0
	GO:0060071	Wnt signaling pathway, planar cell polarity pathway	66.7	GO:0032231	Regulation of actin filament bundle assembly	18.2
	GO:0030215	Semaphorin receptor binding	33.3	GO:0031290	Retinal ganglion cell axon guidance	16.7
	GO:0001748	Optic lobe placode development	33.3	GO:0035160	Maintenance of epithelial integrity, open tracheal system	16.7
	GO:0034446	Substrate adhesion-dependent cell spreading	25.0	GO:0016198	Axon choice point recognition	14.3

2.5. Discussion

2.5.1. The principal processes associated with the brain proteome

The characterisation of a proteome is of great importance as it reveals the characteristic traits of the target organ, or cell, in a non-biased way, allowing us to understand the functioning of a target sample from a biochemical perspective, and identify novel biological roles, previously unexplored. It is also important to understand the bumblebee brain proteome, as it offers insight into the pathways that are necessary for efficient nervous system function, and energy provision, thus highlighting vulnerabilities of the organ, and the limitations of which can impose constraints on information processing capability (Harris *et al.*, 2012).

Our results demonstrate that the bumblebee brain is an organ primarily defined by energy production and catalytic activity processes, destined to feed into transport and localisation of molecules, and into the function and structural reorganisation of synapses. We have seen that about 25% of the total annotated terms account for enzymatic activity and 6.52% account for translation or ribosomal biogenesis. Other terms of interest such as DNA replication and repair (6.44%), mitochondrial biogenesis (4.23%) and membrane trafficking, transport and the exosome (22.7%) are highly abundant in the brain proteome. These values are similar to those of Hernández *et al.* (2012) for the brain of worker honeybees, where energy production and conversion processes account for 6.5% of the brain proteome, signal transduction mechanisms (8.8%), posttranslational modification, protein turnover, chaperones (10.5%) and translation, ribosomal structure and biogenesis to 6.7%. The total number of proteins used in the analysis is also similar with 2,742 proteins identified in their study and 2,574 non redundant proteins in ours, representing an organ highly dependent on energy generation processes, protein synthesis and maintenance.

It is not surprising to find that terms associated with mitochondrial energy processes and oxidative phosphorylation are represented by the greatest number of enrichments in the brain proteome, along with transport and binding of nucleotides, actin and ions. The brain is a very dynamic organ, that is under high energy demand to sustain the continuous synthesis of molecules used in the restructuration of connections, necessary to respond to stimuli (Magistretti and Allaman, 2015). Hence, it is dependent on mitochondrial metabolism as its main energy source (Martinez-vicente, 2017), but also obtains energy from other metabolic processes, including the metabolism of purine and pyrimidine rings, mainly in the form of nucleosides (Fumagalli *et al.*, 2017).

The brain has limited anabolic and catabolic capacity to synthesise these RNA and DNA precursors, so it relies greatly on transport of these molecules through the blood brain barrier (Ipata, 2011). Furthermore, nucleosides are critical for the production of the main constituents of synaptic membranes, or to synthesize nucleic acids, lipids and sugar conjugates, having several functions in addition to helping satisfy the energy demands of the organ, including acting as an extracellular signalling molecule (Ipata, 2011).

The transport dependence of the brain is not only observed in regard to nucleotides. The unique morphology of neurons, with their axons and dendrites, means that molecules and organelles, including mitochondria have to be recruited to distant parts, via axonal transport, to satisfy cellular demands at those sites (Martinez-vicente, 2017). These represent complex molecular events associated with maintaining the neuronal processes of the brain, along with cell signalling, cell division, proliferation and migration (Olivera-Pasilio *et al.*, 2017). Such processes require the participation of several structural proteins such as actins and myosins, associated with transport, but also involved with synaptic organisation and assembly, cell adhesion and cytoskeleton reorganisation, justifying their extensive presence in the brain proteome (Hirokawa *et al.*, 2010; Calábria *et al.*, 2011; Lian and Sheen, 2015) (Figure 2.9, 2.10). In addition to transport, the establishment and restructuration of cell shape, necessary during synaptic plasticity, can also be regulated by the action of some of these structural members, including actin (Lian and Sheen, 2015).

These processes are common to each individual neuron as it receives and passes on information to neighbouring cells through synapses and are thus crucial to the functioning of the brain. Exosomes and any other extracellular or intracellular vesicles also play a very important role in cellular communication, acting in the physiology and development of neural systems, by carrying signalling proteins and both coding and regulatory RNAs, thus facilitating multilevel information transfer, necessary for nerve regeneration, synaptic function and behaviour (Rajendran *et al.*, 2014).

Our proteome data successfully demonstrate the high level presence of these components necessary for efficient nervous system functioning on the bumblebee brain and the high energy demands of this organ, as determined by the enriched energy specific terms (Figure 2.9, 2.10 and table 2.1) observed in the brain proteome.

Enrichment of categories related to metabolism, more specifically lipid metabolism and mitochondria as well as cellular signalling, synaptic organisation, assembly, actin cytoskeleton, translation, exocytosis and ion binding were also described to be

extensively present in the brain proteome of mice (Noya *et al.*, 2019), potentially demonstrating functional convergence in brain cellular composition between mammals and insects.

2.5.2. Age associated changes in the bumblebee brain proteome

Cellular changes in the brain, including: an altered abundance of synaptic and other signalling proteins (synapsin, protein kinase C) and of proteins involved in cellular maintenance and lipid transport (heat shock proteins, peroxiredoxin, and fatty acid binding protein), and the accumulation of residuals from incomplete lysosomal degradation, can be a result of the aging processes and contribute to learning decline in bees (Baker *et al.*, 2012; Münch and Amdam, 2013; Münch *et al.*, 2013). Yet, it remains unclear what factors are characteristic of an aged brain, and how it influences behavioural plasticity.

When a worker bee emerges, it shows no signs of defensiveness or even awareness. A newly emerged bumblebee displays different colour patterns to the adults and even at this stage the bee can be held without protection, as it still does not defend itself or show any signs of complex behaviour. Often when observed in the colonies, these newly emerged bees or callows are observed only seeking places to hide.

The results of the brain age comparison allow us to make inferences that relate proteome changes with behavioural plasticity. These results demonstrate that post emergence, the brain of an adult bumblebee is represented by intensive morphogenic activity, including a high level of genetic information processing, such as DNA replication and repair, transcription, translation, folding, sorting and degradation of newly synthesized molecules, probably involved in the degradation of larval brain connections and formation of the adult brain processes. These enrichments are succeeded by enrichments in carbohydrate, lipid and energy metabolism, as well as environmental information processing as the brain ages.

The metabolic enrichment of KEGG terms highlight a series of processes involving glycolysis/gluconeogenesis, the citrate cycle, pyruvate metabolism, oxidative phosphorylation, fatty acid synthesis and degradation, phospholipid metabolism among others, while the term environmental information processing, represents a number of membrane transporters and several signal transduction mechanisms, including the Ras, the MAPK , the Wnt, cAMP and the mTOR signalling pathways, all of which are very common protein groups that characterise the mature brain (Appendix 2).

The brain maturation is seen structurally by an increase in neuropil areas which are a direct result of strengthening of neurological processes and synapses (Wolschin *et al.*, 2009). Such structural changes can be the drivers of the enrichments in Ras, MAPK, cAMP, mTOR and Wnt pathways found in our results, which are all involved in several cellular transduction processes, including gene expression, mitosis, cell differentiation, growth and survival, thus being crucial for tissue development and activity (Cooper and Hausman, 2013). In addition, boosting Wnt signalling is known to improve synaptic function necessary during aging (Palomer *et al.*, 2019).

Our data displays increased cellular activity and signalling in terms of differentiation and neuromorphogenesis processes, which are already evident in the 5 day old bee brain. This is evidence of rapid brain maturation in young bumblebees, which can perhaps corroborate with the plasticity they show at the behavioural level, in regard to labour specialisation, a response that appears to be driven by the requirement of the colonies (Jones *et al.*, 2013).

An increase in energy associated proteins was also reported in honeybees between foragers and nest bees, a result associated with aging (Wolschin and Amdam, 2007). Due to a high activity of energy producing mechanisms in foragers (older bees), an increase in reactive oxygen species (ROS) is assumed, with an increase in oxidative damage also being expected as a result of aging. Wolschin and Amdam (2007), found an increase in superoxide dismutase activity in foragers and similarly to their study, we also found signs of higher levels of proteins involved in ROS protection, such as the protein superoxide dismutase, in D5 and D20 brains of bumblebees, thus confirming the increase in protective mechanisms to oxidative damage as the bee ages.

Determining whether our results can be used to discriminate between nurse or forager bees and identify the molecular determinants of behaviour in bumblebees is difficult. However, our results corroborate those of previous studies in that an increase in energy associated proteins (Li *et al.* 2016) is observed as a distinction between workers with different colony roles, as a possible factor, driven by age (Wolschin and Amdam, 2007).

The response to a variety of neurotransmitters, neuropeptides and their interactions with their specific receptors, is what determines the expression of complex behaviours, as it drives neurological response. However, the complexity of behaviour output in regards to neurological signalling, is still poorly understood (Klowden, 2013a; Li *et al.*, 2016; Schoofs *et al.*, 2017; Fieber, 2019). Therefore, how the total proteome of D5 and D20 can reflect behavioural differences between bees will not be discussed further here,

and we limit our current interpretation to stating that both are metabolically active brain states, responding to a series of neurotransmitters.

Another known determinant of bee behaviour and sociality, the abundance levels of which are specifically involved in regulating the division of labour in honeybees is vitellogenin (Vg). We detected an increase in the levels of vitellogenin in mature brains of 5- and 20-day old bumblebees, with a higher concentration in the second, where Vg was the protein with the most increased abundance in comparison with D1. In honeybees, vitellogenin also influences social organization and aging rate (Münch and Amdam, 2013; Münch *et al.*, 2015a). Their levels decrease with aging which is accompanied by an increase in juvenile hormones, that are believed to act in accordance with the beginning of foraging in older honeybees (Fahrbach and Velarde, 2009; Münch and Amdam, 2013; Münch *et al.*, 2015). The juvenile hormone is a pro aging agent in *Drosophila melanogaster* and honeybees and Vg is believed to act as a suppressor of its expression. RNA interference-mediated gene knockdown of vitellogenin leads to increased JH levels, speeding up the transition to the short-lived forager stage in honeybees (Flatt *et al.*, 2013).

Additionally, Vg protects cells from oxidative damage, protecting workers and queens from oxidative stress, corroborating with the hypothesis that Vg is a central regulator of honeybee lifespan (Corona *et al.*, 2007; Seehuus *et al.*, 2007; Münch and Amdam, 2013).

Our results demonstrate that the mature bumblebee brain is capable of complex behaviour, as it responds to a diversity of neurotransmitters and avails of a great supply of energy generation processes. The brain at 5 days old shares several physiological markers with the 20 day old bumblebees brain, which suggests rapid maturing and could corroborate with the hypothesis that the rapid maturing of bumblebee brains, allows them to perform complex behaviours at young ages. However, the increased levels of vitelogenin detected in mature bumblebee brains, contradict their aging interaction relationship stablished for honeybees.

It is not yet clear whether the increasing levels of vitellogenin found in the aging bumblebee brain indicates that they are modulators of aging, and negatively correlated with age, as seen in honeybees, or if they have a completely different function. It may be the case that as our experiments did not consider foraging impact on the physiology of the bumblebee brain, the increased levels of vitellogenin, were a response to increased energy and ROS production of a more catalytic active brain, protecting them from oxidative damage and possibly prolonging their life, while keeping workers in tasks associated with colony life.

More research is necessary to determine if vitellogenin expression levels in the bumblebee brain is functionally similar to that in honeybees. Furthermore, it is necessary to better understand the interaction between neurotransmitters and neuropeptides and their receptors, prior to discovering the determinants of labour division, and also investigate if genetic expression and aging, are the factors triggering increase in JH expression and foraging, or if foraging initiation, is the determinant factor of vitellogenin decrease and consequent JH increase, and labour division in the brains of bees.

In this chapter we have provided an in depth analysis of the processes that are changing in the proteome of bumblebee brains, as a result of age associated development. We anticipate that these results can guide future studies that aim at establishing a relationship between the behaviour and the proteome, and that it serves as a foundation for future discussions in this thesis, regariding the impact of neonicotinoids on the brains of mature bumblebees.

2.6. Appendix 2

The following appendix tables are located on the disc provided at the back of thesis or at the link below:

https://drive.google.com/open?id=1ofFp_i77x_3QzmX3eTZDel5gfaGf9i7Z

Table 2.6.1. Complete KEGG mapper reconstruction of the standard bumblebee brain worker proteome.

Table 2.6.2. Complete list of SSDA proteins identified by pairwise comparison student t test between D1 vs D5 proteomes.

Table 2.6.3 Complete list of SSDA proteins identified by pairwise comparison student t test between D1 vs D20 proteomes.

Table 2.6.4. Complete list of proteins components of clusters identified by hierarchical clustering analysis plus proteins found exclusively at the D1 proteome.

Table 2.6.5. Complete list of functional enrichments obtained by STRING analysis on the list of proteins belonging to each cluster identified by hierarchical clustering analysis and the list of exclusive D1 proteins.

Chapter III

A behavioural, histological and molecular characterisation of the effects of acute clothianidin and imidacloprid exposure in *Bombus terrestris*.

3.1 Introduction

3.1.1 Neonicotinoids and their potential role in the decline of pollinating insects

The importance of pollination by insects to the maintenance of the ecosystem and agricultural production is undeniable. It is estimated that 75% of all crop species benefit from insect pollination in one way or another (Klein *et al.*, 2007; Aizen *et al.*, 2009), providing a global service worth $215 billion annually to the food market (Gallai *et al.*, 2009). Nevertheless, current human practices have increased the pressure on several species of pollinators, leading to their decline (Dave Goulson *et al.*, 2015). It is the consensus among scientists that this phenomenon has multiple causes that contribute in different ways to the survivability, and success of species. In regards to bees, major declines were first noted with the farmed honeybee species *Apis mellifera*, where high percentages of colony losses were reported for Europe (25% in 20 years) and the USA (59% in 58 years) over the past number of years (National Research Council USA, 2007; vanEngelsdorp *et al.*, 2008; Potts *et al.*, 2010). The disappearance of honeybees from hives is referred to as colony collapse disorder, which has been associated with decreased food availability, disease and the indiscriminate use of pesticides (vanEngelsdorp *et al.*, 2009).

Although monitoring the decline in wild species has proven difficult, current data for bumblebees and other wild bee species, suggest a substantial decline in populations mainly associated with reduced habitat availability for nesting, access to floral diversity and pathogen contamination, which is increased by commercial rearing of colonies, interaction with pesticides, climate change and competition with invasive species. The decline of pollinators increases the risk for ecosystem stability, food security and human welfare, constituting one of the biggest challenges we face today (Biesmeijer *et al.*, 2006; Potts *et al.*, 2010; Burkle *et al.*, 2013).

Of all the drivers of pollinating insect decline, and specifically that of bees, it is the potential impact of pesticides that are most controversial. When used appropriately pesticides provide a clear economic benefit, by reducing the populations of crop pests in destabilized agricultural environments and increasing crop yield. However, pesticides, especially insecticides, also have a direct effect on beneficial insects, whereas others like herbicides may affect them indirectly, by reducing flower numbers and increasing the toxicity of other pesticides (Goulson *et al.*, 2015; Sánchez-Bayo *et al.*, 2016b). In addition, overuse of insecticides can result in increased persistence in the environment,

therefore increasing the potential risk to bees. Residue analysis of honeybee colonies, their products and constituents, routinely identify large numbers of pesticides and to date a total of 91 different pesticides have been detected (Ostiguy *et al.*, 2019). Assessment of the toxicity, frequency of detection and concentration levels in honeybee hives, has also determined that of all the pesticides reported thus far, the three neonicotinoids thiamethoxam, clothianidin and imidacloprid pose the highest risk to bees on a global scale (Chauzat *et al.*, 2006; Blacquière *et al.*, 2012; Sanchez-Bayo and Goka, 2014).

Neonicotinoids were introduced in the 1990s to minimise the potential risks to beneficial arthropods and to combat pests with resistance to organophosphates (Simon-Delso *et al.*, 2015). Their systemic distribution, which led to the seed coating application, reduced the necessity for spraying, along with the risks for agricultural workers responsible for the applications. By 2005, 16% of the total world pesticide market involved neonicotinoids, making them the main class of insecticide used to treat seeds (77%), and the fastest growing market in the world (Elbert *et al.*, 2008). They are active against sucking and chewing insects, and act by targeting the cholinergic system in the nervous system (Casida, 2018). When applied to soil or seed, the pesticide is absorbed by the roots, and distributed throughout the plant, giving consistent and long-lasting control against pests. When applied to the leaves, it penetrates the lamina, and is distributed acropetally (xylem movement) protecting new shoots (Matsuda *et al.*, 2001; Elbert *et al.*, 2008; Hai-bo *et al.*, 2016; Johnson, 2016).

Today there are eight different neonicotinoids commercially used around the world (Hai-bo *et al.*, 2016). They can be divided into three different classes, according to their chemical structure. The N-nitroguanidines, a group that includes imidacloprid, thiamethoxan, dinotefuran and clothianidin, have a guanine group in its structure ($C{=}NNO_2$). The N-cyanoamidines that include: acetamiprid and thiacloprid have a cyano group (C=NCN) and the nitromethylenes, which includes nithiazine and nitenpyram which have a nitromethyl group ($C{=}CHNO_2$) (Tomizawa and Casida, 2005). Cyano group neonicotinoids are subject to rapid biotransformation which also confers them with lower toxicity compared to other neonicotinoids. The nitroguanidine-type neonicotinoids are the most widely used neonicotinoids in the world, with imidacloprid being at the top of the list, followed by thiamethoxan and clothianidin. Thiamethoxan is actively metabolised into clothianidin (Nauen *et al.*, 2003) justifying our choice to focus on imidacloprid and clothianidin in this chapter (Figure 3.1).

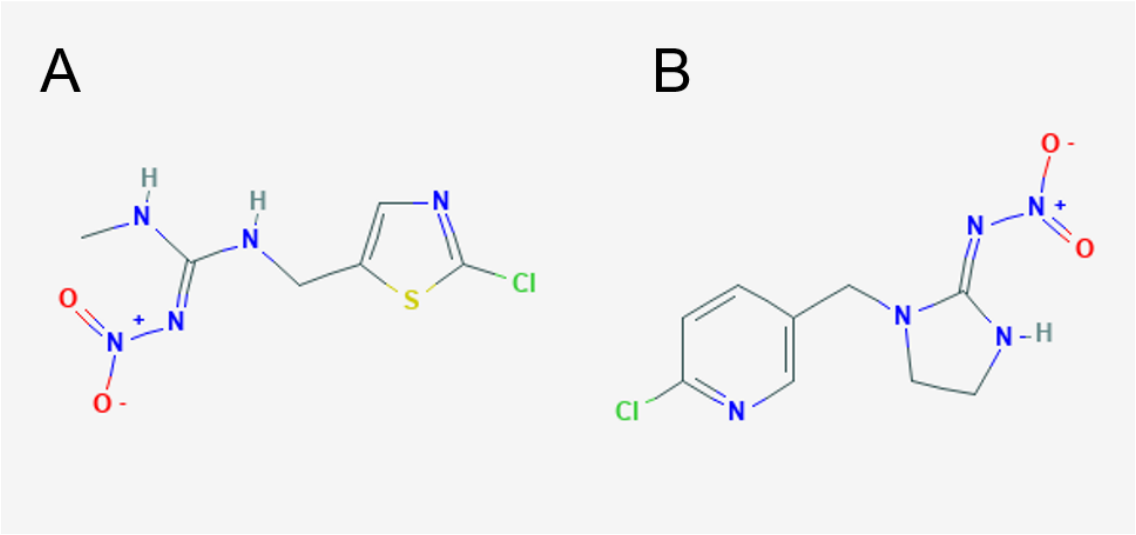


Figure 3.1 Chemical structure depiction for (A) Clothianidin and (B) Imidacloprid. Source PubChem National Center for Biotechnology Information.

Developed by the German company Bayer, imidacloprid was first marketed in 1991. It is the biggest seller among neonicotinoids, and gained registration for over 140 crop uses, in more than 120 countries. With high efficiency, wide insecticidal spectrum, long term effect and low toxicity to mammals, it can be used to treat seeds and soil (Elbert *et al.*, 2008; Hai-bo *et al.*, 2016). Bayer developed clothianidin in 2005, based on a chemical with the same name developed by the Japanese company Sumitomo in 2001. It is used to treat seeds, soil and leaves, and became the world's third biggest seller in 2010. It was shown to be highly effective against Coleoptera, Diptera, Hemiptera and less effective against lepidopteran pests, with a license to be used in 40 different crops (Elbert *et al.*, 2008; Hai-bo *et al.*, 2016). Due to the high toxicity of these chemicals to insects, there has been increasing concern, regarding the potential role that neonicotinoid insecticides may be playing in the decline of pollinators around the world (Blacquière *et al.*, 2012). Traditionally, the measurement of toxicity of insecticides has involved the determination of the lethal dose or concentration on honeybees (Alkassab and Kirchner, 2017). However, LD_{50} studies report widely variable results. For example LD_{50} results reported for imidacloprid using honey bees such as the studies of Nauen and Schmuck (2001) that report LD50 values between 3.7 and 81 ng/bee, while Laurino *et al.* (2013) report 118.74 ng/bee at 24 h and 90.09 ng/bee at 48 h, Decourtye and Pham-Delègue, (2003) report 30 ng/bee at 48 h, Bovi and Orsi (2018) report 107.9 ng/bee and Suchail and Belzunces (2000) that report 5 ng/bee at 24 h and 48 h, which demonstrate that results are highly dependent on a variety of factors, including not only the species tested, but the physiological state of individuals and colonies used.

In addition to mortality, the sublethal effects on behaviour and physiology have been investigated for most neonicotinoids, but for the purpose of this chapter, I will focus on those studies that involve imidacloprid and clothianidin in honeybees and bumblebees. Most of the evidence to date is based on honeybee studies, in which the term neonicotinoid is usually used to represent any of the chemicals in this class, rarely differentiating between chemicals while comparing reported results. The studies have described effects at different organization levels, expanding the endpoints of observation, which used to be restricted to assessment of mortality. Imidacloprid has been found to cause adverse effects in overwintering honeybees exposed to concentrations above 20 ng/g (Dively *et al.*, 2015). The same concentration caused decreased colony growth, colony lethality and reduced foraging activity and effectiveness of the waggle dance, as well as increased trembling, (Kirchner, 1999; Dively *et al.*, 2015). However, when colony growth was assessed by a dose closer to field relevance for nectar and pollen (0.5 to 5 ng/g) no adverse effect was found at the colony level (Faucon *et al.*, 2005). On the other hand when individual effects are considered, imidacloprid exposure has been reported to cause a reduction of haemocyte density, encapsulation processes (Brandt *et al.*, 2016) and expression of immunity related genes in honeybees (Aufauvre *et al.*, 2014). Larval acute exposure to doses higher than 24 ng/bee, caused reduced pupation and emergence rates in laboratory studies (Yang *et al.*, 2012), but the same effect was not corroborated at field level (Pohorecka *et al.*, 2012).

For bumblebees imidacloprid was found to affect colony performance (Laycock *et al.*, 2012), colony growth (Gill *et al.*, 2012a; Whitehorn *et al.*, 2012), and brood production (Laycock and Cresswell, 2013) from concentrations as low as 0.7 ng/g after chronic oral exposure, levels much lower than the lowest observed effect concentration (LOEC) reported for honeybees and of field relevance. Foraging ability and trembling are also reported to be affected when bees are exposed to concentrations from 10 ng/g to 30 ng/g of contaminated pollen (Morandin and Winston, 2003; Gill and Raine, 2014), and reduced male production was described after consumption of contaminated food (10 ng/ml imidacloprid) (Mommaerts *et al.*, 2010). Effects on queen survival and nectar storing are also reported with imidacloprid having significant impact on queens survival from concentrations of 50 ppb (Scholer and Krischik, 2014b). Sublethal effects of imidacloprid on cellular processes and the nervous system of bumblebees will be discussed below.

Clothianidin, like imidacloprid, was not found to affect overwintering success in honeybees when field relevant doses were tested (Cutler and Scott-Dupree, 2007) and did not affect colony performance or development when exposed to concentrations as high as 23 ng/g (Cutler and Scott-Dupree, 2007; Pilling *et al.*, 2013; Rundlöf *et al.*, 2015). However, in bumblebees it was found to reduce colony growth, foraging performance, food storage and reproduction (Larson *et al.*,, 2013; Rundlöf *et al.*, 2015) reducing the survivability of brood, but not the amount of callows in both *B. terrestris and B. impatiens* (Franklin *et al.*, 2004; Scholer and Krischik, 2014a).

When grooming, foraging and motor activities were assessed, as well as learning and memory, deleterious effects were described for both species and both neonicotinoids exposed at field relevant doses (Williamson *et al.*, 2013; Williamson *et al.*, 2014; Manjon *et al.*, 2018). As these individual effects on behaviour are associated with neurophysiological changes in the bee brain they will be discussed further below.

The worldwide attention given to neonicotinoids has been met with political action in some parts of the world. In December of 2019, the European Union will move to permanently banning outdoor use of three neonicotinoids (imidacloprid, clothianidin and thiamethoxam), with France being the only country in the world to ban the use of five neonicotinoids previously authorised in Europe. Farmers worry that this will affect the types of crops produced on the continent, which may increase importations from other parts of the world where the seed treatment is still permitted. Countries including Canada seem to be moving in the same direction, but a final decision has to be made by the end of 2019. In the rest of the Americas there are signs of little change, with neonicotinoids continuing to be licensed in all countries, and used actively in the United States, Brazil and others. Australia has removed household pesticides containing neonicotinoids from shelves but will not be reviewing the use in agriculture by justifying that their honeybee populations are not in decline. To date there is no comprehensive study of the status and trends of pollinators and pesticide impact on pollination services in Africa, with existing studies being limited, and geographically widespread through the continent (Gemmill-Herren *et al.*, 2014). However, in 2018 a program was launched in South Africa to gather information regarding the impact of neonicotinoids in the ecosystem. The initiative involves the participation of 12 countries including Egypt, Sudan, Uganda and Botswana, and aims at bringing specialists from other non-participating countries to consolidate the knowledge in the whole continent. Across the world, it seems that difficult decisions are

being made regarding pesticide regulations. Therefore, if informed policies are to be developed, it is essential that we understand the way these chemicals impact pollinators.

Worryingly, it seems that the new classes of insecticides, likely to replace neonicotinoids, may have similar toxicity effects on pollinators, maintaining the risk. In addition, neonicotinoid contamination is extensive, due to their long persistence in the environment. It is possible to detect trace levels of contamination in water bodies, soil and even in the air (Simon-Delso *et al.*, 2015) with pesticides being found in the environment from concentrations of 0.14 to 18 ppb in 80% of the tested surface waters from nine countries (Blacquière *et al.*, 2012). In addition contamination has even been found in wild plants, found near crop regions that had received treatment (Botías *et al.*, 2016).

Reducing the impacts of neonicotinoid on pollinators will not immediately be solved by banning these chemicals, as they will persist in the environment long afterwards. Furthermore, little movement has been made to curtail their use outside Europe, justifying any future studies that aim at understanding how these chemicals act on non-target insects, from the molecular through to the organismal levels. It is essential that we continue to gather this information, and better understand the effects of neonicotinoid exposure, so that informed and evidence-based decisions about their regulatory status and general use can be made. To achieve this understanding, we must first consider the target system in which these chemicals act, the cholinergic system, and what challenges remain to be clarified, to understand interferences in their mode of action and effects.

3.1.2 The cholinergic system

The cholinergic pathway represents an important excitatory system in insects and involves the neurotransmitter system responsive to acetylcholine. It comprises two types of receptors, muscarinic acetylcholine (mAChRs) and nicotinic acetylcholine receptors (nAChRs). The synthesis of acetylcholine occurs in a two-step pathway which involves the conversion of acetate and coenzyme A into acetyl CoA, by the enzyme Acetyl CoA synthetase. Acetyl CoA and choline are then converted into acetylcholine by the enzyme choline acetyltransferase (Figure 3.2) (Thany *et al.*, 2010).

Acetylcholine is stored at the presynaptic axon, and an increase in its quantity, within the vesicle, triggers mAChR activation, releasing acetylcholine into the synaptic cleft,

mediating synaptic signal transmission. NAChRs are ionotropic receptors, sensitive to acetylcholine and become activated during normal synaptic activity, triggering neuronal depolarization. In a normal scenario, the pathway is completed by the activity of acetylcholinesterase, which breaks down acetylcholine into acetate and choline, releasing the nAChRs and allowing for repolarization of neurons (Thany and Lapied, 2010).

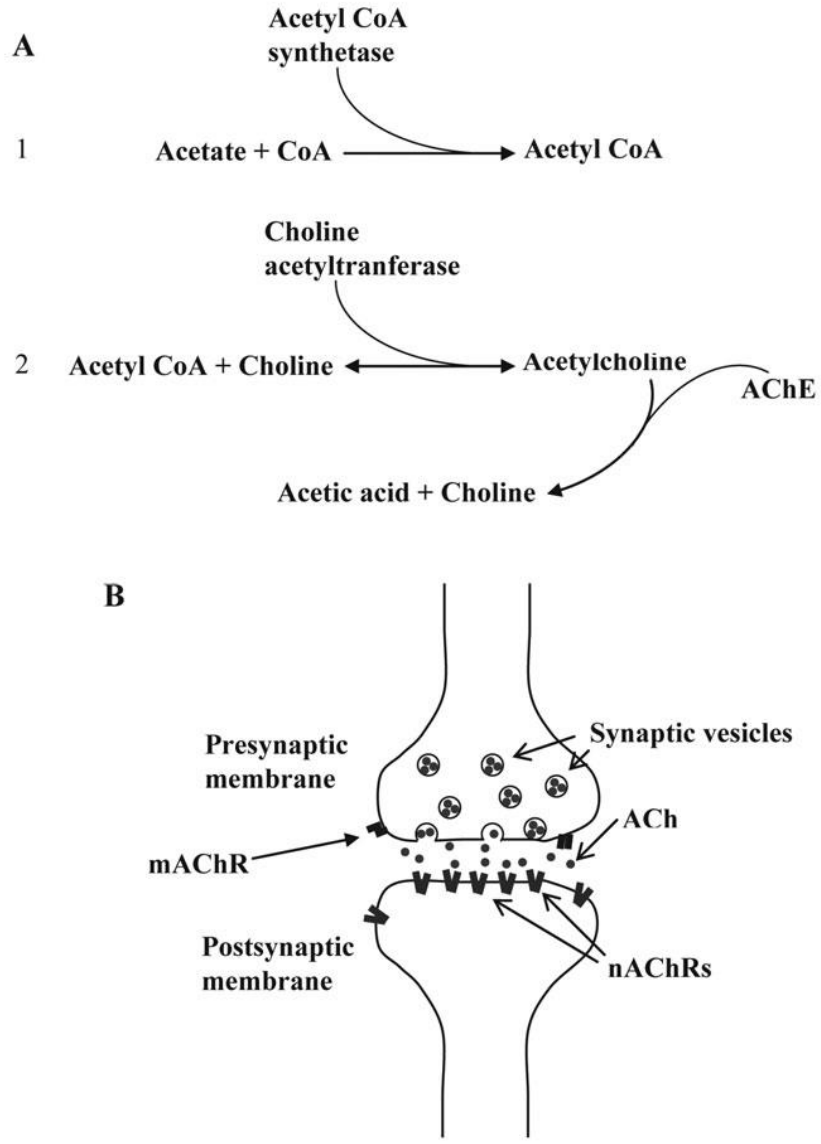


Figure 3.2 (A) Schematic representation of acetylcholine (Ach) synthesis[1] and hydrolysis[2] and (B) its role in the cholinergic synaptic cycle, where neurotransmitter is released from synaptic vesicles in the presynaptic neuron to interact with nAChRs on the postsynaptic membrane of neurons triggering a fast-excitatory response through neuronal depolarization. ACh may also bind to mAChR in the presynaptic membrane which modulates the release of ACh thus forming a negative feedback loop. (Image obtained from Thany and Lapied (2010).

The localization of cholinergic neurons has been investigated by several techniques that targeted the enzymes acetylcholinesterase (AChE) and choline acetyl transferase (ChAT), in both vertebrates and invertebrates. These enzymes are localised throughout the insect CNS, with AChE being found in non-cholinergic regions,

suggesting independent and additional roles outside acetylcholine hydrolysis (Thany *et al.*, 2010).

When it comes to the distribution of the receptors in bees, we rely on honeybee studies that have demonstrated their wide distribution in areas consisting of dense networks of axons and dendrites throughout the brain. They are particularly abundant in the olfactory and visual systems and in the mushroom bodies (Scheidler *et al.*, 1990; Déglise *et al.*, 2002; Strausfeld, 2002; Barbara *et al.*, 2005). Between the two types of cholinergic receptors, nAChRs are more prevalent than mAChRs in the central nervous system of insects, contrary to what is found in mammals (Bicker, 1999; Yasuyama *et al*., 2002; Jensen *et al.*, 2005). Regarding their localisation, in insects, no clear evidence exists of nAChRs presence in presynaptic neurons, which only exhibit receptors with muscarinic profile, suggesting that the release of Ach is modulated by muscarinic receptors, which are present on presynaptic neurons, and form a negative feedback loop that prevents overexcitability of neurons and repeated depolarization (Hue *et al.,* 1989).

NAChRs have a postsynaptic function responding to acetylcholine, which triggers the depolarization of neurons (Thany and Tricoire-Leignel, 2011). They have been characterised into two classes: high sensitive or non-sensitive to known antagonists such as α Bugarotoxin and the nortiterpenoid alkaloid methyllicaconitine (MLA), in the same manner as in vertebrates (Lind *et al.*, 2001). However, contrary to vertebrates, the molecular composition and the stoichiometry of the insect nAChR is still unknown. To try and understand its structure, genomic analyses were conducted and identified 10 nAChR genes in *Drosophila melanogaster* and *Anopheles gambiae* (Jones *et al.*, 2007) and 11 genes in *Apis mellifera* (Jones *et al*., 2006). These genes encode for subunits that can co-assemble forming different subtypes of nAChR, with distinct pharmacological properties (Gauthier, 2010a). The understanding of its structure is of extreme importance since several insecticides target the cholinergic system and the pharmacology of receptors is directly determined by which subunits exist in each subtype.

3.1.3 Diversity and structure of nAChR in mammals and insects

As with mammals, insect nAChRs mediate rapid synaptic transmission, causing excitation and membrane depolarization in postsynaptic neurons in response to acetylcholine or any other agonist (Thany *et al.*, 2007). However, most of our knowledge regarding receptor response, in relation to its structure, derives from studies with

vertebrates, where they are formed by combinations of different subunits. These combinations include alpha subunits (α1 to α10), which all contain cysteine residues (Cys192 and Cys 193) implicated in Ach binding, and the β, γ, δ and ε subunits, which lack such motif (Thany and Tricoire-Leignel, 2011). In addition, a functional receptor in vertebrates can be homomeric (formed by 5 identical alpha subunits) or heterometric (formed by a combination of alpha and any other subunit), but in either, a central cationic channel has to be formed (Figure 3.3) (Gauthier, 2010b). Receptors formed by the α4 and β2 subunits are the most widely distributed type of heteromeric receptors in mammals, while the most common homomeric receptor is formed by 5 identical α7 subunits (Gauthier, 2010b). There are 16 different subunits described for this group, which increases the possibility of the existence of a series of different receptor subtypes (Thany *et al.*, 2007).

As with vertebrates, different genes encoding for receptor subunits have been described for different insect species. Although 11 nAChR subunit encoding genes in *B. terrestris* , have been identified, little is known about their receptors molecular structure and pharmacology (Sadd *et al.*, 2015). Another difficulty in relation to our understanding of the structures of these receptors, is the fact that the nomenclature of subunits in insect nAChRs followed the rule of sequence apparition, and not homology with vertebrate subunits. Making the rationalisation of names, and the understanding of their properties, difficult (Thany and Tricoire-Leignel, 2011).

Insect receptors can however be classified based on their sensitivity to alpha bungarotoxin and other antagonists, as with vertebrate receptors (Thany and Tricoire-Leignel, 2011), although their structure remains unknown.

The elucidation of the pharmacology of the vertebrate receptors came from a series of experiments that used heterologous protein expression in *Xenopus* oocytes. It was shown that α7 assembles into homopentameric ligand-gated ion channels that are cation selective, which rapidly desensitise and shows high affinity to α bungarotoxin (Bertrand, Bertrand and Ballivet, 1992; Séguéla *et al.*, 1993). Other functional receptors expressed on host cells were shown to be formed by pairwise combination of α2, α3 or α4 with β2 or β4, generating heterometric receptors, while α7 and α9 produced homomeric receptors, and α10 alone produced a non-functional receptor subtype (Bertrand *et al.*, 1992; Séguéla *et al.*, 1993; Elgoyhen *et al.*, 1994; Buisson *et al.*, 2000; Nelson *et al.*, 2003; Khiroug, Khiroug and Yakel, 2004), which demonstrates the importance of elucidating structure to understand their pharmacology.

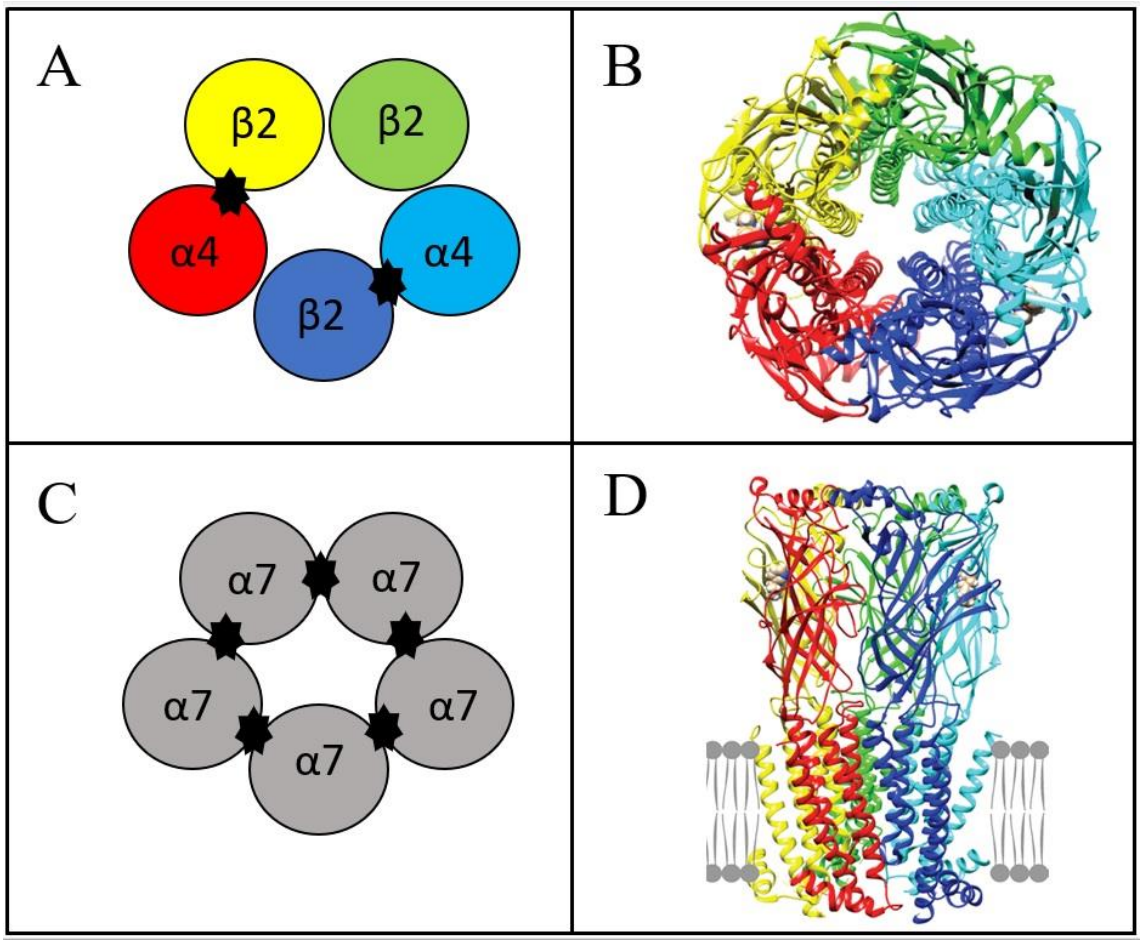


Figure 3.3 Schematic and 3D representations of the general structure of a nAChR. Five subunits must coassemble to form a functional receptor subtype. (A) Schematic representation of a heteromeric receptor formed by the combination of α4 and β2 subunits. Each subunit is represented in a different colour, matching the 3D receptor images. Ach binding sites are depicted with black stars. (B) 3D image of the top view of the nAChR, each subunit is represented in a different colour. (C) Schematic representation of the α7 homomeric receptor, Ach binding sites are depicted with black stars. (D) 3D image of the lateral, or membrane level view of the nAChR, subunits are represented in different colours and the lipid bilayer is represented in gray (modified from Morales-Perez *et al.* 2016).

The expression of insect receptor subtypes in heterologous systems has been highly unsuccessful, with the only expression of a functional insect nAChR having been achieved in the locust *Schistocerca gregaria*, using alpha like subunit (αL1) that formed a homo oligomeric channel, sensitive to alpha bungarotoxin and methyllicaconitine (MLA). Although this receptor showed reduced sensitivity to nicotine when compared to the homomeric receptors formed by the chick α7 subunits (Amar *et al.*, 1995).

It is suggested that insect homomeric nAChR could be pharmacologically different than vertebrate nAChR or that more complex subunits combinations could be required to form a functional insect receptor, as evidence is still scarce. In any case pharmacological profile of insect nAChR associated with subunit composition, has proven difficult to

perform through host cells (Thany and Tricoire-Leignel, 2011). Nevertheless, evidence that different subunit combinations are found in insects is clear by the existence of sensitive and non-sensitive receptors to the antagonists α bungarotoxin and d tubocurarine (Goodman and Spitzer, 1979; Lane *et al.*, 1982). It is also clear that such diversity of subunits may influence their response to chemicals that target the cholinergic system in insects, including neonicotinoid insecticides.

3.1.4 Neonicotinoid mode of action on nAChR

Neonicotinoid insecticides have been shown to act as agonists on post synaptic nAChR, binding irreversibly and causing strong depolarization on synaptic neurons independently of the participation of mAChR (Thany, 2009). The agonist interaction model with the receptor has been developed through affinity labelling, mutagenesis and structural studies that elucidated the role of participating amino acids of three different regions of the α subunits, and of at least three different regions of the non α subunits (β, γ, δ or ε), and these were called the 6 loops of interest (Corringer *et al.*, 2000; Grutter and Changeux, 2001; Sine, 2002). However, it is still unclear if this interaction model can be applied to all animals, due to the high diversity of nAChRs found in the animal kingdom (Liu *et al.*, 2008).

Neonicotinoids can be differentiated based on their chemical structure, which ultimately determines their selectivity (Matsuda *et al.*, 2001; Tomizawa and Casida, 2005). In addition to the chemical structures in the insecticide molecule, each different receptor subunit is also formed by different amino acid loops, and changes in their electrostatic properties, can also influence degree and effect of neonicotinoid binding to the receptor, as shown by studies that through mutations, changed acidic or neutral amino acid residues, to neutral or basic residues in α and β loops, increasing selectivity to neonicotinoid binding (Shimomura *et al.*, 2006; Yao *et al.*, 2008). Although all the six loops are thought to play a role in agonist binding, they have different levels of contribution. The α subunits seem to be the most important (Thany and Tricoire-Leignel, 2011), its 151-site in invertebrates is known to be constituted of different residues that influence intensity of binding, causing different effects depending on species. In *Caenorhabditis elegans* this position has a methionine (Brown *et al.*, 2006). When methionine was introduced through mutagenesis to the same position into an brown planthopper α1 subunit (Y151M) and co expressed with rat β2 using heterologous expression in *Xenopus* oocytes, imidacloprid was found to have antagonist activity on the nAChR receptor, blocking responses to acetylcholine (Zhang *et al.*, 2008). This

demonstrates that it is the structure of the receptor that determines the specificity of the binding, which means that the agonist binding model of neonicotinoids, may no longer be so widely applicable for insects, as we still do not fully understand the diversity of subunits present in this group of invertebrates.

Although neonicotinoids are known to act on the nAChR, their target subunit remains unknown. Furthermore, the subunit composition of insect native and recombinant nAChRs and the agonist site structure, remains to be elucidated (Liu *et al.*, 2008). The diversity of subunits identified by genome sequencing in insect species (nine α and one β in *A. gambiae* (Jones *et al.*, 2007), nine α and two β in *A. mellifera* (Jones *et al.*, 2006), nine α and three β in *B. mori* (Shao et al., 2007) eleven α and one β in *T. castaneum* (Jones *et al.*, 2007) and nine α and two β in *B. terrestris* and *B. impatiens* (Sadd *et al.*, 2015) suggests a complex scenario of potential binding by neonicotinoids. Although progress has been made to understand nAChR structure and insecticide selectivity, more research is required to determine neonicotinoid mode of action on the diversity of receptor structures found in insects, and to correlate such mode of action with effects seem at the ecotoxicological level.

3.1.5 Neonicotinoid effects on the neurological system of bumblebees

In addition to the sublethal colony and developmental effects listed previously, neonicotinoids are known to affect the nervous system of bees triggering detrimental effects on their homing and foraging activities, as well as in learning and memory formation processes (Mengoni *et al*., 2015; Stanley *et al.*, 2016; Tison *et al.*, 2016). These behavioural affects may be explained by cellular and morphological changes at the neuropils, and individual neuronal cells in the brain. Increased apoptosis and autophagy have been reported in the brains of honeybees exposed to neonicotinoids (Wu *et al.*, 2015), with the optical lobes being the most sensitive regions to imidacloprid exposure (de Almeida Rossi *et al.*, 2013; Oliveira *et al.*, 2014). However, apoptosis alone may not account for all effects observed at the behavioural level.

Neonicotinoid exposure has also been reported to cause continuous stimulation of Kenyon cells (KCs) of the mushroom bodies (Moffat *et al.*, 2016), leading to energy depletion, due to mitochondrial dysfunction (Moffat *et al.*, 2015a). This phenomenon has been observed in both honeybees and bumblebees, suggesting a shared toxicity mechanism (Nicodemo *et al.*, 2014; Powner *et al.*, 2016). Decreased synaptic density in

the calyx was reported for adult bees that had been exposed at the larval state to imidacloprid, suggesting interference on brain maturation, which can also have long term effects on the olfactory (Yang *et al.*, 2012), and visual systems (de Almeida Rossi *et al.*, 2013; Oliveira *et al.*, 2014) in adult bees. Such effects have been confirmed, with considerable reductions on KCs neurites outgrowth (Wilson *et al.*, 2013), impairment of visual systems post exposure to imidacloprid (Powner *et al.*, 2016), and reduction in the level of the neurotransmitter release, regulating the protein synapsin in mushroom bodies and antennal lobes, post exposure to thiamethoxam (Tavares *et al.*, 2019a).

Changes in acetylcholinesterase activity, after exposure to clothianidin and imidacloprid (Boily *et al.*, 2013), have also been reported. This enzyme, known to act on nicotine at the synaptic cleft, frees nAChR, allowing for the repolarization of nervous cells. The enzyme cannot act on neonicotinoids, which in turn continue stimulating the neurons (Matsuda and Sattelle, 2004). The constant firing of action potentials in the nervous cells, and the impossibility to return to normal cell functioning can lead to neuronal vulnerability (Moffat *et al.*, 2015a), which has only recently been investigated at the molecular level. Colgan *et al.* (2019) demonstrated the effects of neonicotinoid exposure on the gene expression of queens and workers of *B. terrestris.* They found that clothianidin affects the expression of a greater variety of genes, when compared to imidacloprid, and that both neonicotinoids affect castes differently, with workers being more susceptible to negative effects. The expression of genes associated with mitochondrial function, glucose metabolism, oxidation reduction processes, regulation of developmental growth and neuron projection guidance, were highlighted as exhibiting changes in response to the insecticides after chronic exposure, and ought to be further investigated in future studies.

Although this whole transcriptome profiling study represents an improvement in relation to other studies that have investigated the molecular effects of insecticides on bumblebees, through the assessment of the expression of their direct target sites or on *a priori* metabolic candidate, such as detoxifying enzymes. Given that transcriptional level studies are typically a step removed from the phenotype level and cannot account for post-transcriptional and translational variation and modification, we believe that our proteomic approach represents a superior method to explore the molecular effects of neonicotinoid exposure in bumblebees. At the very least, we anticipate that a different perspective and level of insight will transpire from the proteomic study presented here.

3.2 Chapter aims and hypothesis

Neonicotinoids are known to impair the neurological system of insects at different levels. They are defined as agonists of the postsynaptic nicotinic acetylcholine receptor. Thus, through binding they should activate the nAChR, triggering neuronal depolarization and transmission of synaptic signal.

Therefore, this chapter will investigate the comparative effects of clothianidin and imidacloprid on the neuronal system of the common pollinator *B. terrestris* at the behavioural, cellular and proteomic level through:

1.1 The assessment of the impact of acute neonicotinoid exposure, to different field relevant and sub lethal concentrations of clothianidin and imidacloprid, on the behaviour of bumblebee workers.

1.2 The investigation of the levels of the protein synapsin in the mushroom bodies and antennal lobes of bumblebee workers at 4 h after acute exposure to clothianidin and imidacloprid. Synapsin is associated with neurotransmitter release through their action on synaptic vesicle release and recycling, thus being a good indicative of synaptic activity.

1.3 The characterization of the bumblebee brain proteome after field relevant acute exposure to clothianidin and imidacloprid at three different timepoints within 24h.

This experimental approach was designed to improve our understanding of how different neonicotinoids act on the bumblebee nervous system and determine how their action is translated into individual effects at the behaviour and molecular level.

We hypothesise that as both neonicotinoids bind irreversibly to the receptors, depolarization of cells should be maintained, impairing neuronal function and leading to insect death. Clothianidin and imidacloprid are both characterised as nAChR agonist insecticides. Hence, a similar type of response would be expected at all levels of influence.

3.3 Experimental outline and methods

3.3.1 Colony maintenance *Bombus terrestris audax*

Commercial colonies (two week young research colonies) of *B. terrestris audax* were obtained from Biobest (supplied by Agralan UK) and maintained at standard environmental conditions of 25°C ± 1°C and 60 % ± 5 % relative humidity with continuous darkness or red light and fed a sugar solution (Biobest) *ad libitum* and 2 g of pollen (Biobest) every day (Figure 3.4A). Colonies where the founding queen was found dead or where males started to emerge from pupal cells, were not used for any molecular experiments. Colonies were acclimatised under laboratorial conditions for at least 48 h before beginning the experiments.

3.3.2 Micro colony experiments of pesticide exposure

Workers were removed from four different colonies and assigned to their respective control or treatment groups. Bees were colour-tagged using a water-based paint to allow for colony tracking (Figure 3.5B), before being transferred to a plastic box (w 16,5 cm; h 8,5 cm; d 12 cm) (max 10 bees per box (2 or 3 from each colony)). Autoclaved fine sand was used to cover the bottom of the boxes and pollen and 50 % sucrose (w/v) were provided *ad libitum.* Bees were acclimatised overnight at the same environmental conditions described above, before continuing the experiment.

Acute exposure was performed after a period of 2-hour starvation. Thirty-five microliters of experimental solutions were fed to each bee individually and only bees that fully consumed the solutions were used in the experiments (Figure 3.5A). The procedure control group received 35 µl of a 50 % sucrose (w/v) with <1 % acetone solution to account for the toxicity, if any, of the solvent used for the preparation of insecticide solutions (Organisation for Economic Co-operation and Development., 1998)(*Test No. 247: Bumblebee, Acute Oral Toxicity Test*, 2017). Imidacloprid and clothianidin concentrations ranging from 2.5 ng/ml to 1 µg/ml were tested. Exposed and control bees were returned to their plastic boxes once the micro droplet of the solution was fully consumed and experimental parameters were assessed as relevant at different timepoints according to the chosen technique.

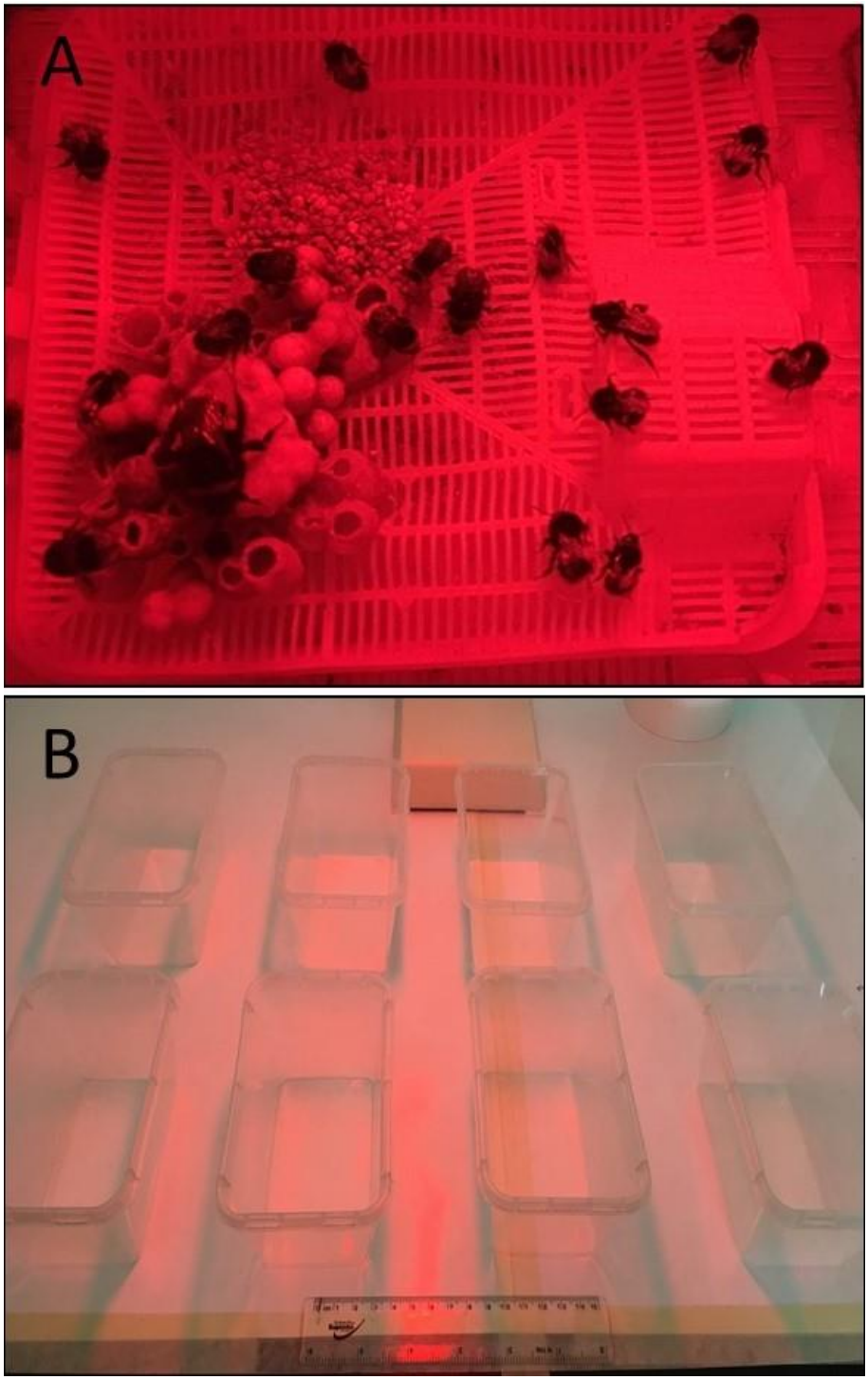


Figure 3.4 (A) Interior view of a young commercial bumblebee colony (Biobest). (B) Plastic boxes set up for individual acute experimental exposure to neonicotinoids (Lids removed).

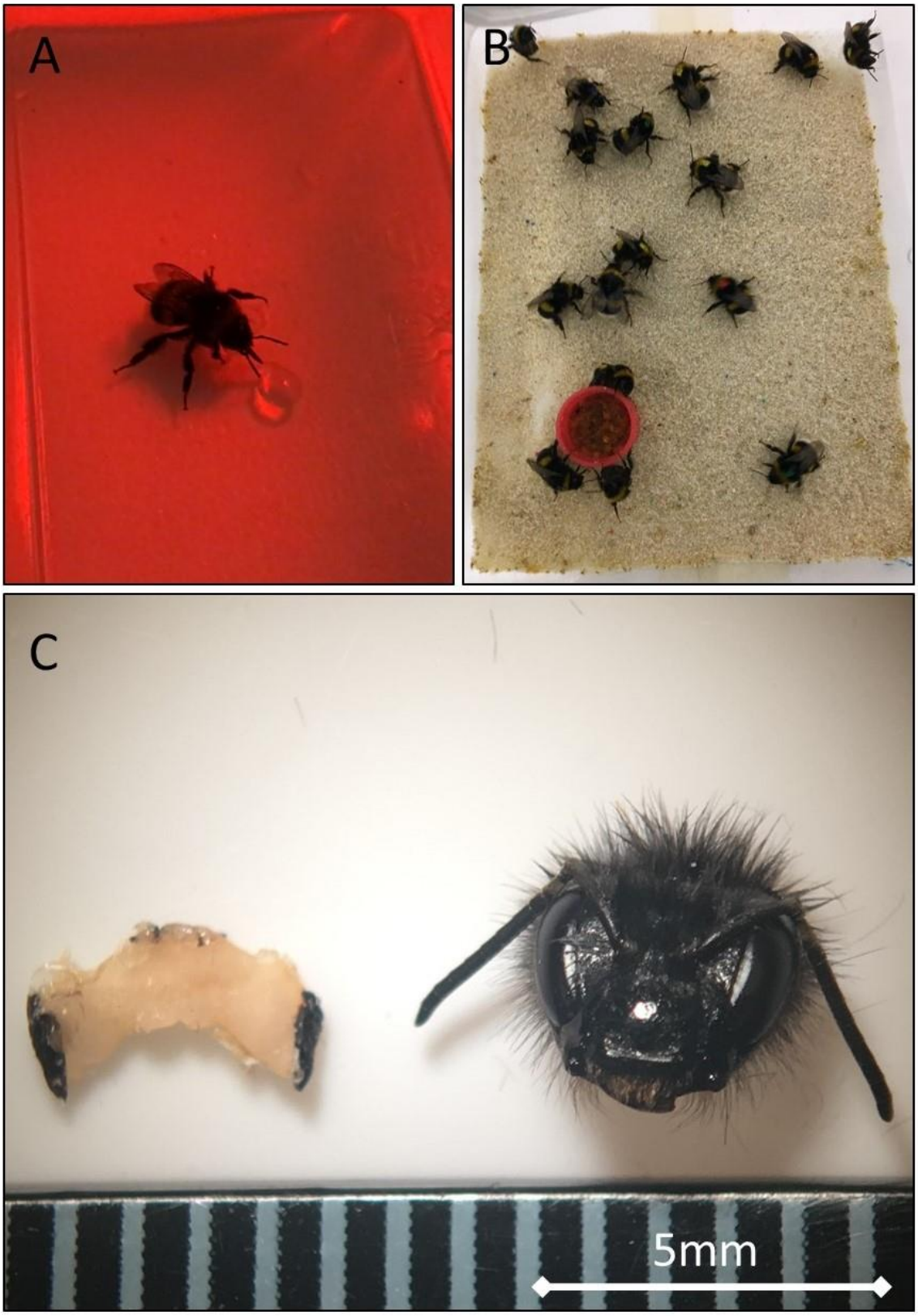


Figure 3.5 (A) Worker bee voluntarily consuming 35 µl of a 50 % w/v sucrose solution contaminated with neonicotinoid. (B) Microcolony set up with fine sand covering the bottom of the box showing colour tagged bumblebees. (C) Bumblebee brain (left) and head (right).

3.3.3 Behavioural analysis

To assess the effects of acute neonicotinoid exposure at the behaviour of workers of *B. terrestris* a protocol adapted from the (OECD, 1998; EPA, 2016) was designed. Specific endpoints of assessment, characterised by discrepancies identified in the behaviour of recently exposed bees, such as immobile behaviour, uncoordinated movement/spasm, hind legs contraction, paralysis, super excitability, involuntary proboscis extension and mortality, were assessed over the course of 48 h, with data collection points at 15, 30 45- and 60-min and 4, 24 and 48 h post ingestion of pesticide solution (Table 3.1). A total of eight different solutions were tested for each neonicotinoid, clothianidin and imidacloprid, with concentrations varying from 0 ng/ml (control), 0 ng/ml with <1% acetone (procedure control), 10 ng/ml, 25 ng/ml, 35 ng/ml, 50 ng/ml, 70 ng/ml, 100 ng/ml to 1 µg/ml for each, and two solutions to test synergism or competition between the neonicotinoids (50 ng/ml imidacloprid + 50 ng/ml clothianidin and 100 ng/ml imidacloprid + 100 ng/ml clothianidin). Dilutions were made in 50% w/v sucrose solution and the highest concentration of acetone was used to prepare the procedure control, making sure that such concentration never surpassed 1% as per the guidelines followed (OECD., 1998; *Test No. 247: Bumblebee, Acute Oral Toxicity Test*, 2017). The experiment was carried out over the course of two years and a total of 540 bees were used (30 per treatment, coming from 4 different colonies each time, in technical triplicates with 10 bees per box). Statistical analysis was performed using Prism GraphPad v 5, applying the Kluskall-Wallis test and Dunn's post hoc on the resulting mortality nonparametric datasets.

Table 3.1 Data collection table for acute toxicological behaviour experiments.

Title of Experiment: **Date:**

Bees per box: 10 **Behaviour test**

Timepoints of observation	15min	30 min	45 min	1h	4h	24h	48h
Treatment:	M ( ), S ( ), P ( ), N( ).	M ( ), S ( ), P ( ), N( ).	M ( ), S ( ), P ( ), N( ).	M ( ), S ( ), P ( ), N( ).	M ( ), S ( ), P ( ), N( ).	M ( ), S ( ), P ( ), N( ).	M ( ), S ( ), P ( ), N( ).
Treatment:	M ( ), S ( ), P ( ), N( ).	M ( ), S ( ), P ( ), N( ).	M ( ), S ( ), P ( ), N( ).	M ( ), S ( ), P ( ), N( ).	M ( ), S ( ), P ( ), N( ).	M ( ), S ( ), P ( ), N( ).	M ( ), S ( ), P ( ), N( ).

Mortality (M): Immobile bees, usually found laying on their backs.
Spasm (S): Crawling Bees, uncoordinated movement.
Paralysis (P): Motionless, on the floor of the test cage, responding to stimuli by moving leg or antenna but not walking, cornered.
No effect (N): Bees having normal behavior
Record the numbers of bees observed with any of the symptoms in brackets.

3.3.4 Confocal microscopy analysis of synapsin levels in bumblebee brains

Brains of bees exposed or unexposed to a unique dose of 0.0875 ng (35 µl of 2.5 ng/ml solution) or 0.35 ng (35 µl of 10 ng/ml solution) of either clothianidin or imidacloprid, were dissected 4 h post exposure and directly transferred into an 1.5 ml microfuge tube containing 50× the volume of the tissue in 4 % w/v paraformaldehyde prepared in 0.1M PBS, pH 7.4, for fixing at 4ºC for 24 h. Samples were then washed twice in PBS (30 min each) and dehydrated gradually (1 hour each), using a series of different ethanol/dd H_2O solutions at concentrations of 50 %, 70 %, 85 %, 95 % and 100 % v/v, and cleared in ethanol/xylenes solutions at concentrations of 50:50 100 % ethanol and xylenes and 100 % xylenes, before being immersed in melted paraffin (60ºC) twice, prior to embedding, again for 1 hour each (samples left overnight in 70% ethanol).

Brain tissues were sectioned at 4 µm using a Leica microtome. Only sections that contained the tissue in its integral form were kept for the analysis and thereafter melted on microscope slides for 20 minutes at 65ºC, washed with xylenes (twice, 5 minutes each) and rehydrated using a concentration series of ethanol and water (100 %, 95 %, 80 % and 70 % ethanol) for a minute each and finally washed with distilled water.

To assess synapsin levels in bee brains after neonicotinoid exposure, a primary antibody anti-synapsin (3C11 (anti SYNORF1)) was purchased from the Developmental Studies Hybridoma Bank at the University of Iowa (http://dshb.biology.uiowa.edu/3C11-anti-SYNORF1_2). An aliquot containing 3 µg/ml was stored at 4ºC until required.

Antigen retrieval was performed on tissue sections using a citrate buffer (10 mM Citric Acid, 0.05 % Tween 20, pH 6). Slides were immersed in buffer at 95-100ºC for 30min, cooled to room temperature, and permeabilised with 0.5 % Triton X-100 in PBS for fifteen minutes on ice, and then washed again with PBS.

Tissues were blocked in 5 % BSA in PBS-Tween (0.05 %) for one hour at room temperature and incubated with 3 µg/ml of anti-synapsin antibody overnight at 4°C. Primary antibodies were then removed, and sections were washed three times prior to the addition of secondary antibody.

Secondary antibody was added as follows; anti-mouse-AlexaFluor 594 (Invitrogen) diluted 1:400 in 5 % BSA in PBS-Tween (0.05%) and DAPI as a nuclear stain (0.5 mg/ml). After staining slides were mounted in PBS and sealed using clear nail varnish.

Analysis was carried out using an Olympus Fluoview Confocal Microscope and Olympus Fluoview FV10-ASW software. Images were captured using a 20× and 40× objective lens (excitation at 405nm and 594nm).

Three brains (biological replicates) were analysed per treatment (control 4h, clothianidin 0.0875 ng /bee 4 h, imidacloprid 0.0875 ng /bee 4 h, clothianidin 0.35 ng/bee 4 h and imidacloprid 0.35 ng/bee 4 h) and 3 measurements each (a, b and c) were taken in 3 different neuropil regions and 8 different subregions: mushroom bodies (lip, collar and basal ring); Pedunculus (Pd1 and Pd2) and Antennal Lobe (Al1, Al2 and Al3) of the bumblebee brain, resulting in 45 samples and 360 measurements.

Statistical analysis was carried out using RStudio. One-way ANOVA tests were applied to measurements taken for each neuropil area and for each individual sub area of the mushroom bodies for comparison.

3.3.5 Sample preparation for mass spectrometry-based proteomics

Eight brains for each treatment (biological replicates) (procedure control, 2.5 ng/ml imidacloprid, 10 ng/ml imidacloprid, 2.5 ng/ml clothianidin and 10 ng/ml clothianidin at 1, 4 and 24 hours post exposure) were removed into 200 µl of 6 M urea, 2 M thiourea buffer, supplemented with a protease inhibitor cocktail (Roche) and immediately snap frozen using liquid nitrogen. Samples were stored at -20 ºC until the dissections were completed. After thawing on ice, brains were homogenised and centrifuged for 5 min at 9,000 x g to pellet any cellular debris.

The supernatant was then removed and quantified using the Qubit™ protein quantification system (Invitrogen), following the manufacturer's instructions. Samples were chosen based on those with most similarity in protein concentration and SDS PAGE profile. Eighty-five micrograms of each sample were precipitated using the 2D Clean Up Kit (GE Healthcare), following the manufacturer's instructions. The resulting protein pellet was resuspended in 50 µl of 6 M urea, 2 M thiourea, 0.1 M Tris-HCl, pH 8.0 buffer, and quantified using the Qubit™ system. 105 µl of 50mM ammonium bicarbonate was added to 20 µl of each sample. Proteins were reduced with 0.5 M of dithiothreitol (DTT) at 56ºC for 20 min and alkylated with 0.55 M iodoacetamide (IAA) at room temperature for 15 min, in the dark. One microliter of a 1% w/v solution of Protease Max Surfactant Trypsin Enhancer (Promega) and a 1 µg Sequence Grade Trypsin (Promega) was added to give a protein:trypsin ratio of 40:1. The protein/trypsin mixture was incubated at 37ºC overnight. Digestion was terminated by adding 1 µl of 100 % trifluoroacetic acid (Sigma Aldrich) and incubation at room temperature for 5 min. Samples were centrifuged at 13,000 x g for 10 min and the supernatant was removed and purified for mass spectrometry using C18 Spin Columns (Pierce), following the manufacturer's instructions. The eluted peptides were dried using a Speedy Vac concentrator (Thermo Scientific Savant DNA120) and resuspended in 2 % v/v acetonitrile and 0.05 % v/v trifluoroacetic acid (TFA). Samples were sonicated for 2 min to aid peptide resuspension followed by centrifugation at 13,000 x g for 5 min. The supernatant was removed and a volume equivalent to 1 µg was loaded onto a QExactive (ThermoFisher Scientific) high-resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system.

3.3.6 Proteomic analysis

Protein profiles were determined in three biological replicates for each control and treatment groups, for each timepoint (n=3 colonies), using label-free, mass spectrometry-based proteomic profiling. Peptides were separated over a 2 % to 40 % gradient of acetonitrile on a 500 mm length EasySpray PepMap column, using a 120 minutes' reverse-phase gradient at a flow rate of 250nL min^{-1}. All data were acquired with the mass spectrometer operating in automatic data dependent switching mode. A full MS scan at 140,000 resolution and range of 300–1700 m/z was followed by an MS/MS scan, resolution 17,500 and range of 200–2000 m/z, selecting the 15 most intense ions prior to MS/MS.

3.3.7 Protein identification and abundance level quantitation

Protein identification and LFQ normalisation of MS/MS data was performed using Max-Quant v1.6.5.0 (http://www.maxquant.org) following the general procedures and settings outlined in Hubner *et al.*, (2010). The Andromeda search algorithm (Cox *et al.*, 2011) incorporated in the MaxQuant software was used to correlate MS/MS data against the SWISS-PROT database for *B. terrestris* (UniProt Consortium, 2015)(entries, downloaded May 2017) and a contaminant sequence set provided by Max-Quant. Prior to the proteomic searches the *Bombus* sequence database was modified so that each protein annotation included the homologous Uniprot ID for *Apis mellifera* and *Drosophila melanogaster*. The following search parameters were used: first search peptide tolerance of 20 ppm, second search peptide tolerance 4.5 ppm with cysteine carbamidomethylation as a fixed modification and N-acetylation of protein and oxidation of methionine as variable modification and a maximum of two missed cleavage sites allowed. False Discovery Rates (FDR) were set to 1 % for both peptides and proteins and the FDR was estimated following searches against a target decoy database. LFQ intensities were calculated using the MaxLFQ algorithm (Cox *et al.*, 2014) razor and unique peptides with a minimum ratio count of two peptides across samples. Peptides with a minimum length of seven amino acids were considered for identification and proteins were only considered identified when more than one unique peptide for each protein was observed.

3.3.8 Proteomic data analysis

Perseus v.1.5.5.3 (http://www.biochem.mpg.de/5111810/perseus), was used for data analysis, processing and visualisation. Normalised LFQ intensity values were used as the quantitative measurement of protein abundance for subsequent analysis. The data matrix was first filtered for the removal of contaminants and peptides only identified by site. LFQ intensity values were $\log_2$ transformed and each sample was assigned to its corresponding group (Procedure control 1 h, procedure control 24 h, 10 ng/ml clothianidin 1 h and 10 ng/ml clothianidin 24 h).

Proteins not found in three out of three replicates, in at least one group, were omitted from the analysis. Principal component analysis was performed on each dataset with all replicates. However, individual t-tests were carried out per timepoint. A data imputation step was conducted to replace missing values with values that simulate signals of low abundant proteins chosen randomly from a distribution specified by a downshift of 2.1 times the mean standard deviation (SD) of all measured values and a width of 0.1 times this SD. Student's t-tests were performed in each timepoint to identify statistically significant differently abundant (SSDA) proteins (-log p value $\geq$ 1.3).

Volcano plots were generated in Perseus by plotting the negative log p-values on the y- axis and $\log_2$ fold-difference values on the x-axis for each pair-wise comparison to visualise changes in the total brain proteome.

The Uniprot ID for *Drosophila melanogaster* for all SSDA proteins was extracted from the identifier string and used to query the STRING v 11 database. Medium network specificity value was applied on the analysis and GO term enrichments obtained for biological process, molecular function and cellular compartments were ranked based on number of genes identified in the dataset, in relation to the total number of background genes for that gene ontology (% coverage).

3.4 Results

3.4.1 Toxicological assays and behavioural analysis

In the experiments described here we have assessed the mortality caused by acute exposure to a series of different concentrations of clothianidin and imidacloprid. In addition, the effects both chemicals had on the behaviour of individual worker bees, and their synergistic effects, were determined in experiments using 30 bees per treatment and exposing them acutely, to 35 µl of insecticide solutions, prepared in 50 % w/v sucrose solutions.

Imidacloprid was found to have no direct short term effect on the mortality of bees within the first hours of the experiment (Figure 3.6A). However, at 48 h post exposure, 6 bees were found to have died at the highest dose tested of 35 ng/bee. No other dose of imidacloprid (from 0 to 3.5 ng/bee) caused lethality, for the duration of this experiment, however, differences in behaviour were observed from doses of 0.875 ng/bee and over (Figure 3.7A).

The behavioural assessment was designed based on guidelines by the OECD and US EPA and allowed for the assessment of paralysis and spasm, as the main symptoms observed for either insecticide. The response to imidacloprid principally involved paralysis, which appeared as soon as 15 min after consumption of the dose with the highest concentration. Inactive bees, found in the corners of the test boxes, were observed for every dose, including and above 0.875 ng/bee at least 30 min after consumption of imidacloprid had occurred (Figure 3.7A). Clothianidin triggered super excitability and spasm, that ultimately lead to death from doses of 0.875 ng/bee and above. Both neonicotinoids caused similar response rates at the highest dose of 35 ng/bee (Figure 3.7A and B), with a consistently increasing and similar number of affected bees at 30- and 60-min post exposure, when the maximum number of affected bees were observed. All other concentrations resulted in directly proportional response curves. However, after the 1h time point (within 4 and 24 h) absent or reduced symptoms were observed, mainly for imidacloprid (Figure 3.7A). This recovery observed for imidacloprid was not seen for clothianidin, which conclusively posed a higher risk to *B. terrestris*, when only mortality is taken into consideration. The approximate LD50 for clothianidin was 1.75– 2.45 ng/bee. However, no LD50 for imidacloprid could be determined. The incidence of paralysis is the principal response to imidacloprid, whereas an increase in uncoordinated movement, leading to death, is primarily observed in response to clothianidin (discussed in further detail below).

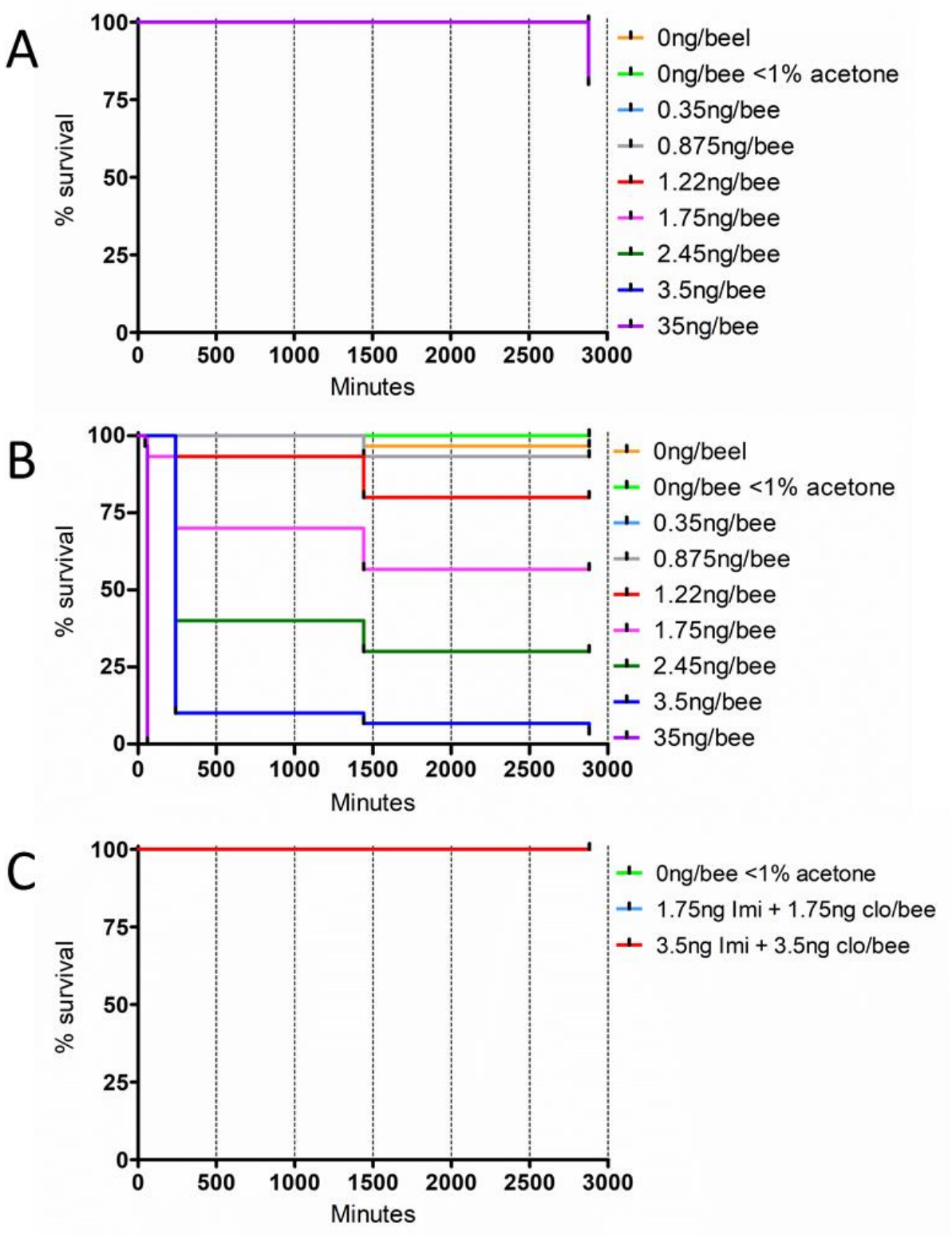


Figure 3.6 Survivability curves based on the number of dead bees found per timepoint and treatment. (A) imidacloprid, (B) clothianidin, (C) synergistic results. Thirty bees were used for each concentration tested, measurements took place at 15, 30, 45, 60, 240, 1440- and 2880-min post exposure. Our results demonstrate that clothianidin is more toxic than imidacloprid to bumblebees, having an estimated LD50 of 1.75– 2.45 ng/bee, whereas the LD50 for imidacloprid could not be determined. When neonicotinoids were applied together no mortality was recorded.

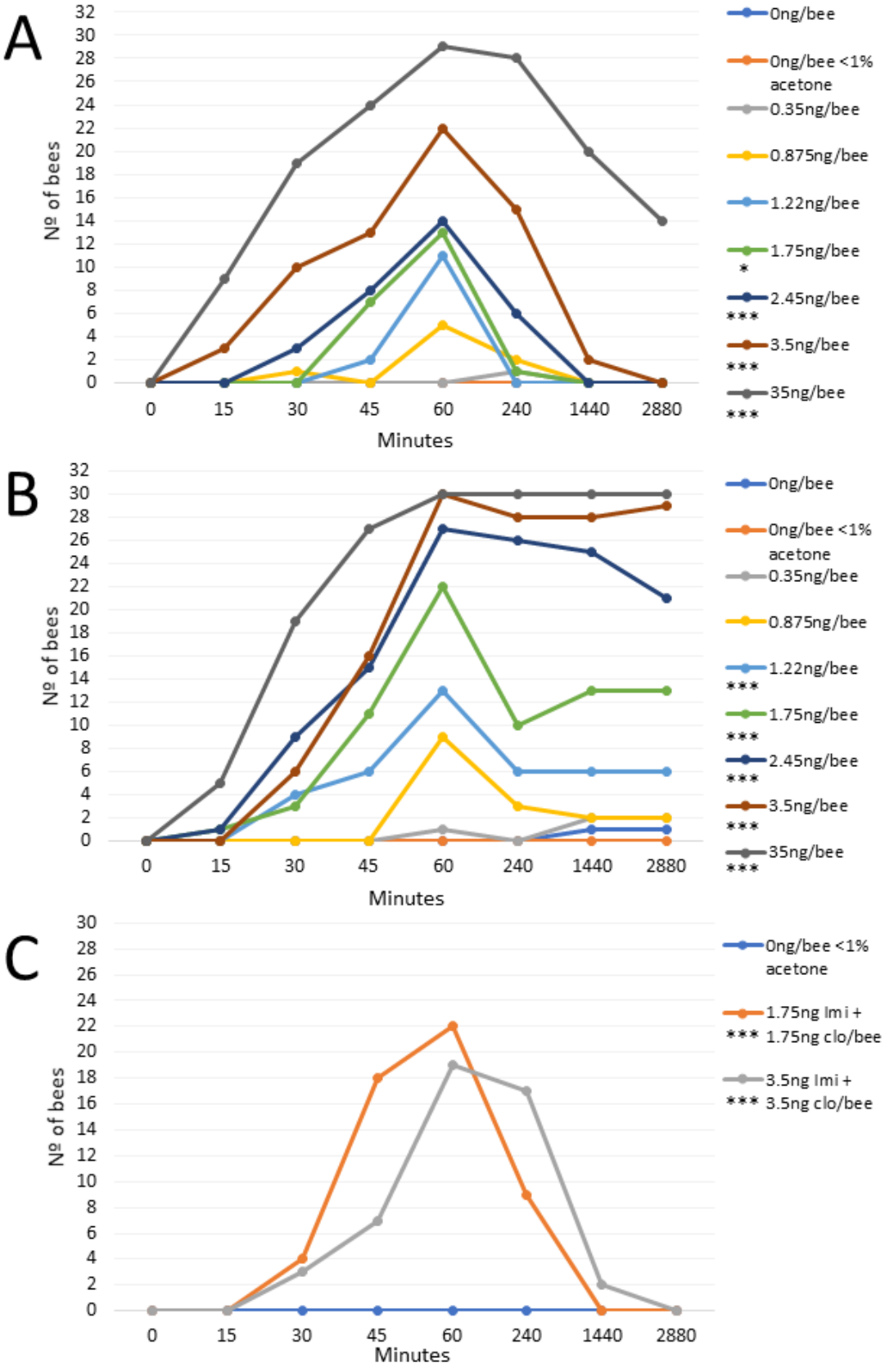


Figure 3.7 Symptoms incidence analysis per timepoint and insecticide. The number of bees displaying specific symptoms in response to (A) imidacloprid, (B) clothianidin and (C) combined pesticide exposure over a period of 48 hours. Any abnormal behaviour was described as either a positive or negative alteration and analysed as a unique category quantitatively, applying a Kruskal Wallis test and a Dunn`s multiple comparison post hoc test (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$.

When exposed to both neonicotinoids at the same time (Figure 3.7C), a synergistic effect was not observed. At the first dose tested of 1.75 ng of each insecticide per bee, the symptom curve obtained was similar to that obtained for clothianidin for the same concentration, with the same number of bees showing symptoms at 60 min (Figure 3.7B) but with an absence in mortality. When the dose was doubled to 3.5 ng/bee for each neonicotinoid, fewer bees showed symptoms, contradicting the hypothesis of directly proportional response, and no mortality was recorded at 48 h.

3.4.2 Assessment of synapsin levels in the bumblebee brain

To gain insight into the impact of the nAChR acting insecticides clothianidin and imidacloprid, on the brain of *B. terrestris*, synapsin levels were assessed in 3 different areas of interest in the bumblebee brain, the mushroom body, the pedunculus and the antennal lobes, using confocal fluorescent microscopy (Figure 3.8). A primary antibody for synapsin was purchased from the Developmental Studies Hybridoma Bank of the University of Iowa and the secondary antibody Alexa 594 (Invitrogen) used for detection was kindly provided by Dr Martina Schroeder (Maynooth University). Bee brains were processed for confocal microscopy 4h after exposure to 2.5 ng/ml and 10 ng/ml of clothianidin or imidacloprid, resulting in a lower and higher total exposure level of 0.0875 and 0.35 ng/bee, respectively, both doses of field relevance. Three measurements were taken in the mushroom body region (lip, collar and basal ring), two at the pedunculus region of the brain and three around the antennal lobes. In total three individuals were analysed per treatment group and 3 different sections were assessed in each, resulting in three biological replicates (1, 2 and 3) and 3 technical replicates (a, b and c) per analysis, giving a total of 360 measurements.

Synapsin was found to be distributed widely throughout the bumblebee brain (Figure 3.9A) but was more concentrated in areas of dense neuronal fibres, and synaptic processing such as the calices (C) in the mushroom bodies, the medulla (M), the lobulla (L) and the antennal lobes (AL). In the other parts of the brain, synapsin was also abundantly distributed, mainly around neurons that connect the medulla and the retina (Figure 3.9E) and around the nucleus of the Kenyon cells (Figure 3.9F).

When exposed to both neonicotinoids, synapsin levels in the brain varied mostly in response to imidacloprid. Synapsin levels increased significantly in imidacloprid exposed bees for both doses tested (0.0875 ng/bee and 0.35 ng/bee), with small differences found between them (Figure 3.10), in all brain regions assessed. The greatest increase was found

at the pedunculus region when in comparison with the controls (Table 3.2 and Figure 3.8), and no statistically significant differences were observed for synapsin levels between the controls and the bees exposed to clothianidin.

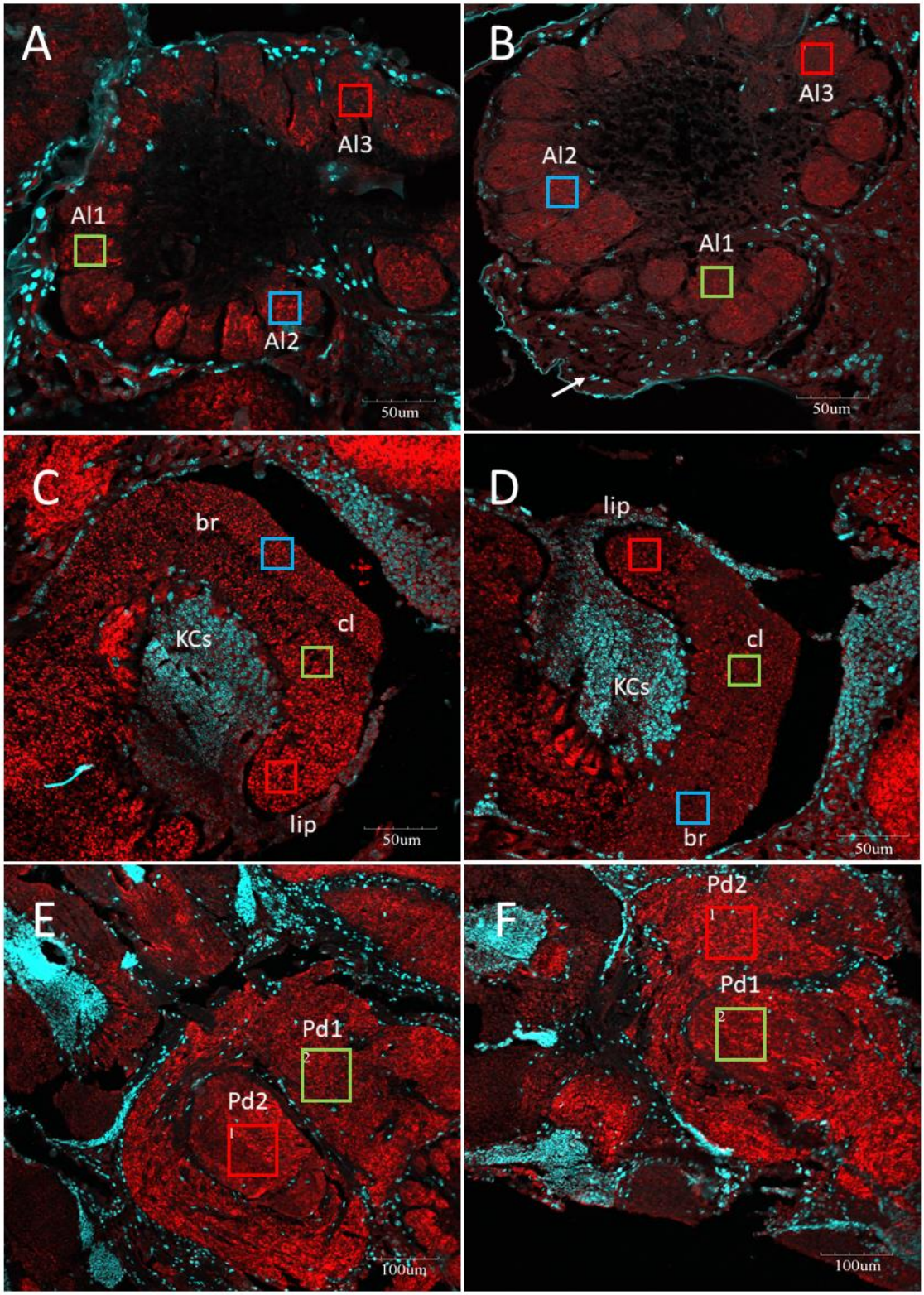


Figure 3.8 Confocal microscopy of data acquisition points of interest for each of the neuropil regions assessed. (A-B) Antennal Lobes Al1, Al2 and Al3, (C-D) Mushroom bodies, KCs = Kenyon cells, lip, cl = collar and br = basal ring, (E-F) Pedunculus, Pd1 and Pd2. Each coloured box represents an area where measurements were taken. Left and right panels are different examples of histological sections used to collect measurements. Synapsin (stained in red with AlexaFluor 594) was found to have significantly increased abundance in all three different areas of the brain, post exposure to both concentrations of imidacloprid but not clothianidin. Nucleus staining in light blue (DAPI).

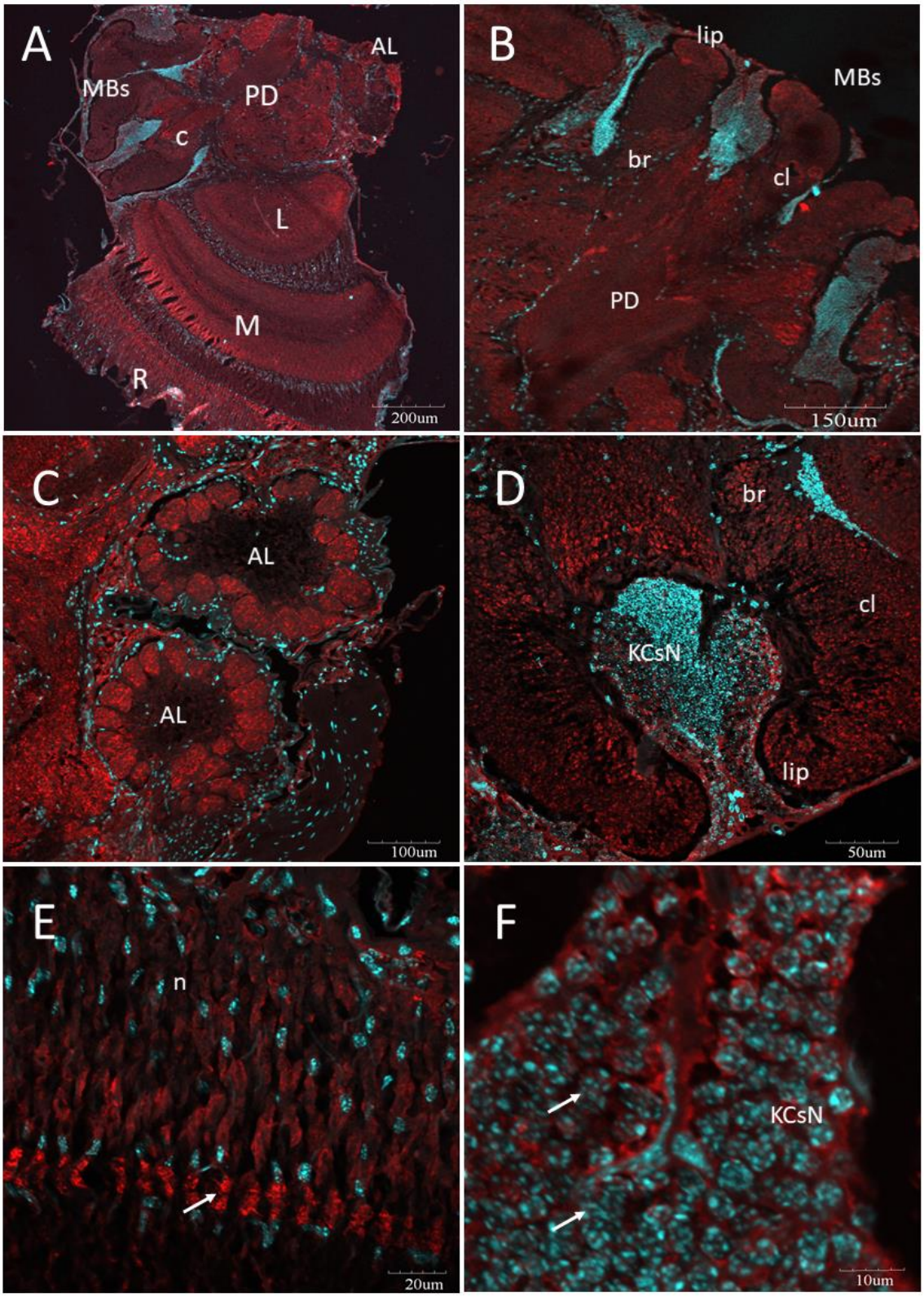


Figure 3.9 Confocal microscopy of synapsin distribution throughout the different areas of the bumblebee brain. (A) Sections containing the left side of the brain highlighting the main areas of neuronal information processing; MBs = mushroom bodies, c = calyx, M = medulla, L = lobula, PD = pedunculus, Al = antennal lobes. (B) close up view of pedunculus and the calices in the mushroom body, comprised of the lips, the br = basal rings and the cl = collar. (C) Antennal lobes. (D) Lateral calyx showing a population of kenyon cells in the centre, KCsN = kenyon cells nucleus. (E) probable inter and sensory neurons, n = cell nucleus, arrow showing higher levels of synapsin between different neurons. (F) KCs nucleus concentrated at the mushroom body, arrows showing condensed chromatin in the nucleus. Red = synapsin staining with Alexa 495, blue = DAPI staining of the genetic material.

Table 3.2 Confocal Fluorescent measurements taken for synapsin levels at all neuropil areas of interest in the bumblebee brain.

Samples	Lip	Collar	Basal_Ring	Pedunculus_1	Pedunculus_2	Al_1	Al_2	Al_3
PC_1_a	451.126	671.097	590.545	839.258	839.833	451.126	671.097	590.545
PC_1_b	693.813	623.221	663.651	753.215	827.459	693.813	623.221	663.651
PC_1_c	672.987	648.57	534.665	644.258	543.29	672.987	648.57	534.665
PC_2_a	877.239	908.593	899.345	633.326	735.643	877.239	908.593	899.345
PC_2_b	828.452	753.01	589.985	564.409	631.082	828.452	753.01	589.985
PC_2_c	904.085	860.112	849.136	920.849	885.264	904.085	860.112	849.136
PC_3_a	492.273	524.083	400.277	685.154	696.887	492.273	524.083	400.277
PC_3_b	411.192	411.678	444.24	854.323	890.115	411.192	411.678	444.24
PC_3_c	680.368	737.806	933.397	719.011	819.033	680.368	737.806	933.397
IMI_2.5_1_a	1220.281	1192.809	1313.004	1535.454	1281.72	1220.281	1192.809	1313.004
IMI_2.5_1_b	933.29	1057.645	1065.415	1345.737	1404.634	933.29	1057.645	1065.415
IMI_2.5_1_c	1252.228	939.294	1293.325	1847.342	1436.398	1252.228	939.294	1293.325
IMI_2.5_2_a	1208.96	1510.428	1137.28	1760.868	1826.399	1208.96	1510.428	1137.28
IMI_2.5_2_b	1247.898	1410.724	1643.13	1389.408	2057.538	1247.898	1410.724	1643.13
IMI_2.5_2_c	874.322	1063.821	967.347	1913.544	1111.583	874.322	1063.821	967.347
IMI_2.5_3_a	874.672	789.314	768.935	1757.669	1521.216	874.672	789.314	768.935
IMI_2.5_3_b	913.491	786.699	921.884	1737.931	1242.661	913.491	786.699	921.884
IMI_2.5_3_c	976.904	1206.466	891.321	1661.293	1670.728	976.904	1206.466	891.321
IMI_10_1_a	1349.949	1162.146	1087.532	2119.445	1823.065	1349.949	1162.146	1087.532
IMI_10_1_b	1163.528	1096.479	1001.103	1471.296	1383.147	1163.528	1096.479	1001.103
IMI_10_1_c	1313.776	1351.028	865.743	1684.956	1841.704	1313.776	1351.028	865.743
IMI_10_2_a	1512.66	1185.708	1415.836	1760.868	1826.399	1512.66	1185.708	1415.836
IMI_10_2_b	1039.065	1138.18	1115.884	1029.289	1335.684	1039.065	1138.18	1115.884
IMI_10_2_c	1502.693	1143.017	1283.196	1691.222	1447.805	1502.693	1143.017	1283.196
IMI_10_3_a	847.525	1315.554	569.556	1262.133	1475.898	847.525	1315.554	569.556
IMI_10_3_b	1348.688	1360.887	1128.937	2227.885	2040.6	1348.688	1360.887	1128.937
IMI_10_3_c	916.538	1314.317	1555.871	1879.395	2099.891	916.538	1314.317	1555.871
CLO_2.5_1_a	741.16	724.152	760.847	1005.969	849.222	857.477	656.312	880.775
CLO_2.5_1_b	590.18	471.634	436.63	1211.051	1205.58	694.284	500.987	630.847
CLO_2.5_1_c	395.088	387.202	405.428	1471.284	1646.572	630.218	658.812	721.193
CLO_2.5_2_a	558.835	541.229	563.57	630.121	620.697	546.973	390.473	573.089
CLO_2.5_2_b	519.778	526.499	671.022	637.365	570.28	500.624	523.058	792.779
CLO_2.5_2_c	581.14	492.637	423.736	426.907	486.632	508.745	597.795	724.453
CLO_2.5_3_a	462.019	338.396	522.306	1115.323	1169.553	517.806	451.982	524.686
CLO_2.5_3_b	419.126	478.84	418.154	1295.745	1097.175	927.292	749.066	643.582
CLO_2.5_3_c	687.899	531.24	648.686	832.555	783.998	604.566	861.999	587.278
CLO_10_1_a	715.315	617.181	582.595	733.549	715.536	416.044	444.296	417.098
CLO_10_1_b	486.294	539.344	775.678	839.374	842.952	588.309	689.63	698.071
CLO_10_1_c	1071.169	902.096	1002.29	865.258	540.085	525.126	680.765	543.927
CLO_10_2_a	972.364	595.329	728.358	1244.865	1073.44	1231.936	1367.992	1238.371
CLO_10_2_b	946.097	664.087	1114.589	796.864	507.696	689.68	738.555	755.478
CLO_10_2_c	405.974	387.233	596.608	1413.017	1140.35	751.038	749.046	792.529
CLO_10_3_a	386.409	330.771	313.236	921.638	953.583	565.406	534.104	514.875
CLO_10_3_b	297.327	269.707	409.081	1044.34	985.346	571.501	522.189	559.046
CLO_10_3_c	630.19	514.48	784.606	1072.825	928.194	752.74	672.695	518.993

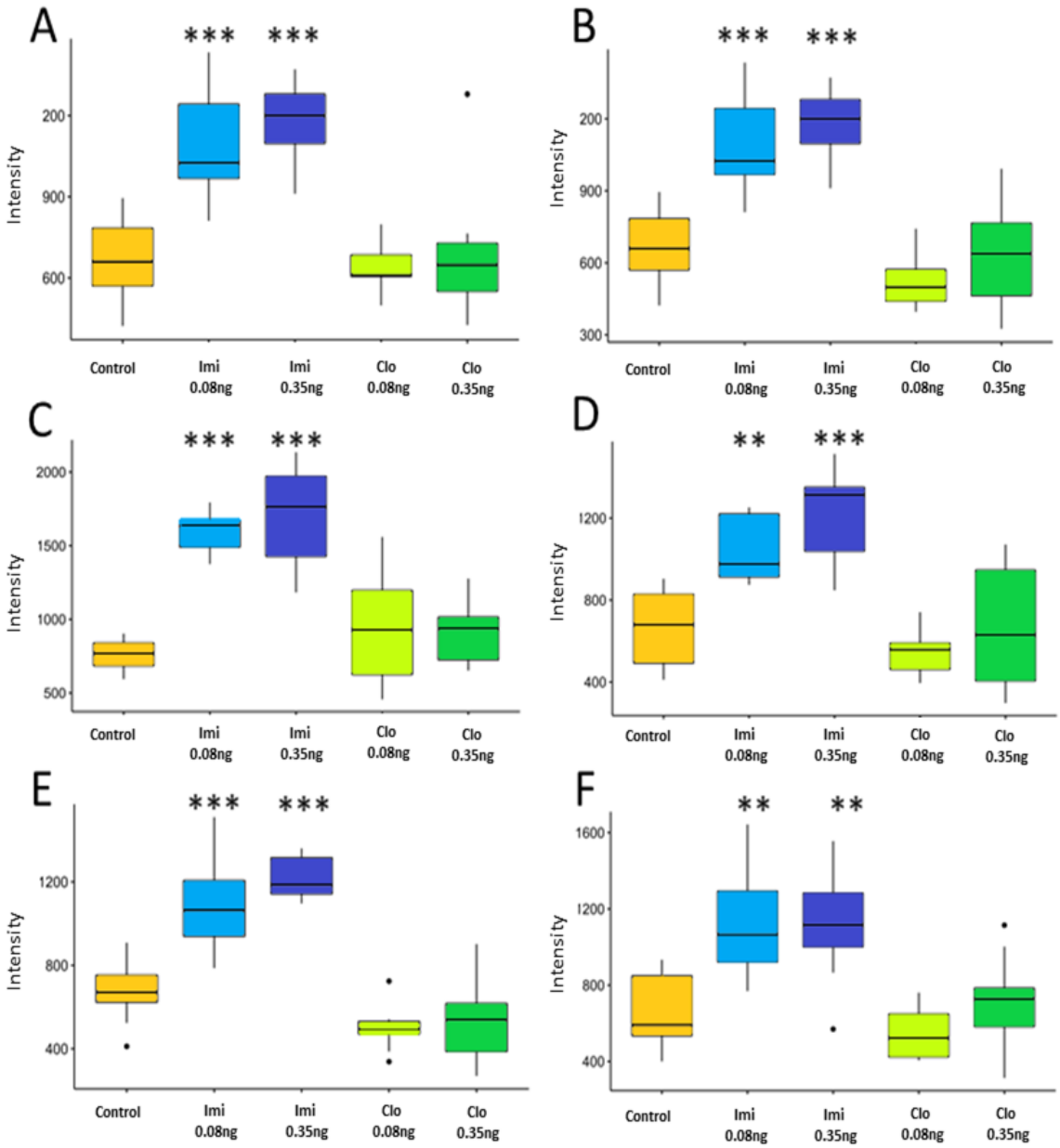


Figure 3.10 Confocal fluorescent intensity measurements for synapsin levels in 6 different regions of the bumblebee brain, (A) Antennal lobe, (B) Mushroom body, (C) Pedunculus, (D) Lip region in the calyx, (E) Collar region in the calyx, (F) Basal ring in the calyx. Synapsin levels significantly increased in the antennal lobes, in all three regions of the mushroom body (lip, collar and basal ring) and in the pedunculus post exposure to imidacloprid. No statistically significant changes were found between control and clothianidin exposed bees. (**) $p \leq 0.01$, (***) $p \leq 0.001$.

3.4.3 Characterisation of the bee brain proteome in response to acute clothianidin exposure

To further investigate the molecular impact of the nAChR acting neonicotinoid clothianidin on the brain proteome of *B. terrestris*, bees were exposed orally to 35 µl solutions of 2.5 ppb (2.5 ng/ml) and 10 ppb (10 ng/ml) of the insecticide in a 50% sucrose

solution (w/v) and compared to bees exposed only to a procedural control of sucrose solution containing < 1% acetone. The selected doses are within the range of field relevance (hereafter the low and high doses of field relevance). After acute exposure to each, the final exposure dose was calculated to be 0.0875 ng/bee for the low dose and 0.35 ng/bee for the high dose. No mortality was recorded, and bee brains were then dissected at 1, 4 and 24 h post exposure. LFQ MS analyses (120 min) were performed on three biological replicates per group (n=18) resulting in the initial identification of 3,047 proteins.

A principal component analysis (PCA) for all proteins identified in all 3 replicates of at least one group did not resolve clear differences between control and treatments (Figure 3.11). The PCA indicates that the treatment did not cause changes in the majority of the brain proteome, suggesting a localised pesticide effect.

Each dose/timepoint was then analysed individually. Proteins not found in all 3 replicates of either, the control or treatments, were omitted, resulting in 1,280, 1,258 and 1,246 proteins in the 1, 4 and 24h timepoints respectively, for the low dose exposure. After exposure to the high dose 1,297, 1,277 and 1,359 proteins remained after relevant filtering steps for the 1, 4 and 24 h timepoints, respectively.

Two sample (treatment vs control) Student's t-tests carried out for all six data sets revealed statistically significant differently abundant (SSDA) proteins in all treatments for each timepoint, which were then visualised through volcano plots (Figure 3.12). Eighty-nine (89), 129 and 78 SSDA proteins (-log p-value ≥ 1.3, equivalent to a p-value ≤0.05) were identified for the groups of bees exposed to 0.0875 ng at 1, 4 and 24 h respectively and 48, 90 and 173 SSDAs for the bees exposed to 0.35 ng/bee at 1, 4 and 24 h (Figure 3.12).

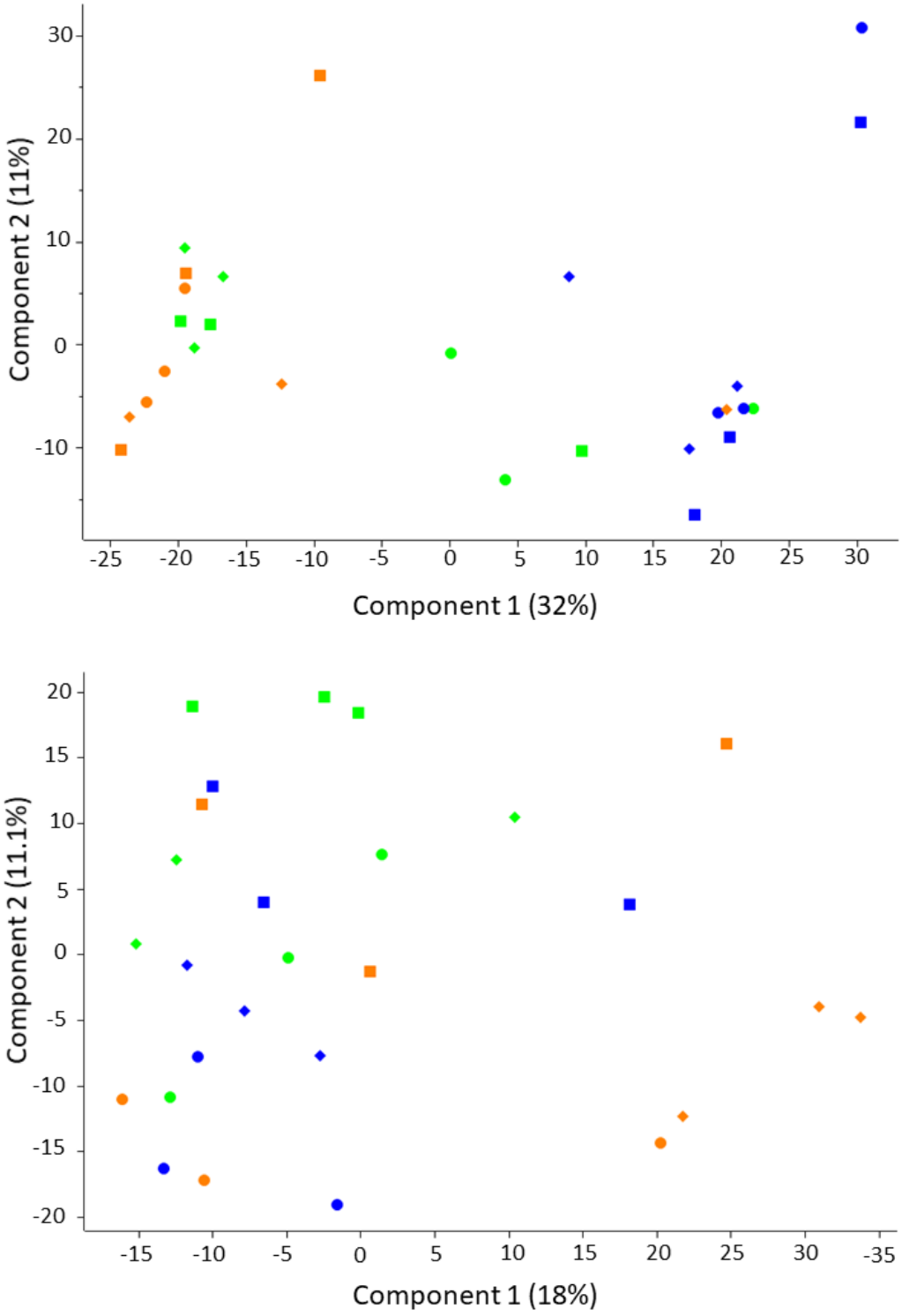


Figure 3.11 Principal component analysis utilizing the Benjamin-Hochberg FDR cut off 0.05 in all identified proteins per experiment. Colours refer to treatment and shapes to timepoints. Green = control, Blue 0.0875 ng/bee, Orange 0.35 ng/bee. Filled Square =1 h, Filled diamond =4 h and Filled circle = 24 h. (Top) clothianidin experiment, (Bottom) imidacloprid experiment. No clear separation between treatments were obtained in either experiment, suggesting that neither clothianidin or imidacloprid causes changes affecting the whole brain proteome of *B. terrestris.*

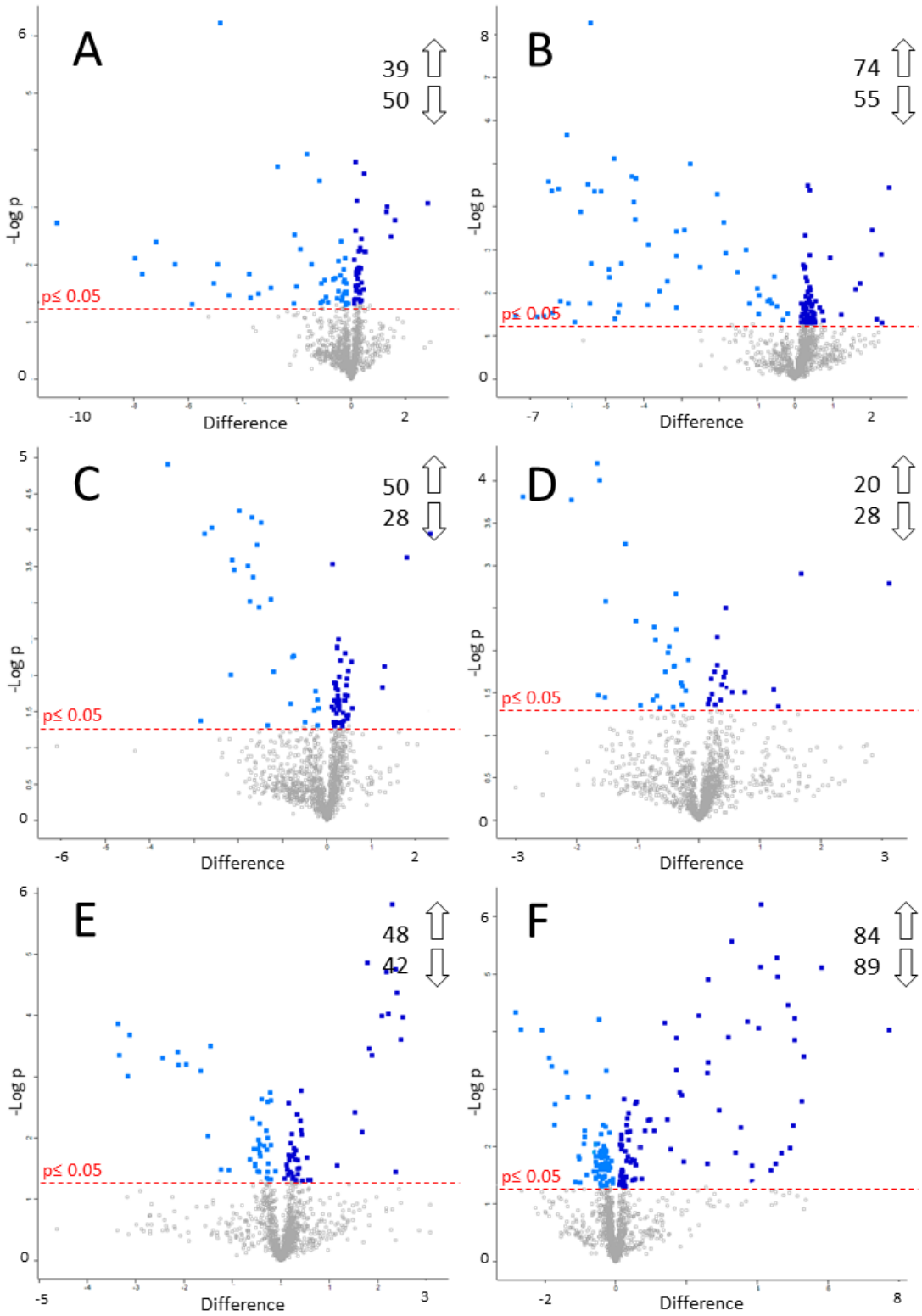


Figure 3.12 Clothianidin Volcano plots derived from pairwise Student t-tests showing patterns of expression of individual proteins in the total brain proteome and SSDA proteins expression ($p \leq 0.05$, red dotted lines). (A) 0.0875 ng/bee 1 h, (B) 0.0875 ng/bee 4 h, (C), 0.0875 ng/bee 24 h, (D) 0.35 ng/bee 1 h, (E) 0.35 ng/bee 4 h, (F) 0.35 ng/bee 24 h. Arrows show number of increased and decreased abundant SSDA proteins. Light blue is used to highlight decreased abundance SSDA protein distribution and dark blue to highlight increased abundance SSDA protein distribution. -log p: -log 10 of the p-value, Difference: $\log_2$ fold difference.

All SSDA proteins are reported in Appendix 3, with the top ten proteins with increased and decreased abundance in each timepoint presented at Table 3.3. At the 1h timepoint, the low dose resulted in increased abundance of a series of proteins including uridine diphosphate glucose pyrophosphatase (XP_003395627.1) with a relative fold change (RFC) of 7 and MAP kinase-activating death domain protein (XP_012172769.1, RFC 3) as the top proteins with increased abundance. Further proteins in the list seem to be associated with signalling regulatory pathways and are displayed in Table 3.3 and in the Appendix 3. Proteins with the greatest decreased abundance for the same dose and timepoint include a myosin heavy chain protein (XP_012166980.1, RFC 1,792) and the tropomyosin-1 (XP_003400287.1, RFC 244). Other structural proteins with similar relative fold changes were also present among the top 10 proteins with decreased abundance, suggesting that at this timepoint a series of structural members were affected (Table 3.3 1h decreased).

At 4 h, the response to the low dose of clothianidin had the protein alpha-tubulin N-acetyltransferase (XP_012166088.1, RFC 5.6) as the protein with most increased abundance and the protein "arginine N-methyltransferase" (XP_003395508.1, RFC 4.96) as the second. Whereas Myosin heavy chain (XP_012166980.1, RFC 166) and tropomyosin-1 (XP_003400287.1, RFC 111) were again the top proteins with decreased abundance at 4h.

At 24 h post exposure, the top proteins with increased abundance were 1-acyl-sn-glycerol-3-phosphate acyltransferase gamma (XP_003395337.1, RFC 5) and regulating synaptic membrane exocytosis protein 1 (XP_012166309.1, RFC 3.5). This time, no structural proteins were found among the top 10 proteins with decreased abundance, instead, the top protein was the V-type proton ATPase subunit e (XP_003401206.1, RFC 12), followed by the acyl-CoA-binding protein (XP_012173041.1, RFC 7.2) (Table 3.3).

In response to the higher dose exposure the protein hsp90 co-chaperone Cdc37 (XP_003394087.2, RFC 8.6) was the top protein with increased abundance at 1 h followed by the MAP kinase-activating death domain protein isoform X1 (XP_012172769.1, RFC 3.2) whereas the protein plasminogen activator inhibitor 1 RNA-binding protein (XP_012166473.1, RFC 7.3), was the top protein with reduced abundance followed by the putative cysteine proteinase CG12163 protein (XP_012163961.1, RFC 4.2).

At 4 h the xaa-Pro aminopeptidase 1 protein (XP_012172282.1, RFC 5.7) was the most increased, followed by programmed cell death protein 5 (XP_003403349.1, RFC

5.5). Whereas the top reduced proteins were tubulin alpha-1C chain (XP_003394986.1, RFC 10.3) and the protein putative fatty acyl-CoA reductase CG8306 (XP_020721715.1, 10.1) with the second most decreased abundance.

At 24 h post exposure the top proteins with increased abundance were the endocuticle structural glycoprotein SgAbd-8 (XP_012172250.1, RFC 211) and the PDZ and LIM domain protein 3 isoform X1 (XP_003398527.1, RFC 56). The protein basic-leucine zipper transcription factor A (XP_012172905.1, RFC 7) and protein RER1 isoform X1 (XP_003394726.2, RFC 6.4), were the top 2 proteins with reduced abundance at 24 h post exposure to 0.35 ng of clothianidin. The top 10 most differential abundant proteins are provided in table 3.4 and the complete list of SSDA is available at appendix 3.

Table 3.3 The top 10 statistically significant (Student's t-tests, $p<0.05$) differentially abundant proteins with increased and decreased abundance based on the LFQ intensities of all proteins identified post-acute exposure to 0.0875ng per bee of clothianidin after 1, 4 and 24h against their respective controls.

	Top 10 Upregulated proteins			Top 10 Downregulated proteins		
	Fold change	Protein name	Protein Id	Fold change	Protein name	Protein Id
1H	7.0	Uridine diphosphate glucose pyrophosphatase	XP_003395627.1	1794.0	Myosin heavy chain	XP_012166980.1
	3.0	MAP kinase-activating death domain protein isoform X1	XP_012172769.1	245.0	Tropomyosin-1	XP_003400287.1
	2.7	Heterogeneous nuclear ribonucleoprotein H	XP_012170365.1	203.7	Paramyosin, long form	XP_012164684.1
	2.4	Glutamine-dependent NAD(+) synthetase	XP_003399614.1	145.3	Myosin regulatory light chain 2	XP_012172352.1
	2.4	60S ribosomal protein L44	XP_003402402.1	88.5	Myosin light chain alkali isoform X1	XP_012172045.1
	1.4	Sodium- and chloride-dependent GABA transporter 1	XP_012164960.1	57.1	Troponin T isoform X1	XP_012164541.1
	1.3	Adenylate cyclase type 8	XP_012168116.1	33.2	Troponin I isoform X1	XP_012173783.1
	1.3	Uncharacterized protein LOC100644414	XP_003399549.1	29.9	Myophilin	XP_003401260.1
	1.3	Guanine nucleotide-binding protein G(s) subunit alpha	XP_003402502.1	27.8	Cytochrome b-c1 complex subunit 6, mitochondrial	XP_003399309.1
	1.2	Arylsulfatase B	XP_003401982.1	22.6	Muscle-specific protein 20	XP_012172948.1
4H	5.6	Alpha-tubulin N-acetyltransferase	XP_012166088.1	166.2	Myosin heavy chain	XP_012166980.1
	4.9	Protein arginine N-methyltransferase 1	XP_003395508.1	111.8	Tropomyosin-1	XP_003400287.1
	4.8	Ras-specific guanine nucleotide-releasing factor 1	XP_012166363.1	99.4	Paramyosin, long form	XP_012164684.1
	4.4	Putative ATP-dependent RNA helicase me31b	XP_003402776.1	91.7	Endocuticle structural glycoprotein SgAbd-8	XP_012172250.1
	4.1	BAG domain-containing protein Samui	XP_003400908.1	86.2	Myophilin	XP_003401260.1
	3.3	Protein alan shepard	XP_012176551.1	84.9	Troponin T isoform X	XP_012164541.1
	3.0	U6 snRNA-associated Sm-like protein LSm8	XP_003401541.1	76.1	Endocuticle structural glycoprotein SgAbd-1	XP_012172264.1
	2.3	Glycine-rich cell wall structural protein 1	XP_020718419.1	73.9	Myosin regulatory light chain 2	XP_012172352.1
	1.9	Cuticle protein 38	XP_012171151.1	65.2	Uncharacterized protein LOC100644880	XP_003394697.1
	1.6	Protein Mo25 isoform X1	XP_003393299.1	63.7	Uncharacterized protein LOC100647860 isoform X1	XP_003398046.1
24H	5.0	1-acyl-sn-glycerol-3-phosphate acyltransferase gamma	XP_003395337.1	12.0	V-type proton ATPase subunit e	XP_003401206.1
	3.5	Regulating synaptic membrane exocytosis protein 1	XP_012166309.1	7.1	Acyl-CoA-binding protein	XP_012173041.1
	2.4	Electrogenic sodium bicarbonate cotransporter 1 isoform X7	XP_020718807.1	6.7	Vacuolar protein sorting-associated protein 29	XP_003402853.1
	2.3	Uncharacterized protein LOC100649546 isoform X1	XP_003399192.1	6.0	Uncharacterized protein LOC100645788 isoform X1	XP_003393207.1
	1.4	Limbic system-associated membrane protein	XP_003393801.1	4.4	Lipid storage droplets surface-binding protein 2	XP_012166752.1
	1.4	Ubiquitin-protein ligase E3A isoform X1	XP_003395884.1	4.4	Mitochondrial import receptor subunit TOM20 homolog	XP_003394034.1
	1.3	Ras-related protein Rab-39B	XP_012169444.1	4.2	Histidine triad nucleotide-binding protein 3	XP_003397417.1
	1.3	Ras GTPase-activating protein-binding protein 2	XP_003403259.1	3.9	Uncharacterized protein LOC110120215	XP_020723823.1
	1.3	Quinone oxidoreductase	XP_003401635.1	3.4	Probable glutamine--tRNA ligase isoform X1	XP_012176427.1
	1.3	Uncharacterized protein LOC100642734 isoform X1	XP_020721142.1	3.3	UPF0554 protein C2orf43 homolog	XP_012165951.1

Table 3.4 The top 10 statistically significant (Student's t-tests, $p<0.05$) differentially abundant proteins with increased and decreased abundance based on the LFQ intensities of all proteins identified post-acute exposure to 0.35ng per bee of clothianidin after 1, 4 and 24h against their respective controls.

	Top 10 Upregulated proteins			Top 10 Downregulated proteins		
	Fold change	**Protein name**	**Protein Id**	**Fold change**	**Protein name**	**Protein Id**
1H	8.6	Hsp90 co-chaperone Cdc37	XP_003394087.2	7.3	Plasminogen activator inhibitor 1 RNA-binding protein	XP_012166473.1
	3.1	MAP kinase-activating death domain protein isoform X1	XP_012172769.1	4.2	Putative cysteine proteinase CG12163	XP_012163961.1
	2.4	Alpha-tocopherol transfer protein	XP_012168727.1	3.1	Glycogen [starch] synthase	XP_003399733.1
	2.3	Protein lethal(2)essential for life	XP_012170000.1	3.1	Gamma-aminobutyric acid receptor-associated protein	XP_003400687.1
	1.6	Ras-related protein Rab-35 isoform X1	XP_012173927.1	3.0	Division abnormally delayed protein	XP_003394661.1
	1.4	Serine/threonine-protein phosphat 2A catalytic sub α isoform	XP_003394796.1	2.9	Inositol polyphosphate 1-phosphatase	XP_012168781.1
	1.3	Sorbitol dehydrogenase	XP_003402903.1	2.8	Multifunctional methyltransferase subunit TRM112	XP_012165415.1
	1.3	Myc box-dependent-interacting protein 1	XP_012174292.1	2.3	Uncharacterized protein LOC100646420 isoform X1	XP_020719731.1
	1.3	Proteasome subunit beta type-3	XP_003400568.1	2.0	Exportin-1	XP_003393170.1
	1.3	Alpha-tocopherol transfer protein	XP_003399181.1	1.9	Galactokinase	XP_003396417.1
4H	5.7	Xaa-Pro aminopeptidase 1	XP_012172282.1	10.3	Tubulin alpha-1C chain	XP_003394986.1
	5.5	Programmed cell death protein 5	XP_003403349.1	10.1	Putative fatty acyl-CoA reductase CG8306	XP_020721715.1
	5.2	Alpha-tubulin N-acetyltransferase	XP_012166088.1	8.9	L-lactate dehydrogenase	XP_003396770.1
	5.1	Alcohol dehydrogenase class-3	XP_003395091.1	8.7	Cytochrome P450 9e2	XP_003393377.1
	5.1	Putative ATP-dependent RNA helicase me31b	XP_003402776.1	5.4	Peptidyl-prolyl cis-trans isomerase 6	XP_003400106.1
	4.9	Cytoplasmic dynein 1 light intermediate chain 2	XP_003402247.1	4.3	Protein transport protein sec31	XP_020721697.1
	4.6	UPF0587 protein C1orf123 homolog	XP_020720608.1	4.3	Protein lap4 isoform X1	XP_012163535.1
	4.5	Protein singed	XP_003402078.1	3.8	TBC1 domain family member 24	XP_012174561.1
	4.2	Histidine triad nucleotide-binding protein 3	XP_003397417.1	3.1	Glutamate receptor 1	XP_012166058.1
	3.6	Isoleucine--tRNA ligase, cytoplasmic	XP_012163547.1	2.8	Ca-transporting ATPase reticulum type	XP_012170503.1
24H	211.0	Endocuticle structural glycoprotein SgAbd-8	XP_012172250.1	7.0	Basic-leucine zipper transcription factor A	XP_012172905.1
	56.2	PDZ and LIM domain protein 3	XP_003398527.1	6.4	Protein RER1 isoform X1	XP_003394726.2
	39.8	Uncharacterized protein LOC100642610	XP_012168226.1	4.2	Endothelin-converting enzyme 1	XP_012171205.1
	38.5	Flexible cuticle protein 12	XP_012175318.1	3.6	Transmembrane protein 53	XP_003402308.1
	33.4	Muscle M-line assembly protein unc-89	XP_012163928.1	3.5	Gamma-aminobutyric acid receptor subunit beta isoform X1	XP_020719229.1
	33.1	Troponin C	XP_012169552.1	3.3	Uncharacterized protein LOC100649516	XP_012172432.1
	32.4	Uncharacterized protein LOC100644880	XP_003394697.1	3.2	Small nuclear ribonucleoprotein F	XP_003398400.1
	30.4	Titin	XP_020719538.1	2.6	Protoporphyrinogen oxidase	XP_003398826.1
	29.2	Uncharacterized protein LOC100646546	XP_003398034.1	2.5	Ras-related protein Rab-8A	XP_012165099.1
	25.6	Twitchin	XP_020721448.1	2.1	Glycine receptor subunit alpha-3	XP_003393576.1

Functional annotation analysis was conducted using the STRING platform v 11.0 (https://string-db.org). Lists of increased and decreased SSDA proteins were analysed individually per timepoint, as a way of visualizing the molecular and biological processes, as well as the main cellular components, being influenced by the response to acute exposure to clothianidin over the course of 24 h. These results are presented in Table 3.5 with the top 5 GO terms enriched for each list of SSDA proteins ranked based on % coverage of the term. The full list resulting from STRING is given in Appendix 3.

The determination and visualization of the GOs provided functional information on the biochemical processes influenced in the *B. terrestris* brain post exposure to clothianidin. At 1 h post exposure to 0.0875 ng/bee, the top 5 GOs enriched from the SSDA proteins with increased abundance, include the biological process response to trehalose (GO:0010353) and four molecular function processes including the glutamate:sodium symporter activity (GO:0015501); amino acid:sodium symporter activity (GO:0005283); acidic amino acid transmembrane transporter activity (GO:0015172) and G-protein beta/gamma-subunit complex binding (GO:0031683), all with GO % coverage being higher or equal to 25 % (Table 3.5). The enrichments from the list of SSDA with reduced abundance at the 1h timepoint contained 135 GO terms, the top 5 of which were all cellular components include; the troponin complex (GO:0005861), the presynaptic periactive zone (GO:0036062), the extracellular matrix component (GO:0044420), the striated muscle thin filament (GO:0005865) and the basement membrane (GO:0005604), all with GO % coverage above 38 % as per table 3.5.

At 4 h the top 5 enriched terms from proteins of increased abundance, were the cellular component myosin V complex (GO:0031475), the molecular function (MF) tropomyosin binding (GO:0005523), the cellular component (CC) AP-2 adaptor complex (GO:0030122), the biological process (BP) regulation of short-term neuronal synaptic plasticity (GO:0048172) and the cellular component vacuolar proton-transporting V-type ATPase, V1 domain (GO:0000221). While enrichments obtained from the decreased abundance SSDA proteins list were again mainly structural terms associated with the troponin complex (GO:0005861, CC), muscle thin filament tropomyosin (GO:0005862, CC), muscle alpha-actinin binding (GO:0051371, MF), muscle myosin complex (GO:0005859, CC) and striated muscle thin filament (GO:0005865, CC), which were all cellular components with the exception of the muscle alpha-actinin binding which is categorised as molecular function.

At 24 h, the top 5 enriched terms from the list of increased abundance SSDA proteins were; the long-term strengthening of neuromuscular junction (GO:0042062, BP), Spectrin (GO:0008091, CC), NADH metabolic process (GO:0006734,BP), spectrosome (GO:0045170, CC) and cytoskeleton of presynaptic active zone (GO:0048788, CC). Whereas the top 5 from the decreased abundance SSDA proteins list were mainly molecular functions, such as the ligand-gated anion channel activity (GO:0099095, MF), chloride channel activity (GO:0005254, MF), and transmitter-gated channel activity (GO:0022835, MF) and the neurotransmitter receptor complex (GO:0098878,CC).

These results demonstrate that at the low dose, clothianidin affects the proteome of bumblebee brains, initially interfering with intracellular transporters and structural proteins that may be responsible for the rearrangement of cellular structures required for optimum neuronal functioning. Alterations in the metabolism of trehalose and functioning of receptors and transporters seem impaired at 1 h. This develops into the upregulation of short term neuronal synaptic plasticity, in association with myosin V and tropomyosin binding, along with the downregulation of other structural complexes like troponin and alpha actinin binding. At 24 h, spectrin seems key for understanding the current cellular state, with the increase in the metabolism of energy and restructuration of the cytoskeleton of the cells, and their receptors, being at the centre of the response.

After the high end of the field relevance exposure to the acute dose of 0.35 ng/bee we did not detect many enrichments at 1 h. Only the molecular function term; unfolded protein binding (GO:0051082, MF), and two cellular components terms; cytoplasm (GO:0005737, CC) and intracellular (GO:0005622, CC) were enriched based on the SSDA list of increased proteins. No enrichment based on the list of proteins with reduced abundance was obtained, which does not allow us to make inferences about functions affected at the gene ontology level, for this timepoint.

At 4 h, the top 5 enrichments based on the increased response are ethanol metabolic process (GO:0006067, BP), chaperonin-containing T-complex (GO:0005832, CC), aminoacyl-tRNA synthetase multienzyme complex (GO:0017101, CC), 'de novo' protein folding (GO:0006458, BP) and protein folding chaperone (GO:0044183, MF). Whereas the processes associated with calcium-transporting ATPase activity (GO:0005388, MF), maintenance of epithelial cell apical/basal polarity (GO:0045199, BP) dorsal closure (GO:0046664, BP), amnioserosa morphology change (GO:0046664, BP), rhodopsin biosynthetic process (GO:0016063, BP) and cadherin binding (GO:0045296, MF) were the top 5 terms enriched from, the list of SSDA proteins with decreased abundance.

At 24 h, the response was characterised by the top 5 upregulated processes muscle alpha-actinin binding (GO:0051371, MF), pyruvate dehydrogenase complex (GO:0045254, CC), acetyl-CoA biosynthetic process from pyruvate (GO:0006086, BP), cytoskeletal anchoring at plasma membrane (GO:0007016, BP), muscle myosin complex (GO:0005859, CC) and by the downregulation of axon cytoplasm (GO:1904115, CC), acetylcholine metabolic process (GO:0008291, BP), positive regulation of lipid transport (GO:0032370, BP), synaptic vesicle transport along microtubule (GO:0099517, BP) and regulation of short-term neuronal synaptic plasticity (GO:0048172, BP).

Thus, the effects triggered by the high dose seem focused on the initial increase of energy processing, although this is not clearly seen by gene ontology enrichments obtained at one hour. When we look at the list of SSDA proteins (Table 3.4) an increase in signalling proteins, belonging to the MAP-kinase and ras pathways, suggest increased metabolic activity. The 4 h timepoint is defined by an increase in energy associated binding and issues with cell adherence and maintenance of calcium levels and cell polarity. At 24 h, an increase in structural agents, signals reestablishment of cytoskeleton anchoring, reshaping and adhesion processes. However, synaptic activity remains downregulated, along with the transport of vesicles and lipids, acetylcholine metabolism and synaptic plasticity.

Table 3.5 Functional enrichment results obtained using the platform STRING based on the list of SSDA proteins identified from the proteome of bees exposed to clothianidin.

	Upregulated			Downregulated		
	GO	Definition	% coverage	GO	Definition	% coverage
0.0875ng/bee 1H	GO:0010353	response to trehalose	66.6	GO:0005861	troponin complex	100
	GO:0015501	glutamate:sodium symporter activity	40.0	GO:0036062	presynaptic periactive zone	66.6
	GO:0005283	amino acid:sodium symporter activity	37.5	GO:0044420	extracellular matrix component	60.0
	GO:0015172	acidic amino acid transmembrane transporter activity	28.5	GO:0005865	striated muscle thin filament	42.8
	GO:0031683	G-protein beta/gamma-subunit complex binding	25.0	GO:0005604	basement membrane	38.4
0.0875ng/bee 4H	GO:0031475	myosin V complex	66.6	GO:0005861	troponin complex	100
	GO:0005523	tropomyosin binding	66.6	GO:0005862	muscle thin filament tropomyosin	100
	GO:0030122	AP-2 adaptor complex	40.0	GO:0051371	muscle alpha-actinin binding	75.0
	GO:0048172	regulation of short-term neuronal synaptic plasticity	28.5	GO:0005859	muscle myosin complex	60.0
	GO:0000221	vacuolar proton-transporting V-type ATPase, V1 domain	25.0	GO:0005865	striated muscle thin filament	57.1
0.0875ng/bee 24H	GO:0042062	long-term strengthening of neuromuscular junction	100	GO:0099095	ligand-gated anion channel activity	25.0
	GO:0008091	Spectrin	66.6	GO:0098878	neurotransmitter receptor complex	13.3
	GO:0006734	NADH metabolic process	40.0	GO:0005254	chloride channel activity	6.0
	GO:0045170	Spectrosome	33.3	GO:0022835	transmitter-gated channel activity	6.0
	GO:0048788	cytoskeleton of presynaptic active zone	33.3	GO:0016860	intramolecular oxidoreductase activity	5.8
0.35ng/bee 1H	GO:0051082	unfolded protein binding	4.1			
	GO:0005737	Cytoplasm	0.3			
	GO:0005622	Intracellular	0.2			
0.35ng/bee 4H	GO:0006067	ethanol metabolic process	66.6	GO:0005388	calcium-transporting ATPase activity	33.3
	GO:0005832	chaperonin-containing T-complex	25.0	GO:0045199	maintenance of epithelial cell apical/basal polarity	25.0
	GO:0017101	aminoacyl-tRNA synthetase multienzyme complex	20.0	GO:0046664	dorsal closure, amnioserosa morphology change	25.0
	GO:0006458	'de novo' protein folding	15.0	GO:0016063	rhodopsin biosynthetic process	20.0
	GO:0044183	protein folding chaperone	14.2	GO:0045296	cadherin binding	18.1
0.35ng/bee 24H	GO:0051371	muscle alpha-actinin binding	75.0	GO:1904115	axon cytoplasm	66.6
	GO:0045254	pyruvate dehydrogenase complex	66.6	GO:0008291	acetylcholine metabolic process	66.6
	GO:0006086	acetyl-CoA biosynthetic process from pyruvate	50.0	GO:0032370	positive regulation of lipid transport	66.6
	GO:0007016	cytoskeletal anchoring at plasma membrane	41.6	GO:0099517	synaptic vesicle transport along microtubule	50.0
	GO:0005859	muscle myosin complex	40.0	GO:0048172	regulation of short-term neuronal synaptic plasticity	42.8

3.4.4 Characterisation of the bee brain proteome in response to acute imidacloprid exposure

To understand how imidacloprid alters the brain proteome over time, and to assess if any sub-phenotypical effects remained 24 h after the field relevant exposure, to the same concentrations tested for clothianidin (0.0875 ng/bee and 0.35 ng/bee), a two samples student t-test (control vs treatment) was also carried out on all proteins identified in the brains of bees exposed to the neonicotinoid imidacloprid.

After appropriate filtering steps, thousands of proteins that were present in all 3 biological replicates of at least one group (treatment or control), for each time point, were identified for 0.0875 ng/bee at 1, 4 and 24 h and for 0.35 ng/bee at 1, 4 and 24 h. Sixty five (65), ninety (90) and one hundred and twelve (112) SSDA proteins were identified at 1, 4 and 24 h post exposure to 0.0875 ng/bee of imidacloprid, and ninety four (94), four hundred and twenty two (422) and eighty seven (87) SSDA at 1, 4 and 24 h post exposure to 0.35 ng/bee of imidacloprid (Figure 3.13).

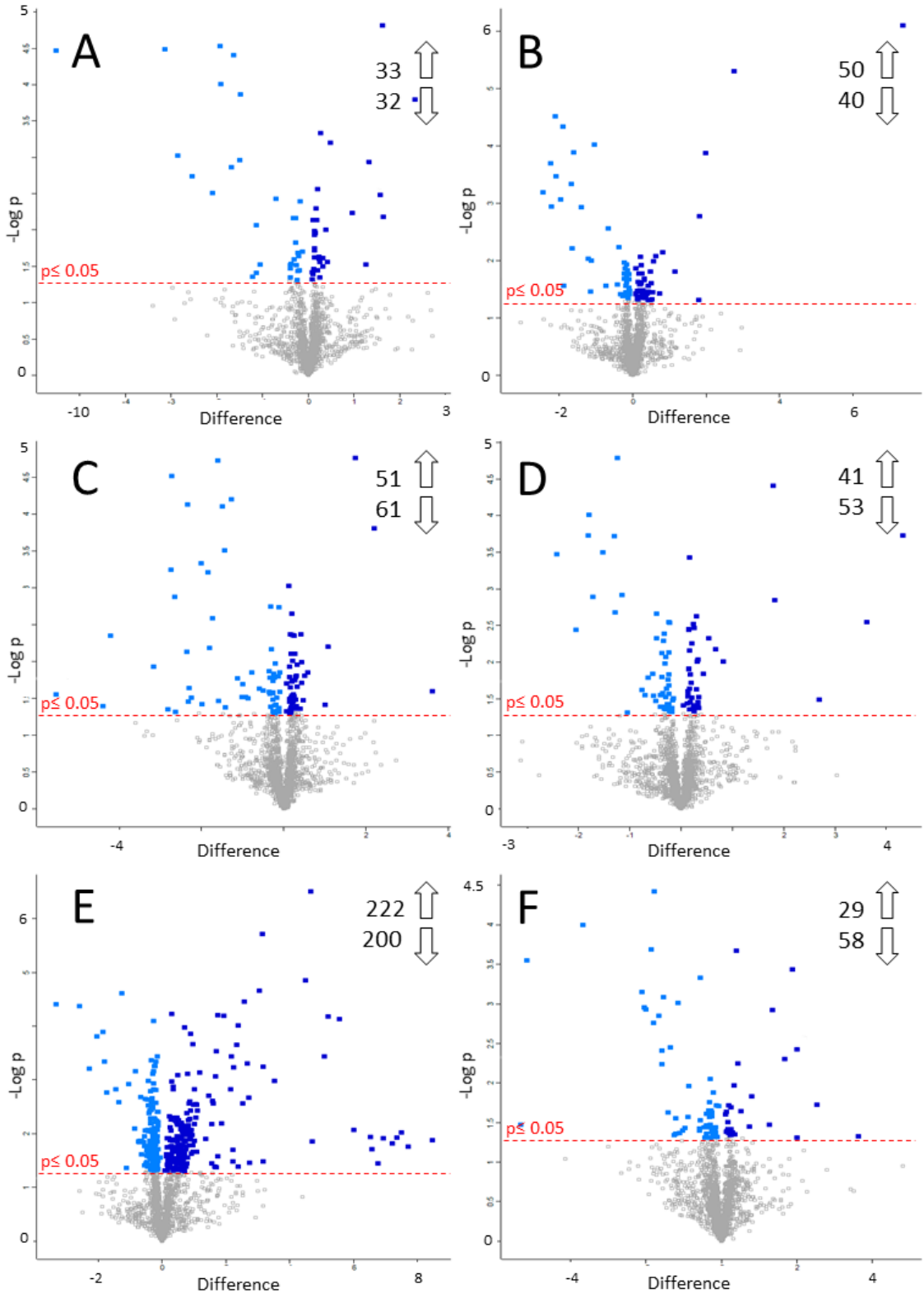


Figure 3.13 Imidacloprid Volcano plots visualizing student t test results for changes in the total brain proteome and SSDA proteins expression. (A) 0.0875 ng/bee 1 h, (B) 0.0875 ng/bee 4 h, (C), 0.0875 ng/bee 24 h, (D) 0.35 ng/bee 1 h, (E) 0.35 ng/bee 4 h, (F) 0.35 ng/bee 24 h. Arrows represent the number of increased and decreased abundance SSDA proteins. Light blue represents decreased abundance SSDA protein distribution and dark blue represent increased abundance SSDA protein distribution.

Uridine 5-monophosphate synthase (XP_012174843.1) and the uncharacterized aarF domain-containing protein kinase 1(XP_003395652.1) are the top increased abundance proteins at 1h, post low dose exposure imidacloprid, with increase RFC of 5 and 4.5 respectively, whereas microsomal glutathione S-transferase 1 (XP_003402656.1), with a decreased RFC of 45.9 and glycine-rich protein DOT1 (XP_012169289.1) RFC of 8.8, are the top 2 proteins with reduced abundance at 1 h (Table 3.6).

At 4 h the top proteins with increased abundance were cytochrome b-c1 complex subunit 6, mitochondrial (XP_003399309.1) and eukaryotic translation initiation factor 3 subunit K (XP_003393301.1) with increased RFC of 165 and 6.7, whereas the top reduced abundant proteins were phosrestin-2 (XP_003394275.1) and protein PRRC2C (XP_012175849.2) with reduced RFC of 5.4 and 4.6.

At 24 h the top increased proteins were beta-ureidopropionase (XP_003403150.1, RFC 12.2) and the uncharacterized protein LOC110119243 (XP_020718517.1, RFC 4.5) and the top decreased were the very long-chain-fatty-acid--CoA ligase bubblegum isoform X2 (XP_003393002.1, RFC reduction of 46), and the 15-hydroxyprostaglandin dehydrogenase [NAD(+)] (XP_012168044.1, RFC reduction of 21).

In response to the higher dose of imidacloprid (Table 3.7), the uncharacterized protein LOC100642544 isoform X1 (XP_003401954.1) and the protein lethal (2) essential for life (XP_012170000.1), were the top 2 increased abundant proteins at 1h, with RFC of 401 and 12.2 respectively. While the uncharacterized protein LOC100646803 isoform X1 (XP_012171776.1) and the potassium voltage-gated channel protein Shaker isoform X1 (XP_012165109.1), were the two top decreased abundant proteins for the same timepoint, with RFC of 5.3 and 4.1, respectively. At 4 h the top 2 increased abundant proteins were regucalcin (XP_003401730.1, RFC 354.6) and the uncharacterized protein LOC100651683 (XP_020719667.1, RFC 207.8), whereas the top 2 decreased abundance proteins were the protein phosphatase inhibitor 2-like isoform X1 (XP_003395341.1, RFC 9.9) and the ATP-dependent RNA helicase DDX46 (XP_020722315.1, RFC 5.9).

At 24 h, the top 2 proteins with increased abundance were beta-ureidopropionase (XP_003403150.1, RFC 12.3), and leucine-rich repeat-containing protein 70 (XP_003394838.1, RFC 5.7), and the top 2 decreased abundance proteins were the very long-chain-fatty-acid--CoA ligase bubblegum isoform X2 (XP_003393002.1, RFC 40.1) and again the microsomal glutathione S-transferase 1 (XP_003402656.1, RFC 35.6).

Table 3.6 The top 10 statistically significant (Student's t-tests, $p<0.05$) differentially abundant proteins with increased and decreased abundance based on the LFQ intensities of all proteins identified post-acute exposure to 0.0875 ng per bee of imidacloprid after 1, 4 and 24h against their respective controls.

	Top 10 Upregulated proteins			Top 10 Downregulated proteins		
	Fold change	Protein name	Protein Id	Fold change	Protein name	Protein Id
1H	5.0	Uridine 5-monophosphate synthase	XP_012174843.1	45.9	Microsomal glutathione S-transferase 1	XP_003402656.1
	3.1	Uncharacterized aarF domain-containing protein kinase 1	XP_003395652.1	8.8	Glycine-rich protein DOT1	XP_012169289.1
	3.0	Dynactin subunit 5	XP_012168646.1	7.2	ATP-dependent Clp protease proteolytic subunit, mitochondrial	XP_012167357.1
	2.9	tRNA selenocysteine 1	XP_012173932.1	5.8	ABC transporter G family member 20	XP_012172596.1
	2.5	Protein CASC3 isoform X1	XP_003402489.1	4.2	RING finger and CHY zinc finger domain-containing protein 1	XP_012165266.1
	2.3	General odorant-binding protein 56d	XP_003402792.1	3.8	Tryptophan--tRNA ligase, mitochondrial	XP_012166805.1
	1.9	Phosphatase and actin regulator 2	XP_012176521.1	3.7	Hornerin	XP_020721218.1
	1.4	Uncharacterized protein LOC100647790	XP_003401188.1	3.2	RING finger protein 121	XP_003399130.1
	1.3	Uncharacterized protein LOC100651819	XP_003401469.1	3.0	H/ACA ribonucleoprotein complex subunit 1	XP_003402120.1
	1.3	Probable galactose-1-phosphate uridylyltransferase	XP_003393491.1	2.8	SPRY domain-containing protein 7	XP_003398026.1
4H	165.0	Cytochrome b-c1 complex subunit 6, mitochondrial	XP_003399309.1	5.4	Phosrestin-2-like isoform X1	XP_003394275.1
	6.7	Eukaryotic translation initiation factor 3 subunit K	XP_003393301.1	4.6	Protein PRRC2C isoform X1	XP_012175849.2
	3.9	General odorant-binding protein 69a-like isoform X3	XP_003398556.1	4.6	Ceramide-1-phosphate transfer protein	XP_003400592.1
	3.5	Uncharacterized protein DDB_G0287625-like	XP_003401515.1	4.3	Dynactin subunit 5	XP_012168646.1
	3.4	RING finger protein 207 isoform X1	XP_020722073.1	4.2	Clustered mitochondria protein homolog	XP_003394619.1
	2.2	FAST kinase domain-containing protein 5	XP_003402672.1	3.9	Manganese-transporting ATPase 13A1	XP_003399527.1
	1.7	Uncharacterized protein LOC100645163	XP_003394861.1	3.7	Putative hydroxypyruvate isomerase	XP_003402136.1
	1.6	Protein FAM117B-like isoform X1	XP_003403010.1	3.6	LOW QUALITY PROTEIN: protein odr-4 homolog	XP_003398574.1
	1.5	Uncharacterized protein LOC100652008	XP_012167732.1	3.1	FACT complex subunit Ssrp1 isoform X1	XP_003395326.1
	1.4	Uncharacterized protein LOC100647232	XP_003399812.1	3.1	Ras-related protein Rap-2a	XP_012172752.1
24H	12.2	Beta-ureidopropionase	XP_003403150.1	46.3	Very long-chain-fatty-acid--CoA ligase bubblegum isoform X2	XP_003393002.1
	4.5	Uncharacterized protein LOC110119243	XP_020718517.1	21.1	15-hydroxyprostaglandin dehydrogenase	XP_012168044.1
	3.3	Transmembrane emp24 domain-containing protein eca	XP_003401219.1	18.4	Nose resistant to fluoxetine protein 6-like	XP_012176310.1
	2.0	Probable cytosolic iron-sulfur protein assembly protein Ciao1 isoform X1	XP_012165271.1	8.9	Probable peroxisomal acyl-coenzyme A oxidase 1	XP_012172746.2
	1.9	COMM domain-containing protein 8-like	XP_003402421.1	7.0	2-acylglycerol O-acyltransferase 1-like isoform X2	XP_003395133.1
	1.4	Uncharacterized protein LOC100645490 isoform X1	XP_003396489.1	6.6	Tubulin alpha-1C chain-like	XP_003394986.1
	1.4	Carbonic anhydrase 1	XP_003395501.1	6.6	Putative serine protease K12H4.7	XP_012165494.1
	1.3	Glutamine synthetase 2 cytoplasmic isoform X1	XP_003397668.1	6.2	Endocuticle structural glycoprotein SgAbd-1-like isoform X1	XP_012172264.1
	1.3	Alpha-2-macroglobulin receptor-associated protein	XP_012163544.1	6.1	Alpha-tocopherol transfer protein-like isoform X1	XP_012168727.1
	1.3	F-BAR domain only protein 2 isoform X1	XP_020719117.1	5.1	Isopentenyl-diphosphate Delta-isomerase 1-like	NP_001267855.1

Table 3.7 The top 10 statistically significant (Student's t-tests, $p<0.05$) differentially abundant proteins with increased and decreased abundance based on the LFQ intensities of all proteins identified post-acute exposure to 0.35 ng per bee of imidacloprid after 1, 4 and 24h against their respective controls.

	Top 10 Upregulated proteins			Top 10 Downregulated proteins		
	Fold change	Protein name	Protein Id	Fold change	Protein name	Protein Id
1H	20.0	Uncharacterized protein LOC100642544 isoform X1	XP_003401954.1	5.3	Uncharacterized protein LOC100646803 isoform X1	XP_012171776.1
	12.2	Protein lethal (2) essential for life-like	XP_012170000.1	4.1	Potassium voltage-gated channel protein Shaker isoform X1	XP_012165109.1
	6.4	Beta-ureidopropionase	XP_003403150.1	3.4	Slit homolog 3 protein-like	XP_003397339.1
	3.5	Uridine 5-monophosphate synthase-like	XP_012174843.1	3.4	Uncharacterized protein LOC100650091 isoform X1	XP_003398954.1
	3.4	Alpha/beta hydrolase domain-containing protein 11 isoform X1	XP_003402554.1	3.3	Tryptophan--tRNA ligase, mitochondrial isoform X1	XP_012166805.1
	1.7	CDK5 regulatory subunit-associated protein 3	XP_003402369.1	2.8	SPRY domain-containing protein 7	XP_003398026.1
	1.5	Protein lethal (2) essential for life-like	XP_003393098.1	2.4	Transmembrane protein 68	XP_003402741.1
	1.4	Protein FAM98B	XP_012175624.1	2.4	Protein Skeletor, isoforms B/C isoform X2	XP_003392952.1
	1.3	Uncharacterized protein LOC100651819	XP_003401469.1	2.3	H/ACA ribonucleoprotein complex subunit 1	XP_003402120.1
	1.2	Leucine-rich repeat-containing protein 15-like isoform X1	XP_003394690.1	2.2	28S ribosomal protein S29, mitochondrial	XP_003393846.1
4H	354.6	Regucalcin-like	XP_003401730.1	9.9	Protein phosphatase inhibitor 2-like isoform X1	XP_003395341.1
	207.8	Uncharacterized protein LOC100651683	XP_020719667.1	5.9	Probable ATP-dependent RNA helicase DDX46	XP_020722315.1
	180.6	Mucin-5AC	XP_012171910.2	4.8	Phosrestin-2-like isoform X1	XP_003394275.1
	163.4	Uncharacterized protein LOC100644055	XP_020718843.1	4.0	TBC1 domain family member 16	XP_012166149.1
	147.8	Glucosylceramidase-like	XP_003402892.1	3.5	Nucleoporin SEH1	XP_003395291.1
	121.8	Pancreatic lipase-related protein 2-like	XP_012168805.1	3.5	F-BAR domain only protein 2 isoform X1	XP_020719117.1
	109.1	Pancreatic lipase-related protein 2-like	XP_003398436.1	3.3	Ras-related protein Rap-2a	XP_012172752.1
	95.7	Venom acid phosphatase Acph-1-like	XP_003394480.1	2.7	Uncharacterized protein LOC100642588 isoform X1	XP_003401715.1
	92.9	Uncharacterized protein LOC100644337 isoform X2	XP_012169771.1	2.5	Uncharacterized protein KIAA1109	XP_020721610.1
	64.0	Esterase FE4-like	XP_003397297.1	2.4	Uncharacterized protein LOC100649490 isoform X1	XP_003393942.1
24H	12.3	Beta-ureidopropionase	XP_003403150.1	40.1	Very long-chain-fatty-acid--CoA ligase bubblegum isoform X2	XP_003393002.1
	5.7	Leucine-rich repeat-containing protein 70	XP_003394838.1	35.6	Microsomal glutathione S-transferase 1-like	XP_003402656.1
	4.0	Uncharacterized protein LOC100644966	XP_003396646.1	12.8	Ubiquitin-conjugating enzyme E2 G2	XP_003402589.1
	3.9	Retinol dehydrogenase 14	XP_012175289.2	4.2	Probable cytosolic Fe-S cluster assembly factor AGAP009023 isoform X1	XP_012164412.1
	3.6	Fibroin heavy chain isoform X1	XP_020719973.1	4.1	NHL repeat-containing protein 2	XP_012172068.1
	3.1	Vam6/Vps39-like protein	XP_003397837.1	4.0	Nuclear cap-binding protein subunit 1	XP_003398727.1
	2.5	Arf-GAP with SH3 domain, ANK repeat and PH domain-containing Protein 2 isoform X1	XP_020720724.1	3.6	tRNA selenocysteine 1-associated protein 1	XP_012173932.1
	2.3	Uncharacterized protein LOC100645428	XP_003402904.1	3.5	Vacuolar protein sorting-associated protein 37B	XP_003397544.1
	1.7	Cytochrome P450 6a2	XP_003400131.1	3.4	ADP-ribosylation factor-binding protein GGA3 isoform X1	XP_003395795.1
	1.6	Trafficking protein particle complex subunit 8 isoform X1	XP_003395280.1	3.1	Methionine--tRNA ligase, mitochondrial-like	XP_003401067.1

GO enrichment analysis on imidacloprid SSDA proteins demonstrate the main biological processes affected by the insecticide, during the first 24 h after exposure (Table 3.8). At 1h post exposure to 0.0875 ng/bee, the top 5 enrichment terms from the list of increased SSDA proteins were 'de novo' pyrimidine nucleobase biosynthetic process (GO:0006207, BP), negative regulation of protein depolymerization (GO:1901880, BP), pyrimidine nucleobase metabolic process (GO:0006206, BP), oocyte microtubule cytoskeleton polarization (GO:0008103, BP) and negative regulation of supramolecular fibre organization (GO:1902904, BP). The only downregulated process at 1 h was catalytic activity (GO:0003824, MF). At 4 h, the top 5 upregulated enrichments were eukaryotic 43S preinitiation complex (GO:0016282, CC), multi-eIF complex (GO:0043614, CC), ESCRT complex (GO:0036452, CC) and anion binding (GO:0043168, MF). Whereas the top 5 downregulated processes were calcium-transporting ATPase activity (GO:0005388, MF), positive regulation of actin filament polymerization (GO:0030838, BP) and mitochondrial intermembrane space (GO:0005758, CC), mitotic spindle (GO:0072686, CC), spindle (GO:0005819, CC).

At 24 h the top 5 enriched processes in response to imidacloprid exposure of 0.0875ng/bee were; cytoskeleton of presynaptic active zone (GO:0048788, CC), presynaptic active zone organization (GO:1990709, BP), NADH dehydrogenase (ubiquinone) activity (GO:0008137, MF), cortical cytoskeleton (GO:0030863, CC), and presynaptic active zone (GO:0048786, CC). The top downregulated processes were mitochondrial fatty acid beta-oxidation multienzyme complex (GO:0016507, CC), long-chain-3-hydroxyacyl-CoA dehydrogenase activity (GO:0016509, MF), cholesterol biosynthetic process (GO:0006695, BP), acetyl-CoA C-acyltransferase activity (GO:0003988, MF), isopentenyl diphosphate biosynthetic process (GO:0009240, BP).

Taken together, the low dose of imidacloprid seems to alter the brain proteome in terms of the molecular pathways associated with protein polymerization, transport and the metabolism of nucleotides, fatty acids and acetyl CoA, in the context of the mitochondria.

After exposure to the high end of the field relevant exposure to imidacloprid, the top enrichments obtained at 1 h, from the list of SSDA with increased abundance were the retromer complex (GO:0030904, CC), 'de novo' pyrimidine nucleobase biosynthetic process (GO:0006207, BP), pyrimidine nucleobase metabolic process (GO:0006206, BP), methionine metabolic process (GO:0006555, BP), aminoacyl-tRNA ligase activity (GO:0004812, MF). And the top downregulated were long-chain fatty acid-CoA ligase activity (GO:0004467, MF), long-chain fatty acid metabolic process (GO:0001676, BP),

regulation of wound healing (GO:0061041,BP), tRNA binding (GO:0000049, MF), endocytic vesicle (GO:0030139, CC).

At 4 h, the top upregulated gene ontology enrichments were the endonucleolytic cleavage to generate mature 3'-end of SSU-rRNA from (SSU-rRNA, 5.8S rRNA, LSU-rRNA)(GO:0000461, BP), GDP-mannose biosynthetic process (GO:0009298, BP), phosphogluconate dehydrogenase (decarboxylating) activity (GO:0004616, MF), GDP-mannose metabolic process (GO:0019673, BP), posttranslational protein targeting to membrane and translocation (GO:0031204, BP). Whereas the top downregulated terms were; mitochondrial proton-transporting ATP synthase, central stalk (GO:0005756, CC), regulation of polarized epithelial cell differentiation (GO:0030860, BP), long-term strengthening of neuromuscular junction (GO:0042062, BP), spectrin (GO:0008091, CC) and neuromuscular junction of somatic muscle (GO:0098527, CC).

At 24 h post exposure to 0.35 ng/bee of imidacloprid, the top upregulated functional enrichments were the apposition of dorsal and ventral imaginal disc-derived wing surfaces (GO:0007475, BP), focal adhesion (GO:0005925, CC), cell-substrate adherens junction (GO:0005924, CC), tethering complex (GO:0099023, CC), cell junction (GO:0030054, CC). And the top downregulated enrichments were acetyl-CoA C-acyltransferase activity (GO:0003988, MF), isopentenyl diphosphate biosynthetic process (GO:0009240, BP), isopentenyl diphosphate metabolic process (GO:0046490, BP), positive regulation of peptide hormone secretion (GO:0090277, BP) and ubiquitin-dependent protein catabolic process via the multivesicular body sorting pathway (GO:0043162, BP).

The response to the higher dose seems to begin similarly to the low dose, with imidacloprid increasing the metabolism of nucleotides and processes of translation while reducing acetyl CoA and fatty acid metabolism, which is maintained until 24 h post exposure. The response evolves to the reduction of endosomal and structural processes of translocation, but cell adhesion and focal adhesion processes are increased

Table 3.8 Functional enrichment results obtained on the platform STRING based on the list of SSDA proteins identified from the proteome of bees exposed to imidacloprid.

	Upregulated			Downregulated		
	GO	Definition	% coverage	GO	Definition	% coverage
0.0875ng per bee 1H	GO:0006207	'de novo' pyrimidine nucleobase biosynthetic process	25.0	GO:0003824	Catalytic activity	0.4
	GO:1901880	Negative regulation of protein depolymerization	18.1			
	GO:0006206	Pyrimidine nucleobase metabolic process	14.2			
	GO:0008103	Oocyte microtubule cytoskeleton polarization	10.5			
	GO:1902904	Negative regulation of supramolecular fiber organization	8.6			
0.0875ng per bee 4H	GO:0016282	Eukaryotic 43S preinitiation complex	50.0	GO:0005388	Calcium-transporting ATPase activity	33.3
	GO:0043614	Multi-eIF complex	40.0	GO:0030838	Positive regulation of actin filament polymerization	11.1
	GO:0036452	ESCRT complex	9.5	GO:0005758	Mitochondrial intermembrane space	9.0
	GO:0043168	Anion binding	1.0	GO:0072686	Mitotic spindle	8.0
	GO:0044444	Cytoplasmic part	0.7	GO:0005819	Spindle	3.2
0.0875ng per bee 24H	GO:0048788	Cytoskeleton of presynaptic active zone	33.3	GO:0016507	Mitochondrial fatty acid beta-oxidation multienzyme complex	100
	GO:1990709	Presynaptic active zone organization	20.0	GO:0016509	Long-chain-3-hydroxyacyl-CoA dehydrogenase activity	100
	GO:0008137	NADH dehydrogenase (ubiquinone) activity	16.0	GO:0006695	Cholesterol biosynthetic process	100
	GO:0030863	Cortical cytoskeleton	13.6	GO:0003988	Acetyl-CoA C-acyltransferase activity	75.0
	GO:0048786	Presynaptic active zone	13.0	GO:0009240	Isopentenyl diphosphate biosynthetic process	75.0
0.35ng per bee 1H	GO:0030904	Retromer complex	28.5	GO:0004467	Long-chain fatty acid-CoA ligase activity	25.0
	GO:0006207	'de novo' pyrimidine nucleobase biosynthetic process	25.0	GO:0001676	Long-chain fatty acid metabolic process	20.0
	GO:0006206	Pyrimidine nucleobase metabolic process	14.2	GO:0061041	Regulation of wound healing	14.2
	GO:0006555	Methionine metabolic process	14.2	GO:0000049	tRNA binding	10.0
	GO:0004812	Aminoacyl-tRNA ligase activity	13.1	GO:0030139	Endocytic vesicle	9.5
0.35ng per bee 4H	GO:0000461	Endonucleolytic cleavage to generate mature 3'-end of SSU-rRNA from (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	100	GO:0005756	Mitochondrial proton-transporting ATP synthase, central stalk	100
	GO:0009298	GDP-mannose biosynthetic process	100	GO:0030860	Regulation of polarized epithelial cell differentiation	100
	GO:0004616	Phosphogluconate dehydrogenase (decarboxylating) activity	66.6	GO:0042062	Long-term strengthening of neuromuscular junction	100
	GO:0019673	GDP-mannose metabolic process	66.6	GO:0008091	Spectrin	66.6
	GO:0031204	Posttranslational protein targeting to membrane, translocation	66.6	GO:0098527	Neuromuscular junction of somatic muscle	66.6
0.35ng per bee 24H	GO:0007475	Apposition of dorsal and ventral imaginal disc-derived wing surfaces	15.7	GO:0003988	Acetyl-CoA C-acyltransferase activity	75.0
	GO:0005925	Focal adhesion	9.0	GO:0009240	Isopentenyl diphosphate biosynthetic process	50.0
	GO:0005924	Cell-substrate adherens junction	8.8	GO:0046490	Isopentenyl diphosphate metabolic process	50.0
	GO:0099023	Tethering complex	4.7	GO:0090277	Positive regulation of peptide hormone secretion	28.5
	GO:0030054	Cell junction	3.2	GO:0043162	Ubiquitin-dependent protein catabolic process via the multivesicular Body sorting pathway	18.1

3.5 Discussion

Investigating the impact of neonicotinoids in bees is not a novel undertaking. Since their introduction in the 1990s, neonicotinoids have had a consistently growing share of the pesticide world market, with their wide-spread use occurring in the USA and Europe from 2004 onwards (van der Sluijs *et al.*, 2013). Today, neonicotinoids are used in more than 120 countries, and are authorised for use on more than 1000 crops. However, since their introduction, a series of new bee disorders have begun to be recorded, with symptoms ranging from bee mortality, to effects on homing and abnormal foraging behaviours (van der Sluijs *et al.*, 2013). Although these symptoms have been described in several studies, the molecular basis or determinants of such behavioural alterations have rarely been explored.

In relation to mortality, the results of our research indicate that the LD50 for clothianidin lies between 1.75 to 2.45 ng/bee and an LD90 of around 3.5 ng/bee in 24 hr. Clothianidin was more toxic to *B. terrestris* in comparison to imidacloprid, demonstrating the occurrence of differential severity between two chemicals from the same family, that are classified as having the same mode of action. Differential toxicity is also reported in mammalian brain cells where clothianidin again was more toxic than imidacloprid (Fotopoulou *et al.*, 2020). The different behaviours between bees exposed to the individual neonicotinoids, clearly provides evidence of a different mode of action in bumblebees, which is corroborated by evidence that clothianidin acts as a super agonist of nAChR in *Drosophila*, whereas imidacloprid, exerts low efficacy activation on such receptors (Jeschke and Nauen, 2008). Possible differences in interactions at the molecular level are then translated into the behavioural level with clothianidin increasing overall bee activity, while imidacloprid reduced it, with exposure to the latter resulting in lethargic behaviour. Such effects may be the result of irregular firing in neurons belonging to the peripheral nervous system in the neuromuscular junction, as movement is controlled by the thoracic ganglia in bees (Ibbotson *et al.*, 2017; Schilling and Cruse, 2020). It is possible that increased activity is a direct effect of increased firing of neurons, resulting from agonistic clothianidin activity, which increases muscle contraction in the limbs causing uncoordinated movement. Whereas imidacloprid can cause reduced activity by having antagonistic or partly agonistic effect on the neurons in the neuromuscular junction, reducing firing. Differential effects on motor function like the ones described in this our study have also been reported in honeybees post 24 h exposure to imidacloprid and clothianidin (Williamson *et al.*, 2014) This demonstration of differential risks posed by each neonicotinoid, may have real ecological consequences, in

that induced hyperactivity caused by clothianidin exposure, could potentially result in increased ecological activity, although, there would be consequences, if energy reserves were utilised at a higher rate than they could be replenished. On the other hand, imidacloprid, could result in reduced performance which could possibly cause bees to become more vulnerable to predators, and other threats in the field.

It was observed, that the toxicological response to acute exposure developed more harshly within 1 hour post exposure, for both neonicotinoids tested, with bees dying, when exposed to lethal doses of clothianidin, or found immobile when exposure to imidacloprid, shortly after consuming the contaminated solutions. In addition, slower responses were observed at lower doses, in comparison to the higher doses tested. This delay in effect, is interesting and potentially significant, given that bumblebees are known to forage for about 57 to 75 min on average, before returning to their colonies (Spaethe and Weidenmüller, 2002). Thus, a delayed sublethal effect has implications beyond bumblebee foraging activity and performance, when in the field, as the effects may only manifest once they have returned to the colony.

Neonicotinoids are known to affect the amount of pollen collected (Gill, Ramos-Rodriguez and Raine, 2012b; Feltham, Park and Goulson, 2014; Stanley and Raine, 2016) and to influence the duration and the motivation towards foraging through effects in the central nervous system (Lämsä *et al.*, 2018). Most of these studies exposed bees chronically, and although similarities of effects can be drawn with the behavioural alterations demonstrated in this study, current behaviour assessment techniques have failed to demonstrate a correlation between acute neonicotinoid exposure, and adverse effects on learning and memory *in vivo*. It is therefore essential that further research is conducted utilising *B. terrestris*, and we need to address the lack of protocols for assessing behavioural responses to acute pesticide exposure, given the importance of this ecologically important semi social species.

In semi field experiments, Stanley and Raine (2016), detected an increase in bee activity following chronic thiamethoxan exposure in *B. terrestris.* Clothianidin is the main metabolite of thiamethoxan, being rapidly produced in insects and plants, and increasing the sensitivity of nAChRs to the insecticide. It is not unreasonable to expect that thiamethoxan acts as a precursor for clothianidin, and that the neurological effects observed in insects, may be the results of exposure to the latter (Nauen *et al.*, 2003). In addition, Lämsä *et al.* (2018) demonstrated that exposure to imidacloprid can reduce bee activity, reducing initiation and length of foraging flights, which corroborates our results showing that imidacloprid has an impact on bee paralysis.

Neonicotinoid impact on honeybee performance, has been attributed to their effects on processes such as visual and odour learning and memory, which in turn influence their navigation, orientation and overall cognitive performance (Decourtye *et al.*, 2007; Aliouane *et al.*, 2009; Williamson *et al.*, 2013; Fischer *et al.*, 2014). The capacity to link a flower to an odour (i.e. associative learning) may appear simple but can be influenced by changes in the efficiency of a single synapse. Cognitive capacity is linked to synaptic density in areas of sensory inputs, and information processing (Chittka, 2017). The Mushroom body is possibly one of the most important of these regions in the social insect brain, acting as a multisensory input area, and functioning as a memory storage region. It is made up of KCs that have dendritic arborizations forming the calyx, which function as the main optical and antennal input areas of the mushroom bodies (the lip as olfactory, the collar as visual and the basal ring as both). Synapses within the calyces are formed by cholinergic boutons, KC dendrites and GABAergic terminals (Ganeshina and Menzel, 2001; Fahrbach, 2006) and the KC axons collect into bundles and form the pedunculus at the base of the calyx (Fahrbach, 2006). Hence, these areas are extremely important when assessing neurological performance in bees.

Therefore, we set out to investigate neonicotinoid effects on synapsin concentration, and the total proteome of the bumblebee brain, in an attempt to improve our understanding of how cognitive performance, through synapsin level alteration, is influenced by this class of insecticide.

Synapsin distribution was assessed using immunolocalization and fluorescent confocal microscopy in the mushroom bodies and antennal lobes of *B. terrestris*. This protein is directly associated with synaptic activity, and our results demonstrate a considerable increase in synapsin abundance, for all neuropil regions assessed, when the bees were exposed to two sub lethal, and field relevant doses of imidacloprid (2.5 and 10 ppb with respective final exposure of 0.0875 ng/bee and 0.35 ng/bee). Alteration on synapsin levels have also been reported post larval thiamethoxam exposure in honeybee pupae, and recently emerged adults (Tavares *et al.*, 2019b). However, studies with bumblebees are novel and necessary, as they improve our understanding of stimuli receptions and memory consolidation in non Apis bees, and because synapsins are important for the regulation of neurotransmitters release.

This conserved family of proteins are predominantly associated with the reserve pool of synaptic vesicles, and their phosphorylation by kinases such as calcium-dependent protein kinases (CaMKs), protein kinase A (PKA), and MAPK/Erk, result in the liberation of these vesicles, inducing neurotransmitter release. In addition to their role in

the phosphorylated form, synapsins have also been implicated in a mechanism that enhances endocytosis, upon continuous and intense neuronal activity, aiding in the recycling, formation and direction of new enlarged vesicles, into the pre synaptic reserve pool (Akbergenova and Bykhovskaia, 2010). This is likely a synapsin dependent mechanism, used to increase synaptic activity in response to continuous stimulation, thus, synapsin deficiency, inhibits activity-dependent formation of both endosome-like structures and vesicles (Akbergenova and Bykhovskaia, 2010). Our results suggest an increase in abundance levels of synapsin as perhaps an attempt to increase synaptic activity by reestablishment of neurotransmitter release, and vesicles recycling.

Recent studies of the *synapsin* gene in *Drosophila*, determined its importance to the adult and larval associative memory and learning processes (Knapek *et al.*, 2010; Sadanandappa *et al.*, 2013). Synapsin acts in associative memory trace formation in the mushroom bodies, and can accumulate at sites of synaptic growth, facilitating budding of new boutons via a cAMP/PKA-dependent pathway (Vasin *et al.*, 2014). They are also necessary in GABAergic local interneurons, facilitating GABA release, from which attenuates excitatory signals, and results in reduced behavioural response. These *in vivo* observations on synapsin function, constitute a substantial advance, as they demonstrate that synapsin-mediated plasticity, usually observed in response to experimentally enforced electrophysiological stimulations, can underlie behavioural learning and memory, induced by sensory experience (Michels *et al.*, 2011).

It is not clear if the increase in synapsin levels observed post exposure to imidacloprid is associated with GABA release and the attenuation of excitatory signals, or to the necessity of producing more candidates to phosphorylation, aiding the release and recycling of neurotransmitter vesicles. More studies are necessary to confirm either case. However, it is clear that synapsin accumulation, and reduction of bee activity, with possible impact upon learning and memory, are clear results from our experiments of imidacloprid acute exposure.

We did not detect alterations on the level of synapsin in clothianidin exposed bee brains as expected, since Tavares *et al.*, (2019a) reported decrease in synapsin levels post exposure to thiamethoxam, a clothianidin precursor. However, this does not mean that the neurophysiology of the brain is not affected by the insecticide, as seen at the behaviour level, clothianidin does inflict changes in bumblebee behaviour, and can often be even more toxic than imidacloprid. To further investigate the effects observed at the behavioural level, we used mass spectrometry-based proteomics to investigate changes in

the total protein expression of bumblebee brains, post-acute field relevant exposure to clothianidin and imidacloprid.

3.5.1 Proteomic changes caused by clothianidin exposure

After one hour, both the low and high doses of clothianidin had altered the bumblebee brain proteome in terms of neurological cellular activity, as signified by differences in transporters and signalling pathway protein abundances. At the lower dose, there is an increase in proteins associated with a response to trehalose (Table 3.5), a non-reducing disaccharide that has been characterised as a neuroprotective agent in animal models of various neurodegenerative diseases (Lee *et al*., 2018). The response to trehalose is increased after exposure to clothianidin, along with the increase of sodium symporter activities associated with the release, re-uptake and recycling of neurotransmitters. This increase in synaptic cycle elements, suggest high synaptic activity during the first hour post exposure to clothianidin. Such an effect is followed however, by a reduction of structural proteins, including myosins and troponins (Table 3.3). GO enrichments for the same timepoint may reflect a deterioration of the presynaptic periactive zone involving the basement membrane and extracellular matrix components (Table 3.5).

The basement membranes are specialised layers of extracellular matrix that cover the basal side of all epithelia and endothelia, the peripheral nerves and other tissues. They are important for tissue morphogenesis, and have a role in mechanical stability, providing physical barriers between different cell types. They are also integral to numerous processes such as cell differentiation, shape, adhesion, survival and migration (Fessler and Fessler, 1989; Timpl, 1996).

At the same timepoint but for the higher dose (0.35 ng/bee), an increase in other neurodegenerative markers, such as the alpha-tocopherol transfer protein and the apoptosis marker MAP kinase-activating death domain protein, were found along with an increase in the endocytosis associated Myc box-dependent protein. In addition, the chaperone Hsp90, responsible for the triage, folding and distribution of proteins is also affected. Hsp 90 has been studied in neurodegenerative diseases, as it can also affect activity and location of motor proteins during axon specification and axon elongation processes. Along with the proteasome subunit beta type-3, which also have increased abundance, we suggest a potential increase in protein degradation, and interference with local stability, which have a direct impact in neuronal polarity mechanisms (Benitez *et al.*, 2014). This was followed by a decrease in galactokinase, GABA receptor associated proteins, glycogen synthase proteins and others. GABAergic associated proteins have

inhibitory function in the brain of insects (Barbara *et al.*, 2005), which suggests that after 1h, there is no mechanism to reduce neurological activity, and that the pressure to maintain energy provision, to an increased level of synaptic transmission, and protein structure and function is increasing.

There is a correlation between the effects caused at both doses at 1h post exposure, subtler signs are observed at the lower dose, but both suggest an increase in cellular synaptic activity.

By four hours, the low dose had induced an increase in the abundance of the vacuolar H+ ATPase, which functions to reduce the pH in neuronal cells, along with AP-2 adaptor complex, and structural protein members, such as myosins and tropomyosins. Increases in these structural proteins, suggest regulation of synaptic plasticity, which is corroborated by the increase in the alan shepard protein, and BAG domain containing protein Samui, which are involved in neuronal differentiation, migration and remodelling (Chen *et al.*, 2014) . High pH is typically associated with increased synaptic activity, while low pH environments are generally inhibitory. The observed changes in proteins involved in the regulation of proton levels, and the increase in neuronal remodelling observed in this study, might suggest an alternative way to reduce the number of neonicotinoids activated terminals, therefore decreasing synaptic activity. A downregulation of other structural components is also observed at the low dose, 4 hours post exposure, specifically a reduction in key elements of organelle and protein transport, processes of key importance to the maintenance of normal neuronal activity.

At the higher dose, many proteins associated with protein folding activity are differentially abundant, including the chaperonin containing T complex, which is essential for the folding of newly translated polypeptides, and axonal transport. Issues with this process may result in misfolded proteins, many of which are associated with neurodegenerative diseases (Chen, 2019). The chaperonin containing T complex, identified here, may form the basis of a neuroprotective mechanism that is triggered by pesticide exposure. The decrease in agents associated with cell polarity, calcium transport and cadherin binding, which are important during cell motility, migration and reorganization, suggest a state in which such activities are being executed to a lower degree, in comparison with the earlier timepoint. This potentially suggests that synaptic activity and neuronal reorganization are being reduced over time, with less activity being found at 4 h than at 1 h. Which is corroborated by the downregulation of microtubule associated transport (tubulin alpha 1c), glutamate receptors, and proteins associated with neuronal projection development, seen at our data. Markers of cell death (programmed

cell death 5), vesicle transport (cytoplasmic dynein) and actin interaction (protein singed), become more evident at this timepoint, as they continued to be upregulated four hours after exposure. These findings suggest that clothianidin exposure results in issues with intracellular transport, and neuronal plasticity, as well as long term effects on other neurotransmitter mediated systems.

Twenty-four hours after exposure to the low dose, cytoskeleton processes (associated with spectrin), and NADH metabolic processes, show increased abundance, whereas proteins associated with receptor and oxidoreductase activity are reduced. The fact that pyruvate dehydrogenase, acetyl coA synthesis and cytoskeleton anchoring at plasma membrane, are upregulated at the higher dose, suggests an increase in energy production, through glycolysis and the Krebs cycle. In addition, transport of synaptic vesicles along the microtubule, the metabolism of acetylcholine and the transport of lipids are reduced for the higher dose.

Therefore, clothianidin exposure leads to an initial increase in proteins associated with endocytosis (1 h and the upregulation of AP-2 adaptor complex at 4 h), which may represent an attempt to incorporate substances at the synaptic cleft. Such a process would also involve the participation of structural proteins such as myosin and tropomyosin complex binding, that are known to mediate cell motility, organelle and protein transport and endocytosis (Behrmann *et al.*, 2012). We suggest that there is increased synaptic transmission and activity during the first hours after exposure. This synaptic activity requires considerable energy (through glycolysis, oxidative phosphorylation etc.) that under continuous hyperstimulation, becomes unsustainable. After time, the maintenance of ion levels, cytoskeleton organization, cell adherence and programmed cell death, become altered, demonstrating the degree of neurological processes being affected. We hypothesise that neuronal cells attempt to develop new connections, to re-establish normal neuronal communication, and try to inactivate and recycle branches permanently bound to the neonicotinoid. This is done through the synthesis of dendritic and axon arborizations, and the transport of structural parts and organelles to new arms of the cells, which demands huge concentration of energy, triggering an increase in alternative pathway of energy generation, like the metabolism of lipids. Such intensive neuronal plasticity processes also require an efficient maintenance system of protein structure and distribution, for efficient neuronal functioning. Such high demands are unsustainable for the cells and markers of cellular stress, apoptosis, negative effects on cell polarity and decreased cellular activity start to become evident at the 4 h timepoint.

After 24h, the synthesis of actin cytoskeleton and mitochondrial elements as well as focal adhesion proteins, possibly indicate some degree of recovery in what concerns the cytoskeleton progression and neuronal motility, or at least a switch back to traditional methods of energy production through the mitochondria. On the other hand, decreased synaptic activity, vesicle and organelle transport remain downregulated, along with elements of axon and synaptic development and maintenance. Suggesting that the brain may be on route to recover but that signs of damage are still apparent.

The brain is the key centre mediating behaviour and cognition in social bees (Chittka and Niven, 2009; O'Donnell *et al.*, 2015) and it contains an abundance of KCss, a neuronal cell type targeted by neonicotinoids due to the high presence of nAChR on their surface membranes (Palmer *et al.*, 2013; Moffat *et al.*, 2016). It is plausible that the molecular results described above, are representative of the cellular disruptions triggered by clothianidin's interaction with its target receptors, and of off-target processes not mediated by nAChRs, but by GABA and glutamate responsive systems. The alteration in protein abundances associated with energy metabolism, cellular structure reorganization and transport, and others, which ultimately affect synaptic functioning, plasticity and cellular damage, help explain the sub lethal effects on cognition, learning and memory (Williamson and Wright, 2013; Stanley *et al.*, 2015; Tison *et al.*, 2019), foraging performance (Gill *et al.*, 2012a; Lämsä *et al.*, 2018) and navigation abilities (Fischer *et al.*, 2014) described in the literature. And to advance our knowledge of pathways affected by clothianidin on bumblebee brains. Neonicotinoid effects on energy equilibrium, biosynthetic and proliferating capacities have also been suggested in mammalian neuron cells through metabolomic studies (Fotopoulou *et al.*, 2020). But to the best of our knowledge the present is the first in depth temporal description on the molecular toxicity of clothianidin to bumblebee brains applying proteomics.

3.5.2 Proteomic changes caused by imidacloprid exposure

Bumblebees exposed to imidacloprid displayed more discrete effects in comparison to those exposed to clothianidin, this was also found to be true for mammalian neuronal cells at the metabolome level (Fotopoulou *et al.*, 2020). Even so, at the proteomic level, exposure to 0.0875 ng/bee of the chemical resulted in a reduction of critical processes including the overall catalytic activity in the brain, one-hour post oral exposure to the neonicotinoid. Although no processes were identified from the list of downregulated proteins by STRING at 1h post exposure, the SSDA proteins with greatest fold changes comprise constituents of the mitochondria, that may be associated with

mitochondrial elongation and transmembrane transporters. The higher dose, resulted in a reduction in endocytic vesicles, tRNA binding and long chain fatty acid metabolic processes, at four hours post exposure, which may suggest a reduction in synaptic activity and energy lipid metabolism. Fatty acid metabolism was also found to be affected in bumblebees after chronic exposure to imidacloprid (Erban *et al.*, 2019a), which the authors suggest to cause disruption in bumblebee communication through defects on lipid homeostasis (Žáček *et al.*, 2013). These effects were followed by an increase at both doses of pyrimidine metabolism, and translation initiation factors along with a down regulation of mitotic spindle processes and protein depolymerization, observed mostly at the lower dose.

Similar processes were observed in the high and low dose by 24 hours after exposure to imidacloprid. These processes primarily involved the activity of the acetyl CoA dehydrogenase, the isopentenyl diphosphate metabolic processes, and the regulation of hormonal processes, including the cholesterol biosynthetic process. A number of structural associated terms were upregulated, including focal adhesion and the tethering complex, post exposure to the high dose, and the presynaptic active zone organization at the lower dose, which may represent a conserved response to both doses.

It seems therefore that imidacloprid gives rise to different effects in comparison to clothianidin, and specifically effects that result in the initial reduction in levels of synaptic activity, and cellular energy fuelling, which is evidenced by the reduction on mitochondrial, endocytic and transmembrane transporter terms. Imidacloprid and clothianidin were shown to differently affect distinct subpopulations of KCs in bumblebee brains (Moffat *et al.*, 2016), and to exert different intensities of activation or inactivation pressures on nAChR (Jeschke and Nauen, 2008), suggesting the potential to affect different cellular pathways which has been corroborated by our proteomic analysis. These pathways represent mainly effects upon energy metabolism, including mitochondrial function (Nicodemo *et al.*, 2014). However, a later alteration in the metabolism of pyrimidines is also suggested. Purines and pyrimidines nucleotides are known to supply essential precursors for RNA and DNA molecules, but they also have many more functions. Pyrimidines are involved in polysaccharides and phospholipid synthesis, detoxification processes, and glycosylation of proteins and lipids ,while purines act as metabolic signals and modulators of brain function (Evans and Guy, 2004). Changes in the metabolism of pyrimidines, such as that suggested by our results, may translate into functional deficits, and be the basis of several neurological pathologies and dysfunctions (Micheli *et al.*, 2011; Fumagalli *et al.*, 2017).

The effects observed at 24h include increased association of structural terms, which can be involved in synaptic organization and communication. However, changes concerning effects at the cholesterol/isoprenoid, and fatty acid synthesis pathways, which downregulate the levels of cholesterol in the brain are detected 24h post exposure to imidacloprid. Defects in cholesterol metabolism have been implicated in neurodegenerative diseases and impact cognitive deficit (Orth and Bellosta, 2012; Zhang and Liu, 2015). If this is also true in bumblebees, our molecular data potentially provides an explanation for how alterations at the proteome level may result in negative effects at the behavioural and colony level in different ways than clothianidin. Moreover the changes in the hormonal and fatty acid pathways are consistent with chronic studies in bumblebees (Sakakura *et al.*, 2001), and indicate a violation of communication in individuals, possibly affecting learning (Wright *et al.*, 2015) due to imidacloprid and its metabolites having a blocking effect on the sterol regulatory element-binding protein and cleavage-activating protein in the mevalonate pathway for cholesterol synthesis (Sakakura *et al.*, 2001; Erban *et al.*, 2019b).

Understanding the sublethal effects of pesticide exposure on beneficial insects is essential in order to assess and mitigate risks posed by different pesticides.

We have for the first time, provided a comparative deep insight into the molecular pathways affected by exposure to neonicotinoid in bumblebees. We have also demonstrated that clothianidin and imidacloprid, differentially affect the brain proteome and provided a molecular perspective of the biochemical state of the brain, at three different timepoints within the 24h, post-acute exposure to these insecticides (Appendix 3), which may underpin both the cellular and behavioural effects previously reported for these pesticides.

It is important to consider that more functional studies are now required to aid our understanding of how these molecular agents interact during exposure. The specific role of synapsin, within the three neuropil regions assessed remain to be further investigated as the alteration levels detected using fluorescent microscopy could not be confirmed by proteomics. Synapsin levels were only found to have their abundances significantly increased after exposure to 0.0875 ng of clothianidin (RFC 1.24) and decreased after exposure to 0.35 ng of imidacloprid (RFC 1.16) (Table 3.7.2 and 3.7.5 Appendix 3). This does not necessarily contradict our microscopy findings, once those measurements were carried out in mushroom bodies, antennal lobes and the pedunculus exclusively. Whereas the mass spectrometer measured synapsin abundance in the whole bumblebee brain, including other neuropils like the lamella and the optical lobes, which account for a

significant portion of the brain, and together can mask the signal of each smaller independent brain region.

The same is true for our proteomic results. It is plausible that different neonicotinoids affect the brain disproportionally, due to the differential distribution of subpopulations of KCs sensitive to each compound. Thus, detecting localized effects on the expression of individual proteins can be challenging as we detected averaged signals across the whole brain.

Furthermore, even in insects, the brain is a dynamic and complex organ. It is constituted by a number of different cell types and has different structures involved in cognitive process regulation (Klowden, 2013a). Such complexity generates complex datasets, the interpretation of which can prove difficult. We are cautious of the potential interpretations provided, but hopeful that our research will serve as a guide to future projects that aim to further explore, and corroborate our findings, as a way of successfully determining molecular bioindicators of pesticide exposure. In addition, elucidating the structures of nAChRs in *B. terrestris,* remains a primary knowledge gap. It has been demonstrated that different chemicals interact with different subpopulations of such receptors, influencing speed and intensity of response. Until we have elucidated this information fully, it will be difficult to determine whether the difference in effects observed in our behavioural and molecular experiments is due to different receptor subunits, that influence patterns of binding (agonist or antagonist effect), sensibility (intensity of activation), desensitisation of receptor depending on neonicotinoid (receptor changes structure and stops responding) or even to neonicotinoid effects on other neurotransmitter systems.

3.6 Appendix 3

The following appendix tables are located on the disc provided at the back of thesis or at the link below:

https://drive.google.com/file/d/1nonpSYgbM2V_mmqYdPvt4kQW4yvl6UZv/view?usp=sharing

Table 3.6.1 Complete list of statistically significant (Student's t-tests, $p<0.05$) differentially abundant proteins (SSDA) with increased and decreased abundance based on the LFQ intensities of all proteins identified post-acute exposure to 2.5ppb of clothianidin after 1h against their respective controls**.**

Table 3.6.2 Complete list SSDA with increased and decreased abundance post-acute exposure to 2.5ppb of clothianidin after 4h against their respective controls**.**

Table 3.6.3 Complete list SSDA with increased and decreased abundance post-acute exposure to 2.5ppb of clothianidin after 24h against their respective controls**.**

Table 3.6.4 Complete list SSDA with increased and decreased abundance post-acute exposure to 10ppb of clothianidin after 1h against their respective controls**.**

Table 3.6.5 Complete list SSDA with increased and decreased abundance post-acute exposure to 10ppb of clothianidin after 4h against their respective controls**.**

Table 3.6.6 Complete list SSDA with increased and decreased abundance post-acute exposure to 10ppb of clothianidin after 24h against their respective controls**.**

Table 3.6.7 Complete list SSDA with increased and decreased abundance post-acute exposure to 2.5ppb of imidacloprid after 1h against their respective controls**.**

Table 3.6.8 Complete list SSDA with increased and decreased abundance post-acute exposure to 2.5ppb of imidacloprid after 4h against their respective controls**.**

Table 3.6.9 Complete list SSDA with increased and decreased abundance post-acute exposure to 2.5ppb of imidacloprid after 24h against their respective controls**.**

Table 3.6.10 Complete list SSDA with increased and decreased abundance post-acute exposure to 10ppb of imidacloprid after 1h against their respective controls**.**

Table 3.6.11 Complete list SSDA with increased and decreased abundance post-acute exposure to 10ppb of imidacloprid after 4h against their respective controls**.**

Table 3.6.12 Complete list SSDA with increased and decreased abundance post-acute exposure to 10ppb of imidacloprid after 24h against their respective controls**.**

Table 3.6.13 Complete list of functional enrichment results obtained on the platform STRING based on the list of SSDA proteins identified post-acute exposure to 2.5ppb clothianidin 1h. Gene ontology terms are ranked based of % coverage of identified and background genes.

Table 3.6.14 Complete list of functional enrichment results obtained on the platform STRING based on the list of SSDA proteins identified post-acute exposure to 2.5ppb clothianidin 4h. Gene ontology terms are ranked based of % coverage of identified and background genes.

Table 3.6.15 Complete list of functional enrichment results obtained on the platform STRING based on the list of SSDA proteins identified post-acute exposure to 2.5ppb clothianidin 24h. Gene ontology terms are ranked based of % coverage of identified and background genes.

Table 3.6.16 Complete list of functional enrichment results obtained on the platform STRING based on the list of SSDA proteins identified post-acute exposure to 10ppb clothianidin 1h. Gene ontology terms are ranked based of % coverage of identified and background genes.

Table 3.6.17 Complete list of functional enrichment results obtained on the platform STRING based on the list of SSDA proteins identified post-acute exposure to 10ppb clothianidin 4h. Gene ontology terms are ranked based of % coverage of identified and background genes.

Table 3.6.18 Complete list of functional enrichment results obtained on the platform STRING based on the list of SSDA proteins identified post-acute exposure to 10ppb clothianidin 24h. Gene ontology terms are ranked based of % coverage of identified and background genes.

Table 3.6.19 Complete list of functional enrichment results obtained on the platform STRING based on the list of SSDA proteins identified post-acute exposure to 2.5ppb

imidacloprid 1h. Gene ontology terms are ranked based of % coverage of identified and background genes.

Table 3.6.20 Complete list of functional enrichment results obtained on the platform STRING based on the list of SSDA proteins identified post-acute exposure to 2.5ppb imidacloprid 4h. Gene ontology terms are ranked based of % coverage of identified and background genes.

Table 3.6.21 Complete list of functional enrichment results obtained on the platform STRING based on the list of SSDA proteins identified post-acute exposure to 2.5ppb imidacloprid 24h. Gene ontology terms are ranked based of % coverage of identified and background genes.

Table 3.6.22 Complete list of functional enrichment results obtained on the platform STRING based on the list of SSDA proteins identified post-acute exposure to 10ppb imidacloprid 1h. Gene ontology terms are ranked based of % coverage of identified and background genes.

Table 3.6.23 Complete list of functional enrichment results obtained on the platform STRING based on the list of SSDA proteins identified post-acute exposure to 10ppb imidacloprid 4h. Gene ontology terms are ranked based of % coverage of identified and background genes.

Table 3.6.24 Complete list of functional enrichment results obtained on the platform STRING based on the list of SSDA proteins identified post-acute exposure to 10ppb imidacloprid 24h. Gene ontology terms are ranked based of % coverage of identified and background genes.

Chapter IV

Investigating lethal and sublethal effects of chronic and acute exposure to clothianidin on the buff tailed bumblebee *Bombus terrestris*.

4.1. Introduction

4.1.1. Bumblebee foraging activity and exposure risks to pesticides

When studying the effects of any toxic chemical on an organism it is important to ask the question, "can exposure really occur in the field?" If the answer is yes, the second question, "How does that exposure occur?" To provide a satisfactory explanation it is necessary to understand the relationship between an organism and its environment, and in the case of bees, foraging behaviour and its characteristics, must be taken into account.

Through the activation of their thoracic muscles, bumblebees can raise their body temperature to above 20ºC, which enables them to forage at low temperatures, near to the freezing point. A correlation with body size also exists, with queens being able to forage at the lowest temperatures, followed by males and workers. Colony age also plays an important role regulating foraging activity, as young colonies tend to produce smaller workers, which can then suffer from reduced foraging, depending on weather conditions. Among the physical factors limiting foraging, it is light and temperature that show the greatest impact, with temperature being the main limiting factor in the morning and both, temperature and light, playing a role in regulating foraging during the evening. Rain is known to decrease activity, even in low intensity, thus incapacitating flight, and reducing the amount of food collected by the colony (Lundberg, 1980).

In contrast to honeybees, bumblebees do not seem to communicate foraging information through movement, or to what has become known as the honeybee "waggle dance", thus resource location seems to rely exclusively on individual exploration of the area. A successful forager transmits the scent of flowers with abundant pollen production to the colony, which then stimulates more foraging (Dornhaus and Chittka, 1999). Although there is no transmission of information through dance in bumblebees, they are more likely to develop optimal foraging routes, as they use trapline foraging strategies, consisting of individuals following memorised routes, known to be profitable, which increases rewards per plant visits and decreases search time (Saleh and Chittka, 2007; Pasquaretta *et al.*, 2017). *B. terrestris* is known to travel more than *B. pascuorum* and *B. muscuorum* which are thought to be regionalists (Osborne *et al.*, 2008). These bees travel distances up to 1.5 km in diverse floral environments, but have been shown to reach distances of 4 km away from their colonies of origin, when resources are scarce (Cresswell *et al.*,2000). Through foraging, bees collect their food to ensure colony growth and survival. However it is also through this indispensable activity to their development,

that bees become exposed to chemical pesticides, used as crop protection products to fight pests (Botías *et al.*, 2015).

B. terrestris fly at speeds of up to 15.7 km/h (Osborne *et al.*, 1999) and the duration of a foraging flight was found to vary between 58.5 ±2 min (Minahan and Brunet, 2018) or from 66 ± 4.6 min to 82 ± 3.7 min when resource availability was taken into consideration (Westphal *et al.*,2006). They can make about 6 foraging trips a day (5.9 ± 0.4) (Minahan and Brunet, 2018) which along with long distances travelled, increases their profitability but also their exposure frequency to crop protection agents.

During the time bees are outside of the colony, they become vulnerable to environmental stressors, including pesticides, and can be exposed via contact or orally. The contact exposure occurs directly when the bees are topically exposed to pesticide particles sprayed out of aircraft, or by contact with other workers and floral parts. The ingestion exposure occurs when bees ingest contaminated pollen, nectar and surface water (Blacquière *et al.*, 2012; Sanchez-Bayo and Goka, 2014). As these contaminants are distributed throughout the environment, and neonicotinoid residues have been found to accumulate to concentrations that reach part per million (ppm) levels in guttation fluids (Alkassab and Kirchner, 2017) it becomes impossible to ignore the risk posed to bees. Exposure becomes even more likely to happen due to the fact that bumblebees make several foraging trips a day, increasing risk and daily exposure based on the number of foraging trips (Sánchez-Bayo *et al.*, 2016a).

After being exposed in the field, bees can also carry the potentially toxic particles back to the colonies, where pesticides become part of the nectar pots used for feeding, and can also be transferred to the wax used to build egg cells, meaning that the entire colony is exposed in addition to individual foragers. This complicates the exposure scenarios that beneficial pollinators are susceptible to, which means that when assessing pesticide effects on beneficial arthropods, a complex scenario of exposure needs to be considered (Desneux *et al.*, 2007; Sánchez-Bayo *et al.*, 2016).

4.1.2. Acute versus chronic exposure assessing damage

In toxicology, acute exposure is defined as a one-time application of a toxic substance, and the respective assessment of its effects during the first hours after exposure (usually up to 24h or 48h), without repetition of application. It is a measurement of short-term effects that can be sometimes restricted to mortality, but include endpoints related to behaviour and physiology (Waller and Allen, 2008). It is important to assess acute exposure, once it allows us to investigate potential negative effects triggered by the

chemical immediately after it is ingested by the organism, without summation of effects or interference from other chemicals. As bumblebees spend about 1 hour or more foraging (Spaethe and Weidenmüller, 2002), it is likely that they suffer from acute exposure, which may influence their individual performance, and homing capabilities immediately after becoming exposed.

In addition, chronic exposure is defined as repeated or continuous exposure to any hazardous substance during a longer period, which may be extended throughout the life of an organism. Assessing chronic exposure of individuals, provides evidence that can be linked not only to homing and memory performance, but also to the overall development of the colony, as chronic exposure usually affects the life span, reproduction, behaviour and physiology of exposed organisms (Waller and Allen, 2008).

The determination of chronic toxicity on bees is limited, because the results of most studies are discussed in general terms, with conclusions being drawn that ignore or fail to consider the nature of the chemical being tested (different insecticides are interpreted as being the same), the level of exposure (chronic or acute and sometimes not mentioning final dose or even length of exposure), and exposure route (oral or contact). Neonicotinoid toxicity has been demonstrated with clothianidin, imidacloprid and thiamethoxam affecting *B. terrestris* colony performance, nest reproduction, provisioning efficiency, foraging performance, fecundity, homing capacity, neuronal vulnerability and mitochondrial function when exposed chronically (Laycock *et al.*, 2012; Larson *et al.*, 2013; Laycock and Cresswell, 2013; Fauser-Misslin *et al.*, 2014; Feltham *et al.*, 2014; Moffat *et al.*, 2015a; Stanley *et al.*, 2016; Colgan *et al.*, 2019). However, differences in exposure length, pesticide type and concentration vary wildly.

To date there is no accepted standard protocol used to assess pesticide damage on bees and replicating the complexity of the natural environment in a controlled laboratory setting is very difficult. Currently the reality of bee testing involves a variety of protocols and methods that not always allow for conclusive evidence of pesticide damage, nor even satisfactory comparison of data and results obtained, as many factors are not standardised, including the mode and intensity of exposure, and the use of field relevant concentrations (European Food Safety Authority, 2012).

The Organization for Economic Co-operation and Development (OECD) through continuous efforts has attempted to develop guidelines that standardise research and the risk assessment of new chemicals. A point often made, is that the volume of sucrose solution used to deliver the pesticide cannot be too small, like the 10µl suggested by van der Steen (2001) as depending on concentration used, the exposure level would be too

high, and not representative of the reality in the field (European Food Safety Authority, 2012).

Mommaerts and Smagghe (2011), proposed a risk assessment method based on tier levels of investigation that include exposure of individuals in microcolonies in the lab environment, to full colonies in the field, aiming at standardising testing to allow comparison between different pesticides in the future. This document, along with the OECD guidelines for bumblebee testing, provide useful insight that should be considered during the design of experiments (*Test No. 247: Bumblebee, Acute Oral Toxicity Test*, 2017).

However, investigating sublethal effects remains a big challenge considering the myriad of exposure scenarios, especially when most research carried out with bees does not account for final exposure level, resulting in difficulties with the comparison of results (European Food Safety Authority, 2012). To account for final exposure in a field relevant manner, it is necessary to consider residue levels found in nature, for the development of protocols that test correct concentrations and use appropriate exposure methods that minimise individual stress.

This chapter will explore three oral exposure scenarios that aimed at investigating tier 1 effects on mortality and sublethal effects of clothianidin exposure on *B. terrestris* workers, using behavioural assessment techniques and expanding the molecular data currently available for this system.

4.2. Chapter aims and hypothesis

The aim of this chapter was to compare the impact of three lengths of exposure to clothianidin on the behaviour, and physiology of workers of *B. terrestris.* We aimed to investigate the differences between acute exposure and chronic low and high intensity exposure, to field relevant doses of the agonist neonicotinoid clothianidin. Acute exposure to clothianidin was compared with chronic exposure during five days in two different settings, one considering low, once a day exposure, and one considering high, or continuous exposure to contaminated sugar solution for 5 days.

The specific aims are:

1. To compare length and intensity of field relevant exposure to clothianidin on the mortality of *B. terrestris* workers.
2. To investigate the effect of clothianidin exposure on activity, learning and memory of bumblebees through feeding experiments.

3. To compare the brain proteome of workers exposed acutely or chronically to clothianidin for five days, at 1 and 24 h after the moment of the last exposure.
4. To analyse the expression levels of selected genes, after acute and chronic continuous exposure to clothianidin, for five days using qPCR.

We hypothesize that toxicity to clothianidin increases with final exposure, which is directly related to exposure length. Thus, increasing the presence of markers of toxicity and damage, the longer and more continuous the exposure.

4.3. Experimental outline and methods

4.3.1. Colony maintenance *Bombus terrestris audax*

Commercial colonies (two week young research colonies) of *B. terrestris audax* were obtained from Biobest (supplied by Agralan UK) and maintained at standard environmental conditions of 25°C ± 1°C and 60 % ± 5 % relative humidity with continuous darkness or red light and fed a sugar solution (Biobest) *ad libitum* and 2 g of pollen (Biobest) every day. Colonies where the founding queen had died (in transit) or where males had started to emerge from pupal cells, were not used for any molecular experiments. Colonies were acclimatised at laboratorial conditions for at least 48h before beginning the experiments.

4.3.2. Micro colony experiments of pesticide exposure

Workers were removed from four different colonies and assigned to their respective control or treatment groups. Bees were colour-tagged using a water-based paint to allow for tracking within colonies, before being transferred to a plastic box (w 16,5cm; h 8,5cm; d 12cm) (max 10 bees per box (2 or 3 from each colony)). Autoclaved fine sand was used to cover the bottom of the boxes and pollen and 50% sucrose (w/v) were provided *ad libitum.* Bees were acclimatized overnight at the same environmental conditions described above, before continuing the experiment.

4.3.2.1. Acute exposure

Acute exposure was performed after a period of 2-hour starvation. Thirty-five microlitres of experimental solutions were fed to each bee individually and only bees that fully consumed the solutions were used in the experiments. The procedural control group received 35 µl of a 50 % sucrose (w/v) with <1 % acetone solution to account for the toxicity, if any, of the solvent used for the preparation of insecticide solutions

(Organisation for Economic Co-operation and Development., 1998)(*Test No. 247: Bumblebee, Acute Oral Toxicity Test*, 2017). A clothianidin concentration of 2.5 ng/ml was tested. Exposed and control bees were returned to their plastic boxes, once the micro droplet of the solution was fully consumed, and experimental parameters were assessed as relevant at different timepoints, according to the chosen technique. Acute exposure was assessed using quantitative real time polymerase chain reaction (qPCR) and mass spectrometry-based proteomics. Brain samples were dissected 1 and 24 h post exposure and immediately homogenised in each respective lysis buffer as described below.

4.3.2.2. Chronic low intensity exposure

Chronic low intensity exposure was performed after a period of 2-hour starvation daily for a period of 5 days. Thirty-five microlitres of experimental solutions were fed to each bee individually and only bees that fully consumed the solutions were used in the experiments. The procedural control group received 35 µl of a 50 % sucrose (w/v) with <1 % acetone solution to account for the toxicity, if any, of the solvent used for the preparation of insecticide solutions (Organisation for Economic Co-operation and Development., 1998)(*Test No. 247: Bumblebee, Acute Oral Toxicity Test*, 2017). Solutions of 2 ng/ml clothianidin concentration were used. Each day exposed and control bees were returned to their plastic boxes once they had consumed the micro droplet and allowed to feed from uncontaminated sucrose solution and pollen *ad libitum.* Individuals that were exposed to chronic low concentration of clothianidin were used in mortality, behavioural and proteomic experiments. Brain samples were dissected 1 and 24 h after the fifth day of exposure and prepared according to section 4.3.5.

4.3.2.3. Chronic continuous exposure

Chronic continuous or high intensity exposure for 5 days, was initiated after a period of 2-hour starvation. Concentrations of 2 ng/ml, 10 ng/ml and 35 ng/ml of clothianidin in 50 % sucrose solution w/v were prepared daily and provided to the test boxes in the feeders *ad libitum.* Each day, solutions were replaced to ensure complete homogenisation. Consumption, bee mortality and activity patterns were also recorded daily. Brains were dissected at 1 h and 24 h after the experiment ceased, once 5 days of exposure were completed, and immediately homogenised. Chronic continuous exposure samples were used for proteomic and qPCR analysis and prepared according to sections 4.3.5 and 4.3.9.

4.3.3. Mortality analysis

To assess the effects of chronic neonicotinoid exposure at the mortality levels of workers of *B. terrestris*, 4 different clothianidin concentrations, and two methods of exposure were chosen. Bees were exposed for 5 days to 2 ng/ml, 10 ng/ml and 35 ng/ml of clothianidin continuously (chronic continuous exposure), and to daily doses of 35 µl of a 2 ng/ml clothianidin concentration (chronic low intensity exposure). Mortality was assessed every 24 hr for a period of 5 days. Dead bees were removed each day, and their absence was taken into consideration when calculating daily sucrose consumption. A total of 30 bees per treatment, divided into 3 technical replicate boxes (n=10 each), were initially used. Dilutions were made in 50 % w/v sucrose solution, and the highest concentration of acetone was used to prepare the procedure control, making sure that such concentration never surpassed 1 % as per the guidelines followed (Organisation for Economic Co-operation and Development., 1998; *Test No. 247: Bumblebee, Acute Oral Toxicity Test*, 2017). Bees that did not consume their daily drops in the low intensity exposure were discarded. Survival curve analysis was performed using Prism GraphPad v.5 and percentage survivability was reported.

4.3.4. Behaviour, learning and memory assessment analysis

Behavioural assessment was carried out through qualitative analysis of activity and quantitative analysis of associative learning, using bumblebee workers that were chronically exposed to clothianidin.

The activity analysis was performed based on observations of the level of movement detected in bees, during the duration of the experiment, in comparison to the procedure control bees of the same batch. The degree of movement was interpreted as a function of the number of bees walking around the cage, the speed of movement and the number of bees showing defensive or flying behaviours. The standard level was obtained from the procedure control bees where most would walk around the box at medium speed, and only one or two would be observed flying (eventually with short duration flights), and 4 or 5 out of 10 would show defensive behaviour, raising their front legs when an object passed nearby. The test of defensiveness was carried out by passing a pencil around bees for 30 seconds. Boxes with the level of activity described above received the mark of three asterisks (***). A box that comprised bees with decreased movement speed and flying, received two or one asterisks. Boxes with bees that showed an overall increase in speed of movement and flying, were scored with up to five asterisks.

To assess possible learning through association or memory activation, we developed a method in which bees subjected to chronic low intensity clothianidin exposure, would be faced with a challenge of finding their sucrose drop. The experiments were carried out daily for five days, after a period of 2-hour starvation each day. The drop served as a reward for starved bees and was always positioned at the centre of a small plastic box, representing a neutral environment. Eight bees were exposed at the same time, and the total duration of exposure was noted and divided by the number of bees that successfully consumed the drop, obtaining an average estimate time, of consumption duration per bee. The experiment was carried out with 6 technical replicates and comparison of the response curve between treatment and procedural control was carried out using Prism GraphPad v.5 (two-way ANOVA with a post hoc Bonferroni test).

4.3.5. Sample preparation for mass spectrometry-based proteomics

Brains from each treatment (not pooled, biological replicates) were removed into 200 µl of 6 M urea, 2 M thiourea buffer, supplemented with a protease inhibitor cocktail (Roche), and immediately snap frozen using liquid nitrogen. Samples were stored at -20 ºC until the dissections were completed. After thawing on ice, brains were homogenised and centrifuged for 5 min at 9,000 x g to pellet any cellular debris.

The supernatant was then removed and quantified using the Qubit™ protein quantification system (Invitrogen), following the manufacturer's instructions. Samples were chosen accordingly to the most similar protein quantities and band patterns obtained on the Qubit, and on an 1D SDS page gel. Eighty-five micrograms of each sample were precipitated using the 2D Clean Up Kit (GE Healthcare), following the manufacturer's instructions. The resulting protein pellet was resuspended in 50 µl of 6 M urea, 2 M thiourea, 0.1 M Tris-HCl, pH 8.0 buffer, and quantified using the Qubit™ system. 105 µl 50 mM ammonium bicarbonate was added to 20 µl of each sample. Proteins were reduced with 0.5 M of dithiothreitol (DTT) at 56 ºC for 20 min and alkylated with 0.55 M iodoacetamide (IAA) at room temperature for 15 min, in the dark. One microliter of a 1 % w/v solution of Protease Max Surfactant Trypsin Enhancer (Promega) and a 1 µg Sequencing Grade Trypsin (Promega, 0.5 µg/ µl stock) was added to give an approximate protein:trypsin ratio of 40:1. The protein/trypsin mixture was incubated at 37ºC overnight. Digestion was terminated by adding 1 µl of 100 % trifluoroacetic acid (Sigma Aldrich) and incubation at room temperature for 5 min. Samples were centrifuged at

13,000 x g for 10 min and the supernatant was removed and purified for mass spectrometry using C18 Spin Columns (Pierce), following the manufacturer's instructions. The eluted peptides were dried using a Speedy Vac concentrator (Thermo Scientific Savant DNA120) and resuspended in 2 % v/v acetonitrile and 0.05 % v/v trifluoroacetic acid (TFA). Samples were sonicated for 2 min to aid peptide resuspension followed by centrifugation at 13,000 x g for 5 min. The supernatant was removed and a volume equivalent to 1 µg was loaded onto a QExactive (ThermoFisher Scientific) high-resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system.

4.3.6. Proteomic analysis

Protein profiles were determined in three biological replicates for each control and treatment group at each timepoint (n=3 colonies), using label-free, mass spectrometry-based proteomic profiling. Peptides were separated on a 2 % to 40 % acetonitrile gradient on a 500 mm length EasySpray PepMap column, using a 180 minutes' reverse-phase gradient at a flow rate of 250nL min^{-1}. All data were acquired with the mass spectrometer operating in automatic data dependent switching mode. A full MS scan at 140,000 resolution and range of 300–1700 m/z was followed by an MS/MS scan, resolution 17,500 and range of 200–2000 m/z, selecting the 15 most intense ions prior to MS/MS.

4.3.7. Protein identification and abundance level quantitation

Protein identification and LFQ normalisation of MS/MS data was performed using Max-Quant v1.6.5.0 (http://www.maxquant.org) following the general procedures and settings outlined in (Hubner *et al.*, 2010). The Andromeda search algorithm (Cox *et al.*, 2011) incorporated in the MaxQuant software was used to correlate MS/MS data against the NCBI database for *B. terrestris* (UniProt Consortium, 2015)(entries, downloaded May 2017, duplicate and redundant sequences were removed resulting in 10584 entries) a contaminant sequence set provided by Max-Quant. Prior to the proteomic searches the *Bombus* sequence database was modified so that each protein annotation included the homologous Uniprot ID for *Apis mellifera* and *Drosophila melanogaster*. The following search parameters were used: first search peptide tolerance of 20 ppm, second search peptide tolerance 4.5 ppm with cysteine carbamidomethylation as a fixed modification and N-acetylation of protein and oxidation of methionine as variable modification and a maximum of two missed cleavage sites allowed. False Discovery

Rates (FDR) were set to 1 % for both peptides and proteins and the FDR was estimated following searches against a target decoy database. LFQ intensities were calculated using the MaxLFQ algorithm (Cox *et al.*, 2014) razor and unique peptides with a minimum ratio count of two peptides across samples. Peptides with a minimum length of seven amino acids were considered for identification and proteins were only considered identified when more than one unique peptide for each protein was observed.

4.3.8. Proteomic data analysis

Perseus v.1.5.5.3 (http://www.biochem.mpg.de/5111810/perseus), was used for data analysis, processing and visualisation. Normalised LFQ intensity values were used as the quantitative measurement of protein abundance for subsequent analysis. The data matrix was first filtered for the removal of contaminants and peptides only identified by site. LFQ intensity values were $\log_2$ transformed and each sample was assigned to its corresponding group with each experimental dataset being analysed individually.

Proteins not found in three out of three replicates in at least one group were omitted from the analysis. Principal component analysis was performed on each dataset with all replicates. However, individual t-tests were carried out per timepoint. A data imputation step was conducted to replace missing values with values that simulate signals of low abundant proteins chosen randomly from a distribution specified by a downshift of 2.1 times the mean standard deviation (SD) of all measured values and a width of 0.1 times this SD. Student's t-tests were performed for each timepoint to identify statistically significant differently abundant proteins (SSDA) (-log p value $\geq$ 1.3).

Volcano plots were generated in Perseus by plotting the negative log p-values on the y- axis and $\log_2$ fold-difference values on the x-axis for each pair-wise comparison to visualise changes in the total brain proteome.

The Uniprot ID for *Drosophila melanogaster* for all statistically significant proteins (SSDA) was extracted from the identifier string and used to query the STRING v 11 database. Medium network specificity value was applied on the analysis and GO term enrichments obtained for biological process, molecular function and cellular compartments were ranked based on number of genes identified in the dataset in relation to the total number of background genes for that gene ontology (% coverage).

4.3.9. Sample preparation for real time quantitative polymerase chain reaction

Following acclimatisation of worker bees as described in section 4.3.1 and clothianidin acute and chronic continuous exposure, as described above, 2 bumblebee brains per treatment (2 procedure controls 2 acute exposure, 2 chronic continuous exposure and 2 chronic procedure control, n=8) were dissected 1h post exposure and immediately homogenised in 300 µl of RNA extraction, lysis buffer/2 mercaptoethanol mixture (GenElute total RNA miniprep kit – Sigma Aldrich). RNA extraction was performed according to the manufacturer instructions for tissue lysates. After extraction, isolation and elution steps, total mRNA was quantified using a NanoDrop1000 (ThermoScientific). cDNA was synthesised from 1 µg of mRNA per strain using the "SuperScript First Strand Synthesis System for RT-PCR" (Invitrogen).

4.3.10. Real time quantitative polymerase chain reaction

The qPCR reaction mixtures were prepared for 13 different genes using the forward as reverse primers present in table 4.1, using a LightCycler 480 SYBR Green I Master mix and a LightCycler 480 qPCR machine (Roche) was used to determine gene expression levels, using a 40S ribosomal protein coding gene as a reference (XP_003393797.1). Bar charts were drawn to represent the mean data of two combined biological replicates and 3 combined technical replicates per strain, ± s.d (n=6). A two-sample unpaired Student t test was used to determine statistically significant values.

Table 4.1 Primer sequences for genes of interest used in the RT-qPCR experiments.

Protein encoding gene name	NCBI accession	Forward primer	GC content	Salt adjusted temperature	Reverse primer	GC content	Salt adjusted temperature	Product size (base pairs)
40S ribosomal protein (REFERENCE GENE)	XP_003393797.1	GTGGACAGAGAGCAAAATCC	50	58.4	GTCGAAGAAGGACATGACG	53	57.5	108
GTP-binding protein	XP_003403094.1	CAGTGAAGTCGCATTACTTCG	48	59.5	GTGTACTCTGTTTGGCGCG	58	59.5	113
Neural ectodermal development factor	XP_020721065.1	CATCAGCACGTTCCTCTAC	53	57.5	GATAGCCAGAGATCGGATTG	50	58.4	106
Actin	XP_003394490.1	CCGTATGGATCGGTGGATC	58	59.5	GAAGCACTTGCGGTGAACG	58	59.5	112
Synaptic vesicle glycoprotein	XP_003398712.1	GCT ATT TCC ATG GTC GCG G	58	59.5	CCACCTATTAGAAGAGCAGC	50	58.4	119
Calcium channel flower	XP_003398827.1	GCTTATTTGGCAGTGGTCTG	50	58.4	CCTCGATATTCGTCGCTGC	58	59.5	111
ATP synthase subunit epsilon	XP_003399456.1	GCTGCCAGACTTGTTAGAC	53	57.5	GTTTACCATCTTTCCATTGGG	43	57.5	103
NADH dehydrogenase	XP_003402322.1	CTTGGGTTGAAGGATCCTTG	50	58.4	CAAACCCGACTAGCATACC	53	57.5	118
Frequenin-1	XP_003396256.1	CGCGTGCGAACTATGTTAC	53	57.5	GAGGTTGTCGGGTCAATTTG	50	58.4	109
Vitellogenin	XP_012163499.1	CGAGGCGAAGAATCTAAAGAC	48	59.5	GCGATGTACTCCTGGTGC	60	58.4	117
Cytochrome P450 9e2	XP_003393865.1	CAAGGCTCAGATGGATTAACC	48	59.5	CGAAAACTTCTGGATCGATCC	48	59.5	109
Myosin light chain	XP_012172045.1	GACCCAGAAGATGACGAAG	53	57.5	GATGGATGTATACCGCATAATG	41	58.4	114
Acetylcholinesterase	XP_012173033.1	GAAAGTAGTAGCGGCACAG	53	57.5	GTATTCGCAGTCAGCGTTTC	50	58.4	121

4.4. Results

To determine the effects of chronic clothianidin exposure on bees, and how these effects differ to those of acute exposure, bumblebees were exposed using three different methodologies. The first group were of bees exposed acutely. In the second, bees were exposed to a unique dose of the neonicotinoid every day, for five days. And the third comprised a higher intensity exposure, with contaminated solution being available *ad libitum* for five days. We were able to assess the changes in bumblebee survivability and feeding behaviour in the presence of the xenobiotic, and to develop a rapid way of inferring learning and conditioning in worker bees. In addition, we also characterized thousands of proteins in the brain proteome and assessed the expression levels of target genes in exposed bees.

4.4.1. Analysis of the toxic potential of clothianidin under three different modes of exposure on the survivability of bumblebee workers

The purpose of this experiment was to address the question of whether clothianidin changes the survival rate of worker bumblebees when exposure frequency is increased. This was achieved by analysing the results of the toxicological experiments that exposed worker bees to clothianidin in three different scenarios. Acute exposure to a unique dose of clothianidin resulted in 100 % survivability of tested individuals. No sublethal effects were observed after 4 h of exposure, independently of the dose tested (Chapter III) and bees that survived the first four hours of the experiment, were considered to have recovered. When the intensity of exposure was increased to once a day for 5 days, using a concentration similar to that used in Chapter III (2 ng/ml), no mortality was recorded (Figure 4.1, purple). These bees had been given a microdroplet of 35 µl a day, during five days, resulting in a daily exposure of 0.07 ng per bee. Bee activity was qualitatively measured in the context of the microcolonies, which considered speed and movement of individuals in the boxes, as well as the number of flying bees and number of individuals that exhibited defensive behaviour. Although bees of the treatment were initially more active, no significant difference was seen between the treatment group of 0.07 ng/bee/day and the procedure control during the rest of the experiment (Table 4.2).

Increasing the frequency of exposure to continuous consumption of the contaminated solution at 2 ng/ml (2 ppb) and 10 ng/ml (10 ppb), resulted in no statistically significant increase in mortality (Figure 4.1). A single death was observed at the 10 ppb exposure,

bringing the percentage of survival to 96.6 % for that group, a level that was sustained until the end of the experiment, 4 days later.

The analysis of the activity suggests that when exposed continuously to clothianidin solutions, at concentrations from 2 ng/ml to 10 ng/ml, worker bumblebees increase their activity in the microcolony environment from the second day onwards. This increased activity is observed predominantly when individuals in the microcolonies are compared with the procedural control and speed of movement, defensiveness and flying, are taken into consideration. The qualitative observation suggests that at those concentrations, clothianidin increases the activity of exposed bees. The period of increased activity lasted for 3 days in bees exposed to 2 ppb but remained until day 5, to bees exposed to 10 ppb (Table 4.2). Such a phenomenon suggests that the higher concentration triggers a longer-lasting effect, but it is not clear if the increase in activity suggests an improvement in bee performance, or a negative adaptation to prolonged xenobiotic exposure.

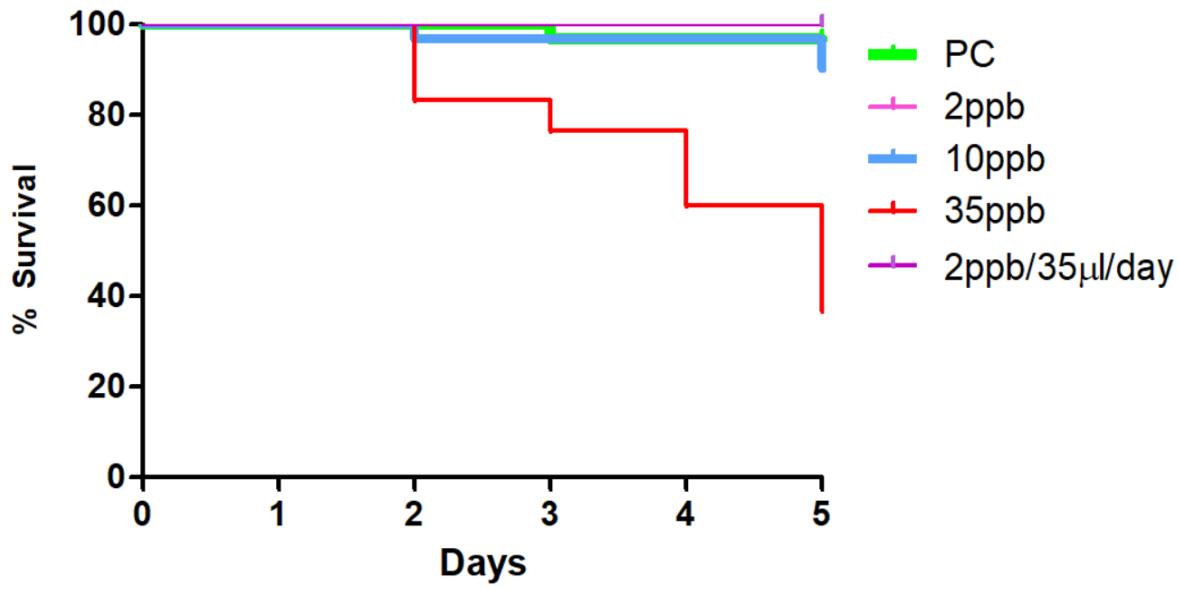


Figure 4.1 Proportion of comparative survival obtained from experiments simulating three scenarios of exposure to clothianidin. PC (green) represents the procedure control group exposed only to the solvent used in experiments (<1% acetone), resulting in the survival of 29 tested subjects (96.6 %). Group labelled as 2 ppb (pink), represents bees exposed to a solution of 2 ng/ml of clothianidin *ad libitum* for 120 hours. Such exposure resulted in the survival of 100% tested subjects (n=30) for 5 days. 10 ppb (blue) represents the group exposed *ad libitum* to a solution containing 10ng/ml of clothianidin for 5 days, 96.6 % of the tested individuals (n=30) survived the 120 hours of exposure. The 35 ppb group was exposed continuously to a solution of 35 ng/bee for 5 days. Exposure resulted in a survival rate falling below 40 % within 5 days of the experiment (36.6 % of 30 individuals tested).

Mortality significantly increased from day 2 onwards, in bees allowed to feed freely from a sucrose solution containing 35 ng/ml of clothianidin, resulting in a 36.6% survival rate at day 5 of the experiment (Figure 4.1). This drastic reduction on survival highlights the lethal potential of clothianidin which also increased the activity of bees from day 2 of the experiment, as seen in smaller concentrations. Some bees showed critical symptoms of intoxication during the first four hours of the experiment and were not considered for the activity assessment. Only individuals that displayed no severe changes in behaviour, such as showing involuntary contractions and spams, were used for the assessment of activity. At 35 ppb, clothianidin increased the movement speed of workers, similar to the 10 ppb treatment group. On the other hand, when activity returned to normal levels, the bees exposed to 35 ppb of clothianidin although active, were less responsive, with none showing defensive behaviour and only one in thirty, was found flying.

Table 4.2 Qualitative assessment of activity in worker bumblebees chronically exposed to clothianidin**.**

Days	1			2			3			4			5		
Replicates	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
PC	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
2ppb/35µl /day	****	***	***	***	***	***	***	***	***	***	***	***	***	***	***
2PPB	***	****	***	***	****	****	***	***	***	***	***	***	***	****	***
10PPB	***	***	***	****	****	****	****	****	****	****	****	****	***	***	***
35PPB	***	***	***	****	****	****	****	****	****	***	***	***	***	***	***

Changes in activity and behaviour were also assessed through the monitoring of feeding. Throughout the duration of the chronic exposure experiments, bees in the procedure control showed a reduction in consumption per day, as the experiment progressed (Figure 4.2 and 4.3). Such reduction was also seen at the treatments of low intensity chronic exposure (0.07 ng/bee/day or 2 ppb/35µl/day)(Figure 4.3) and high intensity chronic exposure to 2 ng/ml or 2 ppb (Figure 4.2) but were not present at the 10 or 35 ppb continuous exposure, which showed maintained or increased daily consumption levels respectively.

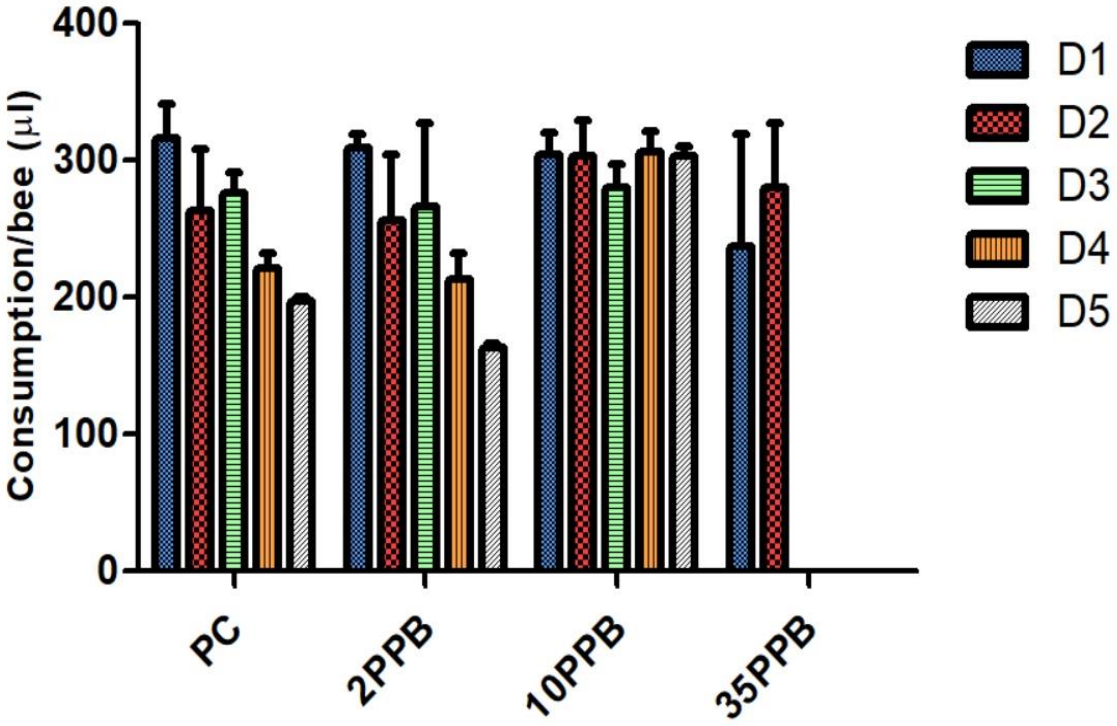


Figure 4.2 Consumption estimation per bee, based on the total volume of 50% w/v sucrose solution consumed daily in each treatment group, of the chronic free feeding experiment. The estimates were calculated based on the total number of bees in each replicate box and each graph bar represents the results of 3 technical replicates. Colours refer to each experiment day, and experimental groups are displayed in the x axis. Decrease in total consumption from day 3 onwards, can be seen in the procedure control (PC) and the 2 ppb treatment group. The average consumption at day 1 was of 316µl per bee for the procedure control and 309.5 µl for the 2 ppb group. For days 2,3,4 and 5 the values are 263, 276, 220 and 196 µl/bee for the PC group, and 256, 266, 213 and 163 µl/bee for the 2 ppb group. Bees given the solution laced with 10ppb of clothianidin showed steady and higher daily consumption than any other group tested (304, 303, 280, 306 and 303 µl/bee at days 1,2,3,4 and 5 respectively. Due to the high mortality levels obtained for the 35 ppb treatment group, no daily consumption was estimated for days 3, 4 and 5 of the experiment. High variability within replicates was seen at day one, resulting in an average consumption of 236 µl/bee and 280 µl/bee at day 2. Based on consumption rate and clothianidin concentration in each solution, a daily exposure rate was calculated for each treatment group (PC = 0 ng/bee of clothianidin; 2 ppb = from 0.326-0.619 ng/bee of clothianidin every 24 h, 10 ppb = from 2.8-3.06 ng/bee of clothianidin every 24 h and the 35 ppb group = 8.26-9.8 ng/bee every 24 h).

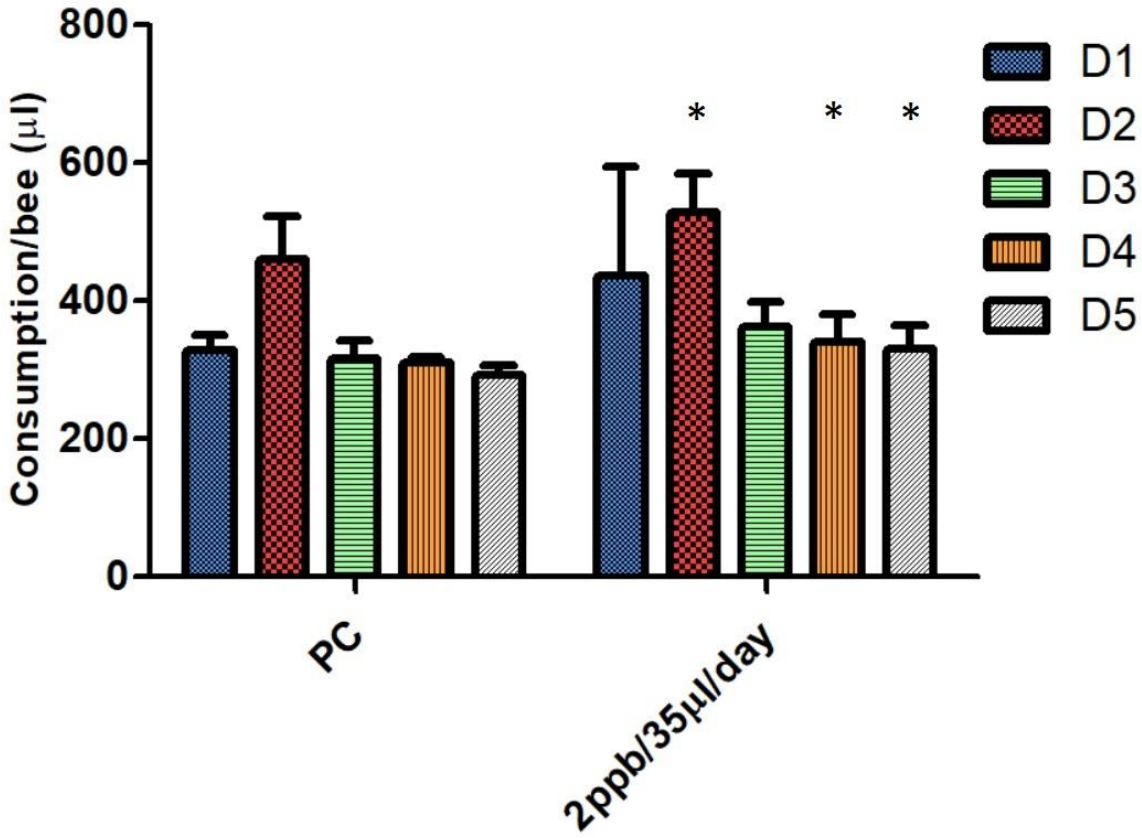


Figure 4.3 Consumption estimation per bee, based on the total volume of uncontaminated 50 % w/v sucrose solution consumed daily for the procedure control, and the group of bees exposed daily to a microdroplet of 35 µl of a clothianidin contaminated solution, at the concentration of 2 ppb (2 ng/ml). At this experiment bees had to be starved for two hours each day prior to being exposed to 35 µl of the test solution, after total consumption of the droplet both groups could feed *ad libitum* from uncontaminated solution, and the volume of such was recorded each day. Decreased consumption (feeding) can be noted during the final days of the experiment, but the highest daily consumption was achieved at day 2. Both treatment groups showed the same pattern of consumption and no statistically significant results were obtained from the comparison between treatments (two-way ANOVA $p > 0.05$). The reduction observed in bees exposed to clothianidin at D2 vs D4 and D2 vs D5 is statistically significant ($p < 0.05$) according to a Bonferroni post hoc test.

4.4.2. Could the chronic low frequency exposure method be used to assess learning, memory and conditioning in bumblebee workers?

The low frequency chronic exposure method involved transferring workers to small plastic boxes, which created a different exposure environment for the bumblebees. After a small acclimatisation period of two minutes, these bees were provided with a microdroplet of the test solution, which was always positioned in the centre of the box. The challenge here consisted of the bees finding and consuming the droplet, which would represent the completion of the task. The exposure was repeated every day for five days, and the time it took for all bees (n=8) in a microcolony to consume the drops individually, was recorded, and used for comparisons between replicates (n=6) and treatments (2).

Our results demonstrate that the test is appropriate when we consider that finding the drop is an activity that requires learning/conditioning, and the potential use of memory. The average time each bee took to find the first drops of sucrose in each group was above 40 and 60 seconds per bee, for the control and the 2 ppb group respectively (Figure 4.4). On the second day however, this time decreased to just over 20 seconds per bee. On the third day, the group being exposed to clothianidin showed a slight jump to over 30 seconds per bee while the time of consumption for the bees exposed to the procedural control solution did not increased, until the fourth day of the experiment, with a slight decrease observed on the last day. The consumption time increase observed on the third day, in the bees exposed to clothianidin, was maintained during day four, and a slight decrease was also seen at day five.

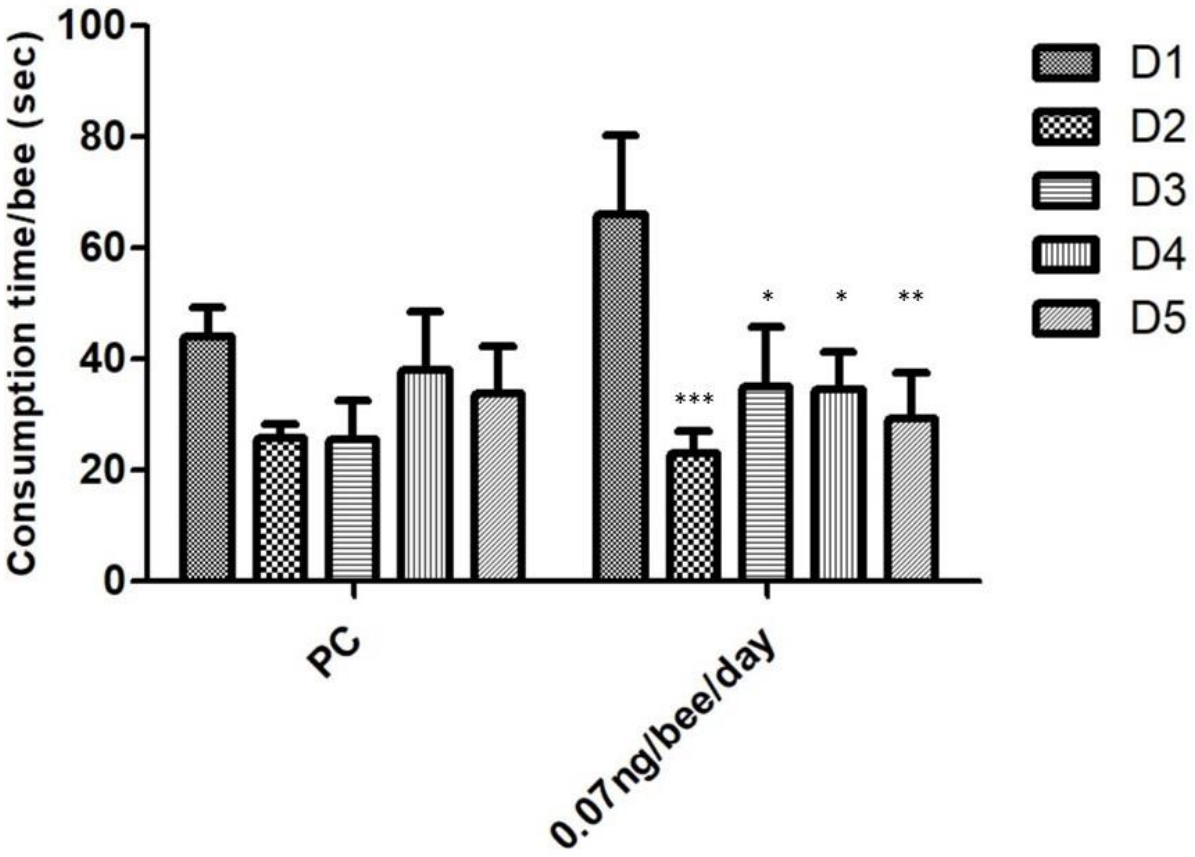


Figure 4.4 Daily consumption rate per bee calculated based on the time taken to expose all bees in each replicate box to 35 µl of the test solution. Each bar represents the average measurement obtained from dividing the total length of exposure by the number of bees per box, in 6 replicates. Different bar textures represent the five days of the experiment. Treatments are displayed on the x axis. This experiment represents an attempt to assess learning, conditioning and memory under clothianidin exposure. The reduction time seen between day 1 and 2 provide successful evidence that bees understand the task and respond by "remembering" what is expected of them when presented with the task for the second time. No statistically significant results were obtained when the treatment response was compared to the control (two-way ANOVA). However, statistically significant post differences were obtained from the post hoc Bonferroni test when Day 2 ($p < 0.001$), 3 ($p < 0.05$), 4 ($p < 0.05$),and 5 ($p< 0.01$) were compared with day 1 of the bees exposed to 0.07 ng of clothianidin per day.

We propose that the task of finding the drops across subsequent days is a simple way to test the bee's capacity to locate the drop each day and indicates the establishment of memory from the previous days. No statistically significant results were obtained from the comparison of the procedure control and the clothianidin exposed values, suggesting, that at the dose and mode of exposure tested, no detectable detrimental effect was observed on the learning and memory of the bumblebees exposed to clothianidin through our assessment method.

Notwithstanding, statistically significant results were obtained within the values for the exposed group, especially when days 1 and 2 were compared, suggesting that this

experimental approach can prove useful for assessing learning, conditioning and memory activation in bumblebee workers.

4.4.3. Utilising molecular data to investigate clothianidin effects on the worker bumblebee brain. Can proteins and genes help us understand how the brain physiology changes under 3 different scenarios of exposure to clothianidin?

Exposure to contaminated solution at the low-end field relevant concentration of 2 ppb (2 ng/ml) of clothianidin was chosen to investigate how the frequency and intensity of exposure alters the brain proteome of *B. terrestris* foragers. The scenarios chosen included; i) a low intensity chronic exposure where bees would receive a drop of 35 µl of the 2 ppb solution each day for 5 days and, ii) a high intensity exposure where the workers were fed the contaminated solution *ad libitum* continuously, for 5 days. A group of bees that received only an acute dose of 2.5 ppb was also analysed for comparison. Each treatment group had its own respective control and the proteomes were characterized for enriched gene ontology terms, as a way of obtaining biologically relevant information on how clothianidin affects the bumblebee brains, in different exposure scenarios.

Over two thousand identified proteins were retained after the filtering steps and used in the analysis of each timepoint and treatment group. Principal component analysis revealed the distribution of each replicate (n=3), according to treatment and timepoint, in relation to two different components. The acute experiment resulted in a clearer separation than the other two chronic experiments (Figure 4.5). Nonetheless, no group was sufficiently separated into a distinct cluster. After acute exposure a greater discrepancy was observed between the treatments at 24 h (Figure 4.5A), the same was true for the chronic continuous exposure (Figure 4.5C), whereas the chronic low intensity exposure, resulted in greater separation between replicates collected 1h after the last exposure, on the fifth day (Figure 4.5B).

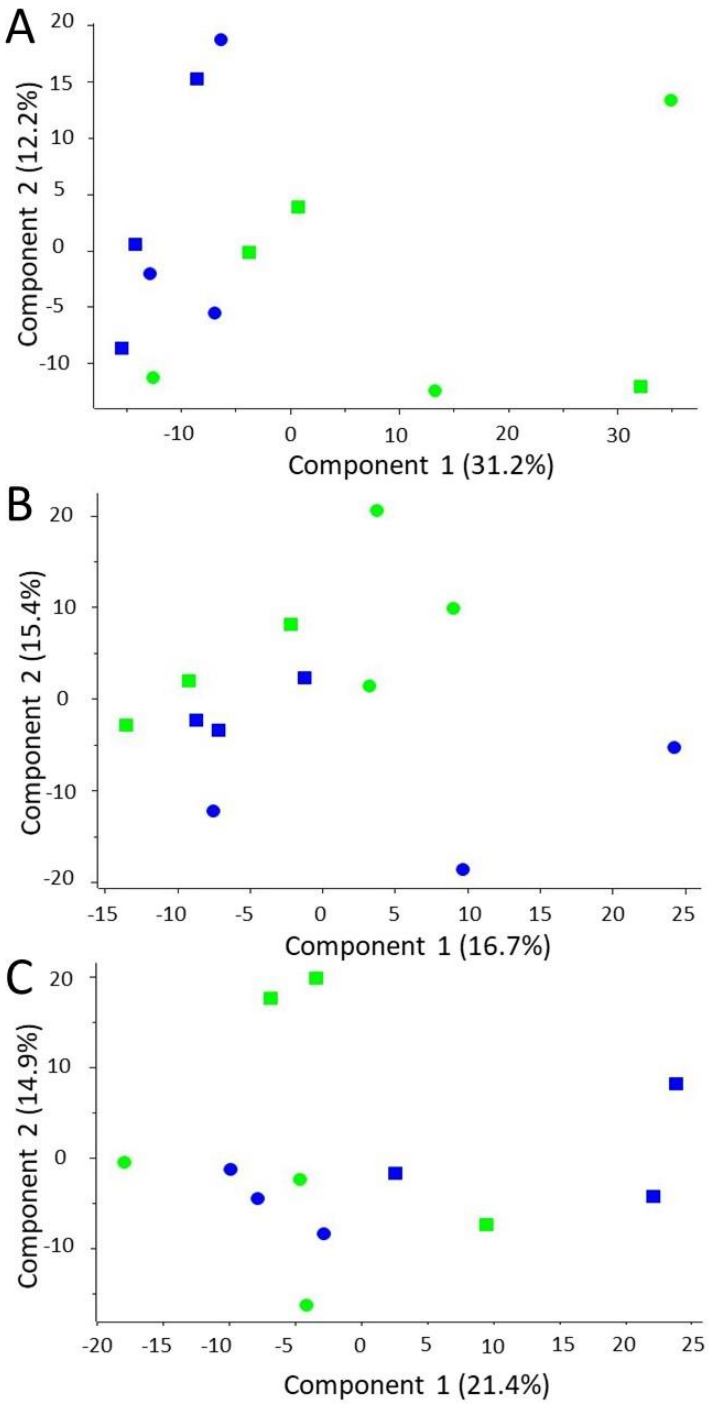


Figure 4.5 Principal component analysis utilizing the Benjamin-Hochberg FDR cut off 0.05 in all identified proteins in each dataset analysed. Colours refer to treatment and shapes to timepoints. Green = procedure control, Blue = clothianidin exposed. The filled squares represent the 1h replicates and the filled circles the 24 h replicates. (A) refers to the experiment with acute exposure to clothianidin, replicates exposed to the neonicotinoid (blue) occupy mainly the left side of the graph while the procedure control replicates are found on the right (green). Replicates are mainly differentiated by component 1 (31.2%). PCAs show greater variability in the replicates within the chronic low frequency exposure (B) and chronic continuous exposure (C) experiments. PCAs show that majority of the proteome does not change in response to the treatment, not separating replicates clearly when all proteins identified (2,011; 2,239 and 2,301 respectively for A,B and C) are considered.

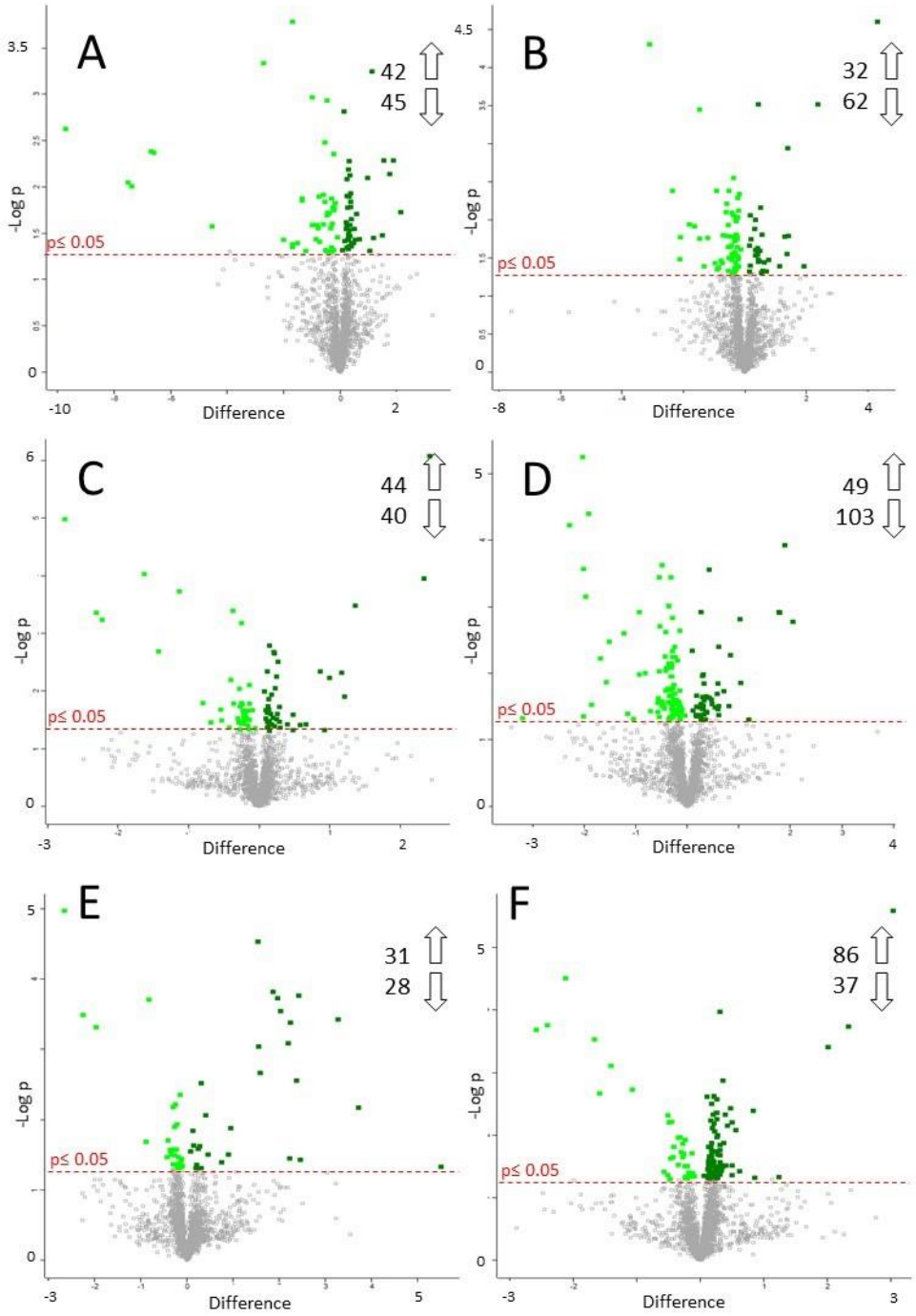


Figure 4.6 Volcano plots visualising the results of Student's t-tests of the LFQ intensities of identified proteins per timepoint and treatment. (A) Acute 2.5 ppb exposure 1 h, (B) Acute 2.5 ppb exposure 24 h, (C) Chronic daily exposure to 5 doses of 2 ppb solution 1 h, (D) Chronic daily exposure to 5 doses of 2 ppb solution 24 h, (E) Chronic continuous 2 ppb exposure for 5 days 1 h, (F) Chronic continuous 2 ppb exposure for 5 days 2 h. Arrows show number of increased and decreased abundance SSDA proteins. Light green is used to highlight decreased abundance SSDA protein distribution, and dark green to highlight increased abundance SSDA protein distribution.

The PCA suggests that the majority of the identified proteome in these experiments does not change in response to clothianidin exposure. To identify possible responding proteins, volcano plots were used to visualize distribution of protein abundances, between treatments and their respective control groups (Student's t-test $p<0.05$). The proteins with statistically significant changes in abundance (SSDA) were then used for gene ontology enrichment analysis. The distribution of SSDA proteins with increased or decreased abundance per timepoint is displayed in figure 4.6 and the top 10 proteins of each treatment and timepoint can be seen in tables 4.3, 4.4 and 4.5.

Eighty-seven SSDA proteins were identified at 1h post-acute exposure, with a relatively equal distribution between proteins with increased (42) and decreased abundance (45) (Figure 4.6A). The protein XP_003396210.1, which is an uncharacterized protein was the protein with most increased abundance, with an increase relative fold change (RFC) of 4.5 in relation to the control. It was followed by Septin-2 (XP_012173415.1, RFC 3.7) and vacuolar protein sorting associated protein 11 (XP_003400665.1, RFC 3.4). The top three proteins with decreased abundance were all structural proteins of the myosin family: myosin heavy chain (XP_012166980.1, RFC 846.4), paramyosin (XP_012164684.1, RFC 181.5) and tropomyosin (XP_003400287.1, RFC 165) (Table 4.3 1h). At twenty-four hours post exposure, the response was defined by a majority of proteins with decreased abundance (62), while only 32 proteins had their abundance increased (Figure 4.6B). Of these SSDA, the top proteins with increased abundance were ankyrin (XP_003402417.1, RFC 19.9), Iron-sulfur cluster co-chaperone protein HscB (XP_003392977.1, RFC 5.5) and arfaptin-2 (XP_003394137.1, RFC 3.8) Unlike the previous timepoint, no structural proteins were observed among the proteins with the most decreased abundance, which included a Zinc transporter 9 (XP_003393925.1, RFC 8.5), shooting 1 (XP_012173555.1, RFC 5.1) and eukaryotic translation initiation factor 2 (XP_003399777.2, RFC 4.3) (Table 4.3 24h).

The chronic low intensity exposure experiment, in which bees received one dose of 35 µl of 2ppb clothianidin contaminated solution per day, resulted in the identification of 88 SSDA proteins at 1h and 152 at 24h (Figure 4.6B and C). Although the 1h timepoint had a relatively equal distribution of proteins, with increased and decreased abundance, the 24h timepoint, had 103 and 49 proteins with decreased and increased abundance, respectively. The proteins tachykinins (XP_003397025.1, RFC 5.3), an uncharacterized protein (XP_012171842.1, RFC 5.1) and the sodium- and chloride-dependent GABA transporter ine (XP_003393654.1, RFC 2.6) had the most increased abundance at 1h. The transmembrane emp24 domain-containing protein bai (XP_003403136.1, RFC 6.8) the

proteins proton-associated sugar transporter A (XP_003397220.1, RFC 5) and the uncharacterized protein YJR142W (XP_012172871.1, RFC 4.7) are the top three proteins with decreased abundance for the same timepoint (Table 4.4 1 h). At 24 h, the chronic low intensity exposure resulted in the increased abundance of the protein exocyst complex component 1 (XP_003392882.1, RFC 4.1), the FACT complex subunit Ssrp1 (XP_003395326.1, RFC 3.7), the molybdenum cofactor biosynthesis protein 1 (XP_012174372.1, RFC 3.5) and on the reduction of the Acyl-CoA delta-9 desaturase (NP_001267818.1, RFC 9.2), Otopetrin 2 (XP_020723208.1, RFC 4.9) and the nuclear cap binding protein (XP_003398727.1, RFC 4.1), which were the top proteins with decreased abundance at this timepoint (Table 4.4, 24 h).

The last experiment involved a chronic continuous exposure, where bees had a high intensity exposure to low level field relevant concentration of clothianidin for 5 days (2 ppb). These samples were also collected 1 and 24h after the last exposure and in total 59 and 123 SSDA proteins, were identified for each timepoint respectively, with the majority of proteins having their abundances increased, in response to clothianidin (31 at 1h and 86 at 24 h) (Figure 4.6E and F). Myosin heavy chain (XP_012166980.1, RFC 45.6), actin (XP_003394490.1, RFC 13.1) and endothelin converting enzyme 1 (XP_012171205.1, RFC 9.7) are the top proteins with increased abundance at 1h, and GTP-binding protein Di-Ras2 (XP_003403094.1, RFC 6.3) , target of rapamycin complex subunit lst8 (XP_003395791.1, RFC 4.7) and tumour susceptibility gene 101 protein (XP_003396690.3, RFC 3.9) are the top decreased abundance proteins (Table 4.5 1 h). Beta glucorinidase (XP_003397976.1, RFC 8.2), Golgi reassembly stacking protein (XP_003396383.1, RFC 5) and tectonin beta-propeller repeat-containing protein isoform X1 (XP_003396363.1, RFC 4), are the top increased abundance proteins at the 24h timepoint, whereas the mitochondrial coenzyme A transporter SLC25A42 (XP_003393261.1, RFC 6), the G-protein coupled receptor B0563.6 (XP_012176061.1, RFC 5.3) and the 5-nucleotidase domain-containing protein 1 (XP_012175462.1, RFC 4.3) are the top decreased abundance proteins (Table 4.5 24 h).

The complete list of SSDA proteins with relative fold changes for each experiment and timepoint are presented in the appendix 4.

Table 4.3 The top 10 statistically significant (Student's t-tests, $p<0.05$) differentially abundant proteins with increased and decreased abundance based on the LFQ intensities of all proteins identified post-acute exposure to 2.5ppb of clothianidin after 1 and 24h against their respective controls.

	Top 10 Upregulated proteins			Top 10 Downregulated proteins		
	Fold change	**Protein name**	**Protein Id**	**Fold change**	**Protein name**	**Protein Id**
1h	4.5	Uncharacterized protein LOC100650600	XP_003396210.1	846.4	Myosin heavy chain, muscle isoform X1	XP_012166980.1
	3.7	Septin-2 isoform X1	XP_012173415.1	181.5	Paramyosin, long form	XP_012164684.1
	3.4	Vacuolar protein sorting-associated protein 11 homolog	XP_003400665.1	165.0	Tropomyosin-1	XP_003400287.1
	2.9	Sodium-coupled monocarboxylate transporter 1	XP_003399925.2	104.3	Myosin light chain alkali isoform X1	XP_012172045.1
	2.8	Vacuolar protein-sorting-associated protein 36 isoform X1	XP_003393640.1	96.7	Troponin T isoform X1	XP_012164541.1
	2.3	Probable leucine--tRNA ligase, mitochondrial	XP_003400899.1	23.1	Uncharacterized protein LOC100647860 isoform X1	XP_003398046.1
	2.2	Nudix hydrolase 8	XP_003396407.1	6.6	Mimitin, mitochondrial	XP_012172182.1
	2.1	Charged multivesicular body protein 1b	XP_003395340.1	4.0	Neural/ectodermal development factor IMP-L2 isoform X1	XP_020721065.1
	2.0	Probable dolichol-phosphate mannosyltransferase	XP_003394983.1	3.2	Uncharacterized protein YJR142W	XP_012172871.1
	1.6	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase epsilon-1-like	XP_012163075.1	3.2	Protein odr-4 homolog	XP_003398574.1
24h	19.9	Ankyrin repeat domain-containing protein 49-like	XP_003402417.1	8.5	Zinc transporter 9	XP_003393925.1
	5.2	Iron-sulfur cluster co-chaperone protein HscB, mitochondrial	XP_003392977.1	5.1	Shootin-1 isoform X1	XP_012173555.1
	3.8	Arfaptin-2	XP_003394137.1	4.3	Eukaryotic translation initiation factor 2 subunit 2	XP_003399777.2
	2.7	Uncharacterized protein LOC100648318 isoform X1	XP_003395536.1	4.3	Signal recognition particle 54 kDa protein	XP_003399441.1
	2.6	Mannose-6-phosphate isomerase	XP_003398360.1	3.5	Ejaculatory bulb-specific protein 3 isoform X1	XP_020719699.1
	2.6	Tetratricopeptide repeat protein 39B-like	XP_012172730.1	3.1	Short coiled-coil protein A isoform X1	XP_012168062.1
	2.5	Sideroflexin-1	XP_003401366.1	2.8	Alpha-tocopherol transfer protein-like isoform X1	XP_012168727.1
	2.2	AP-3 complex subunit beta-2 isoform X1	XP_012169810.1	2.8	Disintegrin and metalloproteinase domain-containing protein 12	XP_012172906.1
	1.7	Glycerol kinase-like	XP_003393370.1	2.5	GPI transamidase component PIG-S	XP_003401873.1
	1.6	Importin subunit alpha-3 isoform X1	XP_012167018.1	2.3	Nuclear pore complex protein Nup50 isoform X1	XP_012175066.1

Table 4.4 The top 10 statistically significant (Student's t-tests, $p<0.05$) differentially abundant proteins with increased and decreased abundance based on the LFQ intensities of all proteins identified in the replicates 1 and 24h after the fifth day of daily chronic exposure to 2ppb of clothianidin (low intensity) against their respective controls.

	Top 10 Upregulated proteins			Top 10 Downregulated proteins		
	Fold change	Protein name	Protein Id	Fold change	Protein name	Protein Id
1H	5.3	Tachykinins	XP_003397025.1	6.8	Transmembrane emp24 domain-containing protein bai	XP_003403136.1
	5.1	Uncharacterized protein LOC100648913	XP_012171842.1	5.0	Proton-associated sugar transporter A-like isoform X1	XP_003397220.1
	2.6	Sodium- and chloride-dependent GABA transporter ine isoform X1	XP_003393654.1	4.7	Uncharacterized protein YJR142W	XP_012172871.1
	2.3	Atrophin-1-like	XP_003400905.1	3.1	Charged multivesicular body protein 1b	XP_003395340.1
	2.2	Protein LZIC-like	XP_012173862.1	2.7	Uncharacterized protein LOC100642796	XP_003393420.1
	2.0	Autophagy protein 5	XP_003394334.1	2.2	Ubiquitin thioesterase OTU1	XP_003397109.1
	1.9	Testis-expressed sequence 2 protein	XP_012171941.1	1.7	Gamma-aminobutyric acid type B receptor subunit 1	XP_012176171.1
	1.8	Conserved oligomeric Golgi complex subunit 7	XP_003397366.1	1.6	Ribosome maturation protein SBDS	XP_003397994.1
	1.6	Pyruvate dehydrogenase phosphatase regulatory subunit, Mitochondrial-like isoform X1	XP_012171680.1	1.5	Glucosylceramidase isoform X1	XP_003402889.2
	1.5	Cytochrome c	XP_003394648.1	1.5	Transketolase isoform X1	XP_003393706.1
24H	4.1	Exocyst complex component 1	XP_003392882.1	9.2	Acyl-CoA delta-9 desaturase	NP_001267818.1
	3.7	FACT complex subunit Ssrp1 isoform X1	XP_003395326.1	4.9	Otopetrin-2 isoform X1	XP_020723208.1
	3.5	Molybdenum cofactor biosynthesis protein 1 isoform X1	XP_012174372.1	4.1	Nuclear cap-binding protein subunit 1	XP_003398727.1
	3.4	LysM and putative peptidoglycan-binding domain-containing protein 1	XP_012167894.1	4.1	Chitinase domain-containing protein 1	XP_012169613.1
	2.3	Myeloid leukemia factor isoform X1	XP_003399033.1	4.0	TraB domain-containing protein isoform X1	XP_003401714.1
	2.1	Leucine-rich repeat and calponin homology domain-containing Protein 1 isoform X1	XP_012166113.1	3.9	G-protein coupled receptor moody isoform X1	XP_003398466.1
	2.0	Uncharacterized protein LOC100651497	XP_003399683.1	3.8	Uncharacterized protein LOC100643711 isoform X1	XP_003400670.1
	1.8	Calexcitin-2	XP_003397062.1	3.6	Hydroxymethylglutaryl-CoA synthase 1	NP_001295236.1
	1.8	Protein obstructor-E isoform X1	XP_003394139.1	3.2	Coatomer subunit zeta-1 isoform X1	XP_003400828.1
	1.6	Uncharacterized protein LOC110120215, partial	XP_020723823.1	3.0	Ejaculatory bulb-specific protein 3-like isoform X1	XP_003402013.1

Table 4.5 The top 10 statistically significant (Student's t-tests, $p<0.05$) differentially abundant proteins with increased and decreased abundance based on the LFQ intensities of all proteins identified in the replicates 1 and 24h after the fifth day of continuous *ad libitum* chronic exposure to 2ppb of clothianidin (high intensity) against their respective controls**.**

	Top 10 Upregulated proteins			Top 10 Downregulated proteins		
	Fold change	Protein name	Protein Id	Fold change	Protein name	Protein Id
1H	45.6	Myosin heavy chain, muscle isoform X1	XP_012166980.1	6.3	GTP-binding protein Di-Ras2	XP_003403094.1
	13.1	Actin	XP_003394490.1	4.7	Target of rapamycin complex subunit lst8	XP_003395791.1
	9.7	Endothelin-converting enzyme 1 isoform X3	XP_012171205.1	3.9	Tumour susceptibility gene 101 protein	XP_003396690.3
	5.5	Probable peroxisomal acyl-coenzyme A oxidase 1	XP_003401606.2	1.9	Trans-Golgi network integral membrane protein TGN38 isofX1	XP_003397609.1
	5.4	Protein SCAI	XP_003396866.1	1.8	Cysteine-rich with EGF-like domain protein 2	XP_003403018.2
	5.2	Opsin, blue-sensitive	XP_003400385.1	1.4	NADH dehydrogenase	XP_003397313.1
	4.7	5-nucleotidase domain-containing protein 1	XP_012175462.1	1.3	Glutathione S-transferase	XP_012163604.1
	4.7	ATP-binding cassette sub-family D member 3	XP_003403063.1	1.3	AP-2 complex subunit sigma	XP_012173383.1
	4.6	Pantothenate kinase 3 isoform X1	XP_012172283.1	1.3	Tubulin gamma-2 chain	XP_003399824.1
	4.1	ERI1 exoribonuclease 3	XP_012168566.1	1.2	Cytoplasmic phosphatidylinositol transfer protein 1	XP_003403112.1
24h	8.2	Beta-glucuronidase	XP_003397976.1	6.0	Mitochondrial coenzyme A transporter SLC25A42	XP_003393261.1
	5.0	Golgi reassembly-stacking protein 2	XP_003396383.1	5.3	G-protein coupled receptor B0563.6	XP_012176061.1
	4.0	Tectonin beta-propeller repeat-containing protein isoform X1	XP_003396363.1	4.3	5-nucleotidase domain-containing protein 1	XP_012175462.1
	2.4	DNA fragmentation factor subunit alpha-like	XP_003402640.1	3.2	PITH domain-containing protein GA19395	XP_003399719.1
	1.8	BET1 homolog	XP_012175654.1	3.0	Galectin-6-like isoform X1	XP_003397897.1
	1.8	Endothelial differentiation-related factor 1 homolog	XP_003396800.1	2.6	Sequestosome-1	XP_012175274.1
	1.5	Carcinine transporter isoform X1	XP_020721872.1	2.1	UPF0568 protein C14orf166 homolog	XP_003401630.1
	1.5	Phosphomannomutase	XP_003399165.1	1.5	Peptidyl-prolyl cis-trans isomerase 6	XP_003400106.1
	1.4	Protein mago nashi homolog	XP_012176347.1	1.4	Transferrin	XP_012172140.1
	1.4	Cysteine and histidine-rich domain-containing protein	XP_003395114.1	1.4	Uncharacterized protein LOC100647232	XP_003399812.1

The gene ontology enrichment analysis permitted the assignment of biological meaning to the sets of statistically significant differentially abundant proteins and provided an overview of the cellular and molecular processes and mechanisms that were changing in response to clothianidin exposure, at a given timepoint and exposure level.

At one hour after acute exposure to clothianidin, an upregulation in vesicle mediated transport processes, endocytosis and exocytosis as well as a behavioural response to ethanol was observed. Cellular components associated with the striated muscle thin filament, contractile fibre part, myosin complex and sarcomere, as well as the biological function muscle contraction, appear as downregulated terms (Table 4.6). The response changes at 24 h where the main upregulated proteins are associated with the mitochondrial intermembrane and the regulation of ATPases, and the downregulated terms belong to a more diverse range of processes involving actin filament depolymerization, DNA endonuclease activity, rhabdomere membrane biogenesis, proteasome core complex and the downregulation of components of the cytosolic small ribosomal subunit (Table 4.6).

The low intensity chronic exposure also affected the mitochondria, as well as translation and the metabolism of fatty acids, as seen by the upregulation of processes associated with the mitochondria intermembrane space, the proton-transporting two-sector ATPase complex, the early endosome, the rhabdomere and the translation regulator activity and nucleic acid binding, as well as the downregulation of the mitochondrial electron transfer flavoprotein complex, the integrin-mediated signalling pathway, fatty acid elongation and fatty acid beta-oxidation, as part of cellular lipid catabolic processes (Table 4.6).

At 24 h, only two processes were determined to be upregulated enriched pathways, one involving the mitochondrial matrix, and the other endodermal digestive tract morphogenesis. The top 5 downregulated processes however, involved the reduction of components of the sentin complex, and the COPI and COPII vesicle coats and budding system, suggesting affects in the transport between the rough endoplasmic reticulum and the golgi apparatus (Table 4.6).

Table 4.6 The top 5 increased and decreased functional enrichments obtained from the STRING analysis and based on the list of SSDA proteins identified from the proteomes of bees exposed to clothianidin simulating three different scenarios and intensity of exposure. Gene ontology terms are ranked based of % coverage of identified and background genes.

	Upregulated			Downregulated		
	GO	Definition	% coverage	GO	Definition	% coverage
Acute exposure to 2.5ppb 1h	GO:0006904	vesicle docking involved in exocytosis	8.8	GO:0005865	striated muscle thin filament	28.6
	GO:0007034	vacuolar transport	6.5	GO:0006936	muscle contraction	16.7
	GO:0030100	regulation of endocytosis	5.7	GO:0044449	contractile fiber part	10.5
	GO:0048149	behavioral response to ethanol	5.5	GO:0016459	myosin complex	10.3
	GO:0060627	regulation of vesicle-mediated transport	4.1	GO:0030017	Sarcomere	10
Acute exposure to 2.5ppb 24h	GO:0042721	TIM22 mitochondrial import inner membrane insertion complex	50	GO:0030042	actin filament depolymerization	33.3
	GO:0042719	mitochondrial intermembrane space protein transporter	33.3	GO:0003906	DNA (apurinic or apyrimidinic site) endonuclease activity	28.6
	GO:0045039	protein insertion into mitochondrial inner membrane	20	GO:0045313	rhabdomere membrane biogenesis	28.6
	GO:0060590	ATPase regulator activity	16.7	GO:0019773	proteasome core complex, alpha-subunit complex	16.7
	GO:0044743	protein transmembrane import into intracellular organelle	9.5	GO:0022627	cytosolic small ribosomal subunit	14.0
Daily chronic exposure to 2ppb (low intensity) 1H	GO:0005758	mitochondrial intermembrane space	9.1	GO:0017133	mitochondrial electron transfer flavoprotein complex	66.7
	GO:0033178	proton-transporting two-sector ATPase complex, catalytic domain	9.1	GO:0007229	integrin-mediated signaling pathway	40
	GO:0005769	early endosome	6	dme00062	Fatty acid elongation	12.5
	GO:0016028	Rhabdomere	5.7	GO:0006635	fatty acid beta-oxidation	10
	GO:0090079	translation regulator activity, nucleic acid binding	5	GO:0044242	cellular lipid catabolic process	6.7
Daily chronic exposure to 2ppb (low intensity) 24H	GO:0061031	endodermal digestive tract morphogenesis	100	GO:0031105	septin complex	66.7
	GO:0005759	mitochondrial matrix	3.0	GO:0005940	septin ring	60
				GO:0090114	COPII-coated vesicle budding	40
				GO:0030126	COPI vesicle coat	37.5
				GO:0030127	COPII vesicle coat	33.3
Continuous *ad libitum* chronic exposure to 2ppb 1H	GO:0071329	cellular response to sucrose stimulus	28.6	GO:1990124	messenger ribonucleoprotein complex	66.7
	GO:0060361	Flight	22.2			
	GO:0003824	catalytic activity	0.5			
	GO:0044237	cellular metabolic process	0.5			
	GO:0044238	primary metabolic process	0.5			
Continuous *ad libitum* chronic exposure to 2ppb 24H	GO:0071569	protein ufmylation	66.7	GO:0005227	calcium activated cation channel activity	28.6
	GO:0035062	omega speckle	50	GO:0051184	cofactor transmembrane transporter activity	28.6
	GO:0090443	FAR/SIN/STRIPAK complex	40	GO:0005905	clathrin-coated pit	22.2
	GO:0005092	GDP-dissociation inhibitor activity	33.3	GO:0015101	organic cation transmembrane transporter activity	22.2
	GO:0019774	proteasome core complex, beta-subunit complex	27.3	GO:0016063	rhodopsin biosynthetic process	20

STRING analysis of the SSDA proteins resolved a broader effect after chronic continuous exposure to clothianidin (see Appendix 4 for the complete STRING output). Proteins with increased abundance, had terms enriched for cellular response to sucrose stimulus, flight, catalytic activities and other cellular metabolic processes, while the only term enriched in the decreased abundance proteins was the messenger ribonucleoprotein complex (Table 4.6). At 24 h, processes involving protein ufmylation, a newly identified ubiquitin-like protein which is part of a conserved cascade associated with endoplasmic reticulum stress responses, and an increased abundance of terms related to the omega speckle, the FAR/SIN/STRIPAK complex, GDP-dissociation inhibitor activity and the proteasome core complex were observed, while the activity of calcium, and other organic cation activated channels and the cofactor transmembrane transporter activity, the clathrin coated pit, and rhodopsin biosynthetic processes were downregulated (Table 4.6).

To evaluate the expression of genes of interest which would allow to obtain further information regarding the physiological state of bee brains after exposure to clothianidin qPCR analysis was conducted. The candidate genes were chosen based on 2 parameters: Firstly, the capacity to correlate their expression intensities with the significant alteration in protein expression levels, corroborating results detected by proteomics. And secondly, based on the opportunity they offered to further assess neurophysiology and detoxification mechanisms in bumblebee's brains. Of the twelve genes investigated (Table 4.1), 8 had their expressions reduced in response to clothianidin, independently of type of exposure. These were the protein encoding genes for the GTP binding protein (Figure 7A&B), acetylcholinesterase (Figure 7C&D), frequenin-1 (Figure 8A&B), calcium channel flower (Figure 8C&D), NADH dehydrogenase (Figure 8E&F), neural ectodermal developmental factor (Figure 9C&D), ATP synthase (Figure 9E&F) and synaptic vesicle glycoprotein (Figure 10C&D). No gene had increased expression under both types of exposure, or in the continuous exposure alone, apart from cytochrome p450, which had discreet non statistically significant upregulation post continuous chronic exposure (Figure 10 E&F). The protein encoding genes for myosin light chain (Figure 10A&B), actin (Figure 9A&B) and vitellogenin (Figure 7E&F), had increased expression after acute exposure to clothianidin but decreased after chronic continuous exposure.

The comparison between the proteomics and the qPCR results provided conflicting results in 30% of the cases. The neural ectodermal developmental factor, actin, calcium channel flower and myosin light chain proteins, demonstrated opposite levels of transcript and protein abundance, in at least one type of exposure or timepoint. The remaining genes had expression levels that either corroborated the protein abundance

levels, or were not found in the volcano plots, due to their absence in proteomics replicates which caused them to be possibly filtered out of the analysis.

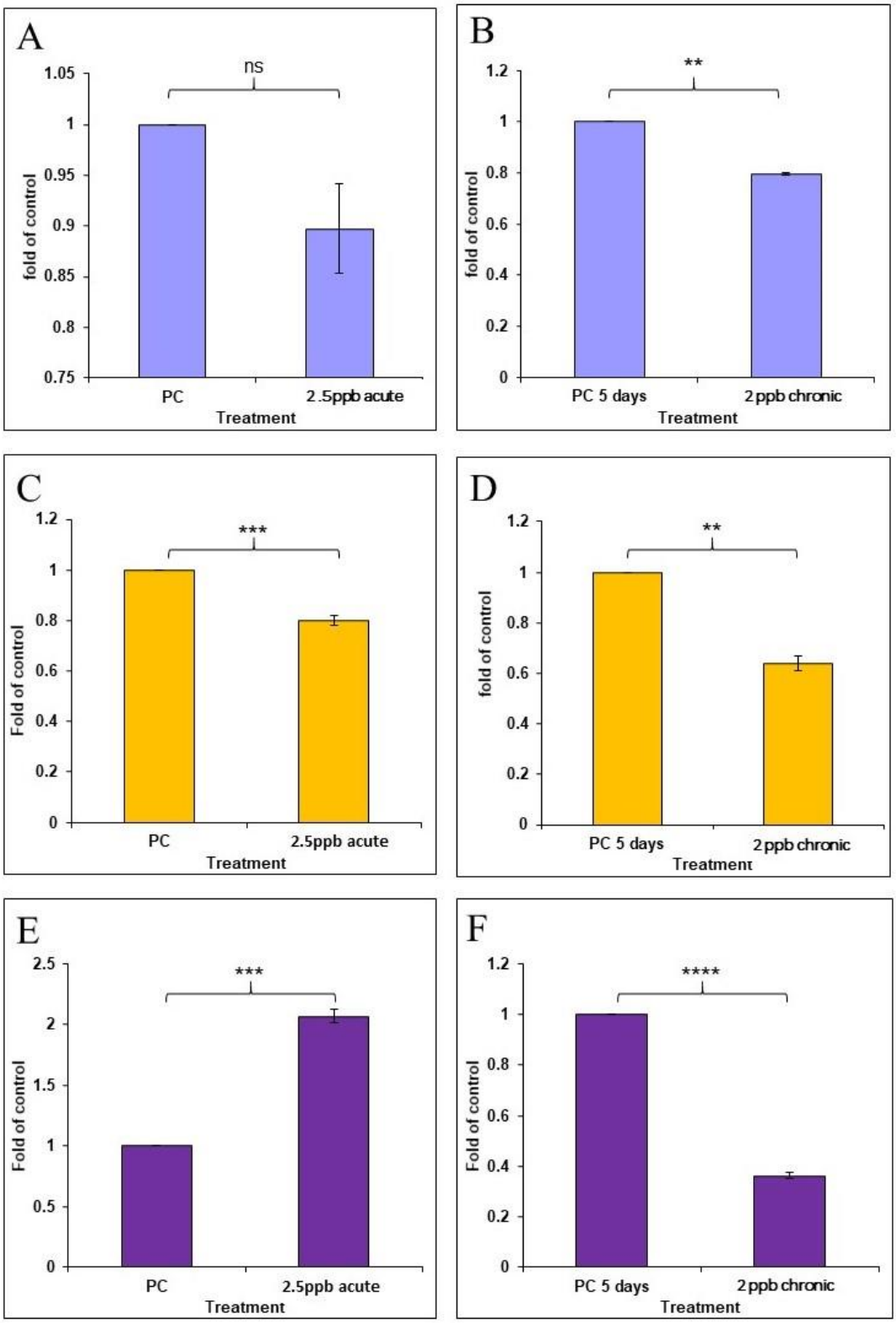


Figure 4.7 Regulation of various genes of interest in response to acute and chronic continuous exposure to clothianidin. (A&B) Expression levels of the protein encoding gene for the GTP-binding protein (XP_003403094.1). (C&D) Expression levels of the protein encoding gene for acetylcholinesterase (XP_012173033.1). (E&F) Expression levels of the protein encoding gene for vitellogenin (XP_012163499.1). For each qPCR experiment, 2 independent biological replicates and 3 technical replicates were used (N = 6). P-values were calculated by performing unpaired Student's t-tests (* = $p < 0.05$, ** = $p<0.01$, *** = $p<0.001$).

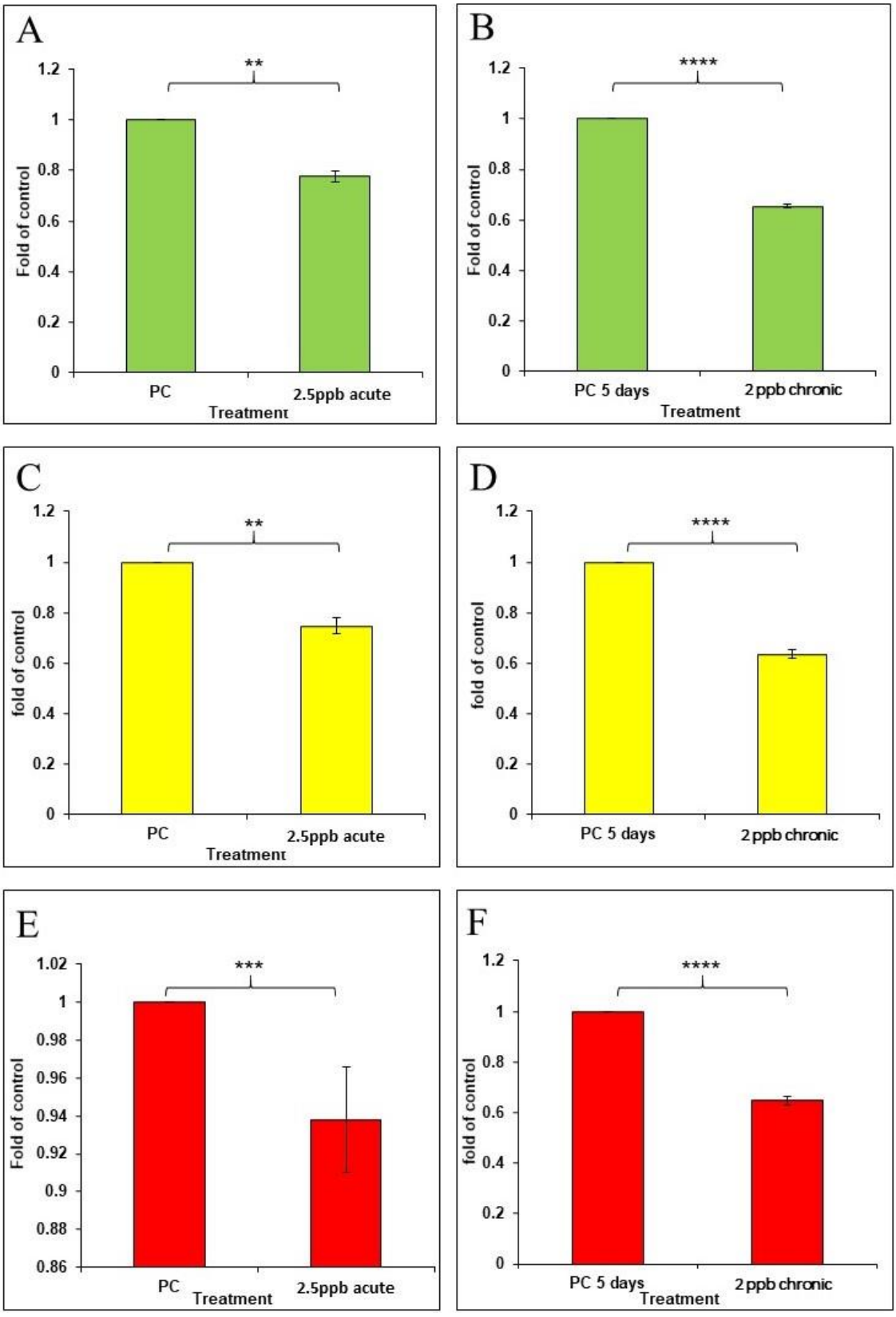


Figure 4.8 Regulation of various genes of interest in response to acute and chronic continuous exposure to clothianidin. (A&B) Expression levels of the protein encoding gene for frequenin-1(XP_003396256.1). (C&D) Expression levels of the protein encoding gene for the protein calcium channel flower (XP_003398827.1). (E&F) Expression levels of the protein encoding gene for NADH dehydrogenase (XP_003402322.1). For each qPCR experiment, 2 independent biological replicates and 3 technical replicates were used (N = 6). P-values were calculated by performing unpaired Student's t-tests (* = $p < 0.05$, ** = $p<0.01$, *** = $p<0.001$).

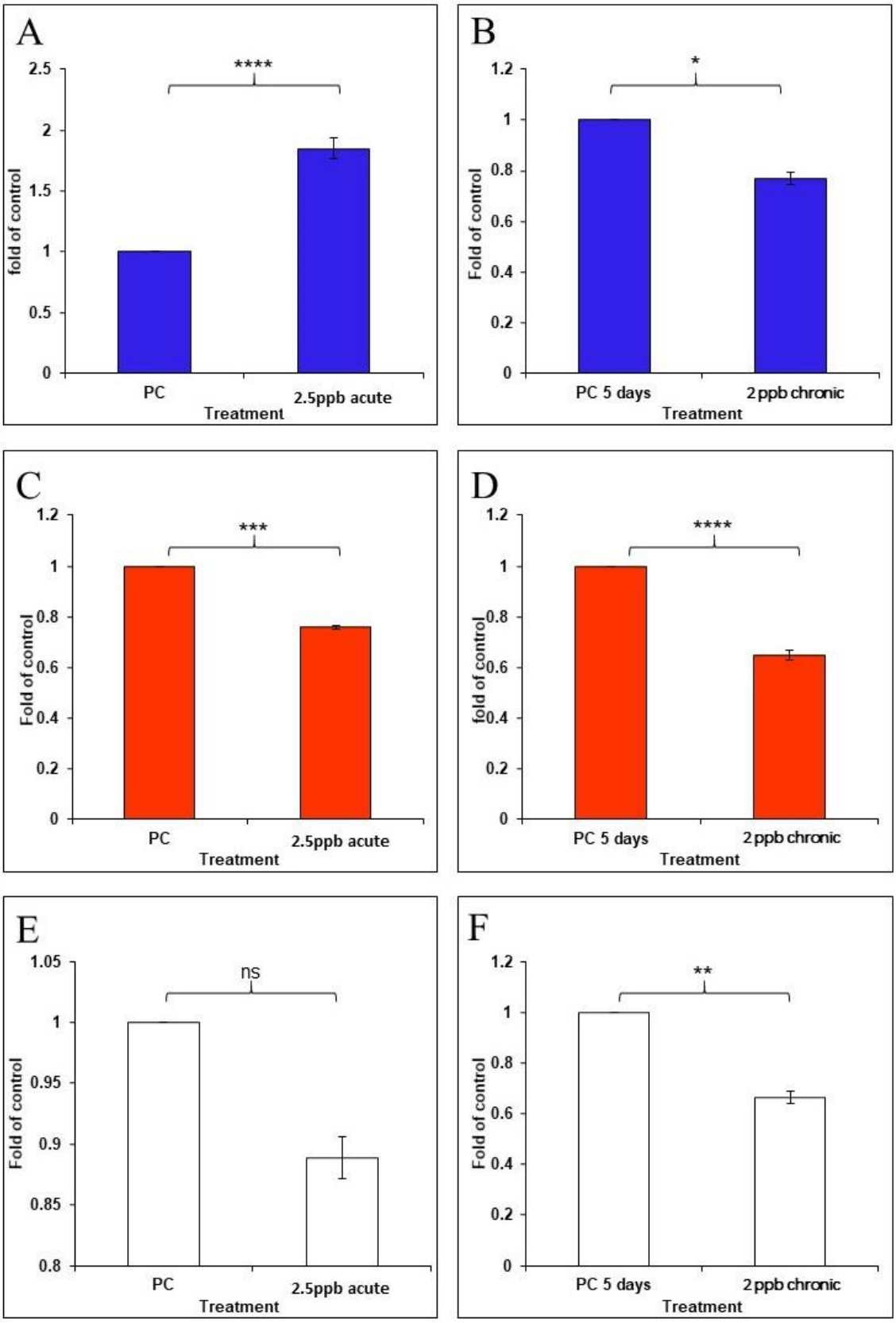


Figure 4.9 Regulation of various genes of interest in response to acute and chronic continuous exposure to clothianidin. (A&B) Expression levels of the protein encoding gene for protein actin (XP_003394490.1). (C&D) Expression levels of the protein encoding gene for the neural ectodermal developmental factor (XP_020721065.1). (E&F) Expression levels of the protein encoding gene for ATP synthase subunit ε (XP_003399456.1). For each qPCR experiment, 2 independent biological replicates and 3 technical replicates were used (N = 6). P-values were calculated by performing unpaired Student's t-tests(* = $p < 0.05$, ** = $p<0.01$, *** = $p<0.001$).

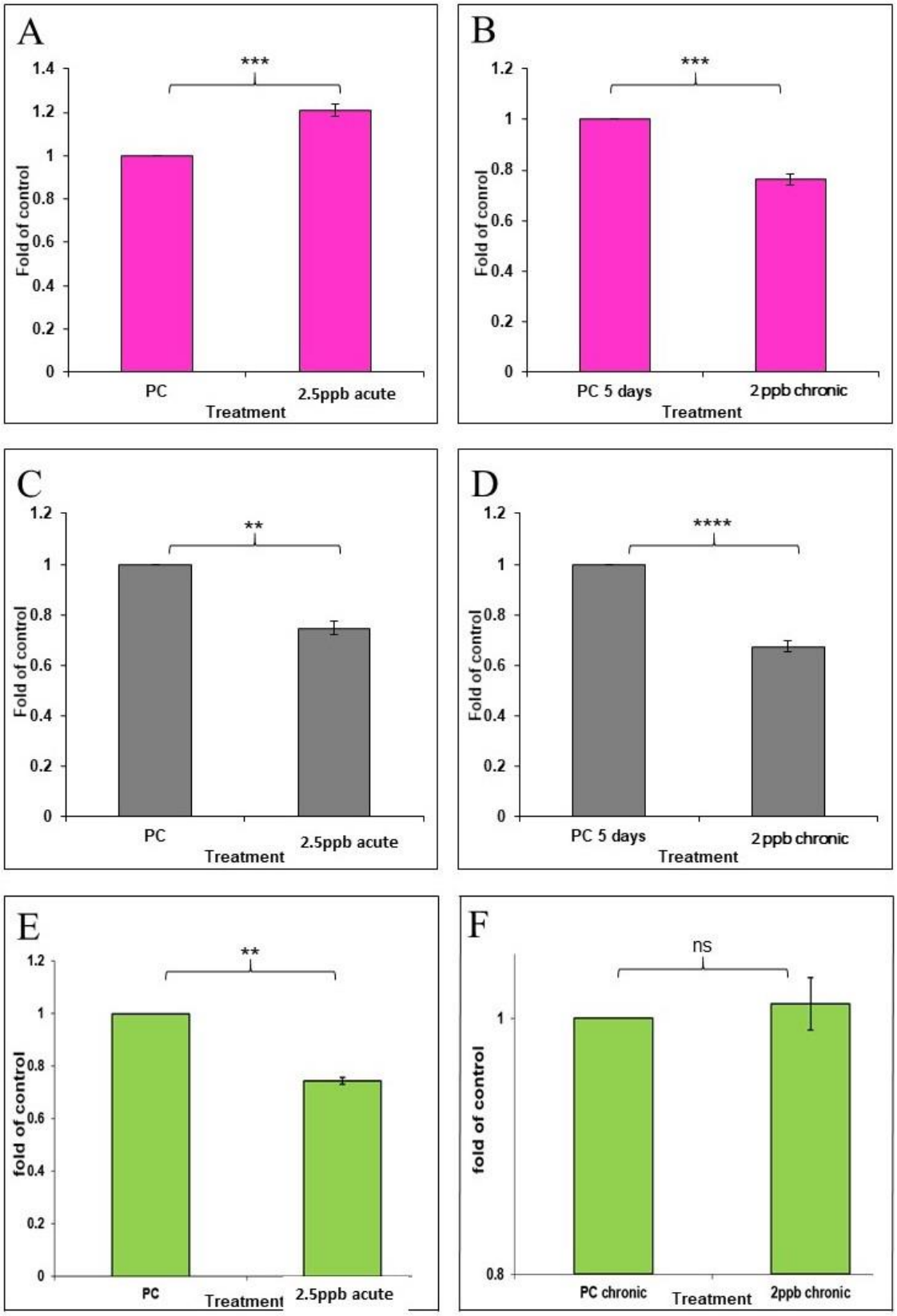


Figure 4.10 Regulation of various genes of interest in response to acute and chronic continuous exposure to clothianidin. (A&B) Expression levels of the protein encoding gene for protein myosin light chain (XP_012172045.1). (C&D) Expression levels of the protein encoding gene for the synaptic vesicle glycoprotein (XP_003398712.1). (E&F) Expression levels of the protein encoding gene for cytochrome p450 9e2(XP_003393865.1). For each qPCR experiment, 2 independent biological replicates and 3 technical replicates were used (N = 6). P-values were calculated by performing unpaired Student's t-tests (* = $p < 0.05$, ** = $p<0.01$, *** = $p<0.001$).

4.5. Discussion

The complexity of exposure scenarios faced by bumblebees makes it very difficult to address how they are influenced by the presence of xenobiotics in the environment. There are different levels of investigation, and the lack of standard exposure methods makes the interpretation, and comparison of results difficult, providing unclear conclusions to a current problem that may have catastrophic consequences to crop production and ecosystem maintenance.

In this chapter, we present our attempts to address three different exposure scenarios based on the frequency to which bumblebee workers, would be in contact with clothianidin, through oral exposure. The experiments were designed to mimic scenarios in which foragers would collect and feed on contaminated nectar/pollen at different concentrations. These represented scenarios where bees had access to, and ingested; i) uncontaminated food sources, ii) clothianidin-contaminated food only once a day, for a period of 5 days and iii) clothianidin-contaminated food continuously, for the same amount of time. We then compared both chronic exposure scenarios with bees that had only been exposed to a similar dose once. Data was obtained at the behavioural and molecular level and compared between bees exposed to similar doses but different exposure scenarios.

Bees were also continuously exposed to higher concentrations of clothianidin to understand how they respond to the chronic increase of insecticide concentration for 5 days.

4.5.1. The effects of chronic clothianidin exposure on the mortality and learning capabilities of worker bumblebees

The importance of understanding chronic exposure on bumblebees goes beyond effects on the individual level. Chronic toxicity usually results from the summation of deleterious effects produced by each dose and can be manifested sometimes by a reduction in the life expectancy of organisms. The availability of clothianidin in the environment has been demonstrated by experiments that quantified its presence in pollen (0.3 to 88 ppb) (Nikolakis *et al.*, 2010; Stewart *et al.*, 2014), nectar (0.5 to 16 ppb) (Pohorecka *et al.*, 2012; Rundlöf *et al.*, 2015), guttation water (0.6 to 31.9 ppm) and surface water (0.01 to 4.6ppb) (Blacquière *et al.*, 2012; Sánchez-Bayo and Hyne, 2014), all of which represent routes of oral exposure to bumblebees, some more than the others (Goulson *et al.*, 2015; Alkassab and Kirchner, 2017).

Divergence in the exposure duration and the endpoints used to assess effects were determined, which in chronic exposure are usually limited to field studies that assess colony performance, rather than having a focus on the individual bees.

The results of this study demonstrate a reduction in adult longevity from day 2 when bees are exposed to concentrations of 35 ppb clothianidin, but there was no increase in mortality when bees are exposed to 10 ppb for 5 days. This suggests that there is an exceedance threshold below which worker bees are able to survive for at least 5 days of constant exposure.

Negative effects on bee populations and performance are frequently reported for higher doses. However, investigating the effects lower doses have on bee physiology deserve further attention as dose-dependent effects in behaviour and physiology are also reported in a threshold dependent manner (Di Prisco *et al.*, 2013; Scholer and Krischik, 2014a; Tison *et al.*, 2016; Cook, 2019).

According to the literature, bees could only be exposed orally to higher concentrations of clothianidin in highly contaminated nectar, or more dangerously, if they become exposed to guttation drops (Bonmatin *et al.*, 2015). Therefore, the concentrations chosen for our experiments do not represent the worst-case scenario but are within the limits of field relevance. The results produced here are of extreme importance and demonstrate that our doses have the potential to impact bees in the field. Effects of clothianidin exposure on adult longevity have been investigated in *B. impatiens*, with deleterious effects on longevity described for queens exposed to 20 ppb for a minimum of 6 weeks (Scholer and Krischik, 2014a), a much longer exposure than the one used in our experiments. Queens are larger and more robust in comparison to workers, which means that they are also more resilient to environmental stressors, which may explain why they are able to cope with an exposure to 20 ppb clothianidin for such a long period. We believe that the longer the duration of chronic exposure the more toxic clothianidin becomes, therefore requiring smaller concentrations to provoke negative effects.

Our results do not indicate that an increase in mortality occurs when bees are exposed to 10 ppb or 2 ppb continuously for 5 days, or to 2 ppb with daily doses (low intensity). However, the 10 ppb exposure, clearly changed the daily consumption pattern and the level of activity of exposed bees. It has been suggested that bumblebees are not only able to detect neonicotinoids in sugary solutions, but that they might have a preference for feeding on contaminated sources (Kessler *et al.*, 2015; Arce *et al.*, 2018). This cannot be corroborated by our results, respective to the group that was exposed continually to 2 ppb of clothianidin, as this concentration showed the same consumption pattern as the

procedural control bees. However, bees that were provided a solution laced with 10 ppb clothianidin, maintained a steady consumption above 300 µl/bee per day. Although we were not able to monitor the consumption of bees exposed to 35 ppb over the 5 days of the experiment, an increase in the volume consumed from day 1 to 2 was observed. More research is necessary to fully elucidate these observations, but it seems possible that exposed bees seek to ingest more solution when contaminated, to increase their energy input. It is necessary to understand why this is the case, as it presents exposed bees with a potential catch, in that ingestion of these insecticides, results in an increase in neuronal function and energy expenditure, which ultimately, forces the bee to feed more to obtain energy. However, this endeavour results in bees expending more energy, due to the increase in activity triggered by the exposure to the agonist neonicotinoid in the first place. Neonicotinoid alterations on nutritional aspects of bees may represent the insecticides interference with endocrine and neurophysiological pathways, determining observed effects that are compound and dose dependent.

The increase in activity observed in the 10 and 35 ppb exposed groups may be a result of the continuous stimulation, provided by the neonicotinoid, which is not as clear at lower doses, perhaps due to a threshold that has not been exceeded, type of effect that has also been described in honeybees (Cook, 2019). We hypothesize that the reduction in activity at around day 4 for the 10 ppb exposed group, is either due to the upregulation of detoxification mechanisms, or to the fact that at that time, the insecticide or its metabolites, may be affecting the bee negatively, compromising its physiology, which translates into changes in their activity, as observed at the 35 ppb. Furthermore, from day 2 onwards, the concentration of 35 ppb increased lethality on the population of tested bumblebees. It is possible that differential individual consumption occurs, meaning that some bees might have consumed more of the solution than others, which explains why some died and others did not. In addition, the increase in activity in surviving bees, may be a result of a better dosage distribution throughout the day or their consumption in 24h. The increase of activity at 35 ppb was also marked by a decrease in responsiveness, which may cause bees to become less efficient and more vulnerable to predators in the field. The reduction in foraging performance was demonstrated by Fauser-Misslin *et al.* (2014) in *B terrestris*, after long term chronic exposure to concentrations above 1.5 ppb of clothianidin. Our results show effects on bee activity at higher concentrations, with no effect being detected for the chronic exposure to 2 ppb.

Although our exposure length was much shorter, we were attempting to demonstrate results at the individual level rather than to assess their effects on the colony level as was

the case by Fauser-Misslin and colleagues (2014). The majority of bumblebee workers live about 20 days post exposure (>80%) when provided with unlimited food sources (Smeets and Duchateau, 2003). During that time, it is not clear how much of it they spend foraging. Thus, by reducing the duration of the experiment we hoped to reduce the effect of natural death in the experiment, as only forager bees that are expected to be older than newly emerged workers, were used.

To further assess the possible negative influence of clothianidin on the behaviour of *B. terrestris* post chronic exposure, we applied a method to test changes in the time of localisation and consumption of a drop of sucrose solution, as an attempt to test learning and memory in bumblebees. In our experiments, workers responded much better to the task the second time they were presented with the drop, or task, showing a considerable reduction in time needed to locate the sucrose drop. This reduction on the second day was observed in both the control and treatment groups, suggesting that the experiment can successfully be used to assess impacts on learning.

During the subsequent days, clothianidin exposure seemed to result in an increase in the time required by bees to find the drops, especially on days 3 and 4, however such measurements were not statistically significant when compared with the control (two-way ANOVA). The increase observed in day 3 for clothianidin exposed bees is not observed in the control group. However, at day four, both treatments show an increase in bee response time, and a slight decrease on day 5. As seen in the activity analysis for 10 ppb, after day 4, the behaviour of exposed bees returned to a normal state, similar to what is observed in the control, even though the consumption levels of the contaminated solution were not reduced.

Behavioural changes associated with learning and memory in bees have been mainly investigated through the proboscis extension response assay (Bitterman *et al.*, 1983), or through microchip tagging and navigation trajectory recording experiments (Decourtye *et al.*, 2011). Both approaches make use of well-established protocols that allow robust analysis to be made in regard to assessing olfactory conditioning and memory effects of insecticide exposure in bees. Although our method requires extensive validation before being compared with the two mentioned above, it showed promising results and stands as a novel way of assessing memory and conditioning of individual bumblebees in a laboratory setting.

More research is required to determine if increase in detoxification pathways after the first 3 or 4 days post the beginning of continuous exposure can be used to explain absence of negative effects at respective timepoints.

To investigate whether molecular level changes to clothianidin exposure correlated to, or could explain the behavioural changes observed in this study and others, including effects on foraging, learning, memory and homing performance described in the literature (Stanley *et al.,* 2015; Stanley and Raine, 2016; Stanley *et al.,* 2016) mass spectrometry based proteomics and quantitative polymerase chain reaction was used to characterize the brain proteome and target genes expression, respectively.

4.5.2. The characterisation of the brain proteome of worker bumblebees post chronic exposure to clothianidin and the investigation of target gene expression

The characterisation of the brain proteome through mass spectrometry-based proteomics post exposure to clothianidin, presents a means of expanding our knowledge about the molecular processes affected by neonicotinoid exposure, in the common pollinator *B. terrestris.* Our experiments simulated different scenarios of exposure, capturing different states of the brain biochemistry, and molecular landscape, after the insecticide had been introduced. To date, very little is known about the cellular processes that are affected by these xenobiotics. The results of the acute exposure experiment suggest an increase in endocytic and exocytotic activity one-hour post ingestion of 0.087 ng of clothianidin. This may represent increased processing of neurotransmitter vesicles in the cholinergic system which is the main target of this chemical (Vehovszky *et al.*, 2015). As clothianidin behaves as a super agonist of the system (Brown *et al.*, 2006) it is expected that increased activity would be observed after consumption. On the downregulated side, a series of muscle related components are reported after gene ontology enrichment using STRING analysis. We suggest that such enrichment is a result of multiple structural proteins in the set of decreased abundance SSDA proteins. These proteins are members of the myosin, paramyosin and tropomyosin families, which are known components of muscular systems, but also have important functions in intracellular transport and participate in the organization of the highly dynamic neuronal cytoskeleton (Blitz and Fine, 1974; Hirokawa *et al.,* 2010). The largescale reduction in these components (150 relative fold difference in some cases) in exposed bee brains, suggest that structural reorganisation and intracellular transport processes are highly compromised. Twenty-four hours after clothianidin exposure a considerable increase in mitochondrial inter and inner membrane components, and elevated ATPase activity, are observed. Such effects on the main cellular energy producing machinery, after pesticide

exposure have long been reported. For instance Moffat and collaborators (2015b) demonstrated that clothianidin exposure triggers rapid mitochondrial depolarization increasing neuronal risk in Kenyon cells. In addition, genes associated with mitochondrial function have also been shown to be differently expressed, in response to clothianidin on the transcriptional level (Colgan *et al.*, 2019). Although the two mitochondrial associated protein encoding genes reported by Colgan and collaborators are not present in our list of SSDA proteins for the acute experiment, several other mitochondrial associated proteins were determined to be differentially abundant.

In addition, the effects reported here demonstrate increased stress on the inner membrane of the mitochondria, impacting the electron transport chain directly, indicating a potential increase in the production of energy through aerobic respiration. A decrease in actin filament depolymerization was also observed at 24h post-acute exposure, in addition to other cellular components, such as the proteasome core complex, cytosolic small ribosomal subunits, rhabdomere membrane biogenesis and DNA endonuclease activity. Taken together these results suggest that there is a reduction in cargo transport, cellular motility, and environmental sensitivity through reduction of photoreception, as evidenced by effects on the rhabdomere.

The proteomic analysis of chronic exposed bees suggests a sustained effect after five days, again, with increased abundance in components of the mitochondria intermembrane space, ATPase activity, rhabdomere, early endosome and translation, All these effects indicate an increase in cellular activity and metabolism, where translation feeds into the production of more proteins, and places a demand on energy output. The upregulation of early endosome generation, also suggests the formation of vesicles, that may later be used to transport cargo into proteolytic active lysosomes, which degrade excess materials in the brain cells (Kulkarni and Maday, 2018). In addition, the mitochondrial electron transfer flavoprotein complex, integrin mediated signalling pathways, the metabolism of fatty acids and the catabolism of other cellular lipids are also affected, being downregulated at 1h. Changes in the use of energy after chronic clothianidin exposure are also suggested by Colgan et al. (2019) with increased pressure on mitochondrial processes and changes in the metabolism of lipids. Furthermore, we found signs of interference with integrin signalling pathways, which is described as a potential risk for neurological disorders due to their roles modulating ion channels, interactions with the extracellular matrix and cytoskeleton filaments reorganization (Wu and Reddy, 2012).

The response at 24h was marked by an increase in components of the mitochondrial matrix, and by a decrease in COP I and COP II signalling interference, with the transport

of proteins between the Golgi complex and the rough endoplasmic reticulum. COP II proteins are required for the export of newly synthesized proteins from the endoplasmic reticulum, and COP I proteins mediate a retrograde transport pathway recycling proteins from the Golgi complex to the ER (Duden, 2003).

The proteome changes in response to our third exposure scenario, the continuous chronic exposure, appear more complex than previously reported in our other experimental groups. Signals of increased omega speckles, which are nucleoplasmic structures distributed in the interchromatin space of cells, in close proximity to chromatin, are evident in our data set and are suggested to appear as a sign of cellular recovery from stress (Singh and Lakhotia, 2015). In addition, signs of interference with complexes associated with a number of cellular functions like the FAR/SIN/STRIPAK complex and an increase in GDP-dissociation inhibitor activity suggest changes on synaptic plasticity, involving formation and neurotransmission (Ikonomov *et al.*, 1998; Ishizaki *et al.*, 2000). An increase in proteasomal components and a decrease in cation channels activity and clathrin coated pits, that participate in receptor mediated endocytosis, were also observed, and its importance on synaptic cycle is corroborated by Milosevic (2018).

The assessment of target gene expression levels through qPCR aimed at providing corroboration for the theory that clothianidin affects the energy metabolism and changes the dynamics between structural members, responsible for cytoskeleton reorganization, and neurotransmitter secretion in the bumblebee brain cells. Out of 12 genes tested, 8 had their expression levels reduced in response to clothianidin and only actin, myosin light chain and vitellogenin showed increased abundance after acute exposure. Reductions in energy metabolism gene expression were also observed for NADH dehydrogenase and ATP synthase. The structural proteins actin and myosin light chain also have their transcript levels altered by clothianidin exposure. However, their expression was opposite to what was observed at the translational level. It is possible that an increased degradation of these proteins calls to an increase in gene expression for actin and myosin. However, it is possible that a lag effect or a micro RNA-interference on translation processes hampers the expression of these elements on the protein level.

The expression levels of transcripts used to assess neuronal physiology capability were also assessed. These included acetylcholinesterase (AChE), which is associated with learning, and its activity has been shown to be directly dependant on mRNA expression level (Shapira *et al.*, 2001). The gene encoding for the enzyme had reduced expression post exposure to clothianidin, in both treatments (acute and chronic continuous exposure).

However, acetylcholinesterase is not part of the list of SSDA proteins that showed changes in abundance post treatment. The enzyme is not capable of hydrolysing clothianidin, as it does for acetylcholine, freeing the receptors and allowing for repolarization of neurons (Buszewski *et al.*, 2019). It is unsurprising therefore to observe no change in response to the neonicotinoid.

Vitellogenin expression was also assessed due to its possible role in the regulation of social behaviour, which includes division of labour and aging in honeybees (Münch *et al.*, 2015b) but as yet undetermined function in bumblebees (Amsalem *et al.*, 2014). The transcript levels of vitellogenin were severely increased after acute exposure, which was corroborated by increased protein abundance at the proteome level. Nevertheless, chronic exposure to clothianidin decreased the levels of vitellogenin. The role of vitellogenin as a possible 24 h biomarker of neonicotinoid exposure is corroborated by the increase of protein and transcript levels in the brain of honeybees (Christen., *et al.*, 2019) but further investigation is required to confirm its biomarker potential in exposures of longer duration.

A member of the cytochrome p450 protein family was also assessed to investigate if chronic exposure to clothianidin would trigger cytochrome p450 detoxification. No changes in its transcript levels were detected, potentially indicating that this protein does not play a role in clothianidin detoxification, as previously suggested by Manjon *et al.* (2018) who questioned the efficacy of cytochrome p450 CYP9Q subfamily as detoxifiers of N-nitroguanidine neonicotinoids.

The expression level changes, observed between the transcripts of the controls and treatments, were smaller than those seen at the protein abundance level. According to Vogel and Marcotte (2012) there are a series of factors apart from the varied post transcriptional modifications, that are not even well defined yet, that may also explain this variation. These include protein stability, that makes these molecules last longer, and the fact that protein levels vary over a much wider range than the levels of the corresponding mRNAs, indicating extensive regulation of protein metabolism, and especially synthesis (Schwanhüusser *et al.*, 2011). Translation also seems to be a faster process than transcription in eukaryotes (Ingolia *et al.*, 2011; Vogel and Marcotte, 2012*)*, producing several copies of the same protein every hour, and sometimes using the same transcript.

Low correlation between mRNA and protein levels has been widely reported, and apart from considering technical difficulties, like the scale of the comparison (size of the organs), and if mRNA and proteins were quantified from the same samples or not, it is

also necessary to consider that low correlation is expected in situations of cellular differentiation or stress response (Liu *et al.,* 2016). Translation is a high energy expenditure process, while transcription is a much cheaper process in relation to energy usage (Warner, 1999). This means that the production of proteins is subject to the availability of energy, in addition to protein necessity. Protein-mRNA correlations also must take into consideration the fact that the cellular machineries catalysing the reactions of protein biosynthesis are limited (RNA polymerase and ribosomes), and not equally distributed throughout the cell. Notably, the complexity of tissue means that their cellular composition differs. Thus, this complexity could explain the incorrect or incomparable estimates of mRNA and protein levels (Liu *et al.,* 2016).

In conclusion, this chapter brings forth an in depth characterisation of molecular effects triggered in the brain of bumblebees post chronic exposure to clothianidin, whilst highlighting its impact, even at sub lethal field relevant levels, on cellular transport and mobility, environmental sensing and energy production and metabolism. It compares powerful molecular biology techniques and reveals the complexity of the xenobiotic response obtained in each level of analysis, which calls for caution in the interpretation of results, for the same reasons discussed in the previous chapter.

Regarding the behaviour analysis a critical threshold exists, where mortality levels can be kept to a minimum and slightly increased patterns of activity can be seen once the threshold is not surpassed. Assessing if such increase in activity can be beneficial to bumblebee workers remains to be investigated at the behavioural level, but we have demonstrated that when exposed, bees have critical molecular processes in their brains impaired by the neonicotinoid, which affects overall cellular communication and homeostasis. These results can now add to the knowledge bank used to understand neonicotinoids effects on beneficial pollinators and should be further questioned by projects that will make visual validation of protein expression levels, such as antibody tagging through western blotting, fluorescent microscopy analysis, targeted proteomics or even metabolomics studies, should availability of sample material allow. Only then, we can successfully determine the molecular bioindicators of pesticide exposure in bees and develop ways to mitigate their negative effects.

4.6 Appendix 4

The following appendix tables are located on the disc provided at the back of thesis or at the link below:

https://drive.google.com/file/d/1g5uN5Mro8pbhkhfxe_IPdS7sL8xNnpQx/view?usp=sharing

Table 4.6.1 Complete list of statistically significant (Student's t-tests, $p<0.05$) differentially abundant proteins (SSDA) with increased and decreased abundance based on the LFQ intensities of all proteins identified post-acute exposure to 2.5ppb of clothianidin after 1h against their respective controls**.**

Table 4.6.2 Complete list SSDA with increased and decreased abundance post-acute exposure to 2.5ppb of clothianidin after 24h against their respective controls**.**

Table 4.6.3 Complete list of SSDA with increased and decreased abundance post-chronic exposure to 2ppb of clothianidin for 5 days, samples collected after 1h.

Table 4.6.4 Complete list of SSDA with increased and decreased abundance post-chronic exposure to 2ppb of clothianidin for 5 days, samples collected after 24h.

Table 4.6.5 Complete list of SSDA with increased and decreased abundance post-chronic free feeding exposure to 2ppb of clothianidin for 5 days, samples collected after 1h.

Table 4.6.6 Complete list of SSDA with increased and decreased abundance post-chronic free feeding exposure to 2ppb of clothianidin for 5 days, samples collected after 24h.

Table 4.6.7 Complete list of functional enrichment results obtained on the platform STRING based on the list of SSDA proteins identified post-acute exposure to 2.5ppb 1h. Gene ontology terms are ranked based of % coverage of identified and background genes.

Table 4.6.8 Complete list of functional enrichment results obtained on the platform STRING based on the list of SSDA proteins identified post-acute exposure to 2.5ppb 24h. Gene ontology terms are ranked based of % coverage of identified and background genes.

Table 4.6.9 Complete list of functional enrichment results obtained on the platform STRING based on the list of SSDA proteins identified post-chronic exposure for five days to 2ppb 1h. Gene ontology terms are ranked based of % coverage of identified and background genes.

Table 4.6.10 Complete list of functional enrichment results obtained on the platform STRING based on the list of SSDA proteins identified post-chronic exposure for five

days to 2ppb 24h. Gene ontology terms are ranked based of % coverage of identified and background genes.

Table 4.6.11 Complete list of functional enrichment results obtained on the platform STRING based on the list of SSDA proteins identified post-chronic free feeding exposure for five days to 2ppb 1h. Gene ontology terms are ranked based of % coverage of identified and background genes.

Table 4.6.12 Complete list of functional enrichment results obtained on the platform STRING based on the list of SSDA proteins identified post-chronic free feeding exposure for five days to 2ppb 24h. Gene ontology terms are ranked based of % coverage of identified and background genes.

Chapter V

Identifying potential biomarkers of neonicotinoid exposure in the bumblebee brain proteome

5.1. Introduction

5.1.1. Biomarkers

Biomarkers are broadly defined as any substance in an organism, the presence/absence or abundance of which can indicate perturbations caused by disease, infection or exposure to environmental contaminants (xenobiotics) (Gupta, 2014). They have been commonly used in clinical research, as primary endpoints in clinical trials for a considerable number of years, providing us the confidence of accurate diagnosis, prognosis and treatment (Strimbu and Tavel, 2010). One of the most important criteria for a biomarker is that it can be accurately and reproducibly measured, consistently representing or correlating to the condition/effect being screened for (Gil and Pla, 2001). Although biomarkers, which may be molecular, biochemical, cellular, physiological, pathological or behavioural in nature, predominantly relate to human toxicology, their definition and application has been expanded to include indicators of environmental exposure in any animal species. Currently, a biomarker may be defined as any measurement that reflects an interaction between a biological system and a potential hazard, which may be chemical, physical or biological (Strimbu and Tavel, 2010; Gupta, 2014). This broadened view of biomarkers now includes molecular interactions that signal physiological, or biochemical changes at the cellular level, through to changes in pulse or blood pressure, or even behaviour (Strimbu and Tavel, 2010).

The relationship between toxic levels within different organisms and the triggered toxic response is complex, and depends on several factors that include the toxicokinetic properties of the chemical (dose response relationship), and the biological and environmental factors that can influence the baseline of response, as well as genetic factors which ends up contributing to species specific differences, in relation to biomarker expression (Gil and Pla, 2001; Gupta, 2014).

Furthermore, biomarkers are also usually subdivided by their degree of specificity and class, resulting in biomarkers of exposure, effect and susceptibility (Gil and Pla, 2001). In regards to specificity, some cellular effects are seen in response to a variety of xenobiotics such as DNA damage, that is the result of exposure to a variety of compounds including, nitrosamines, aromatic amines, chemotherapeutic agents and pesticides representing a nonspecific biomarker, whereas the alteration in acetylcholinesterase (AChE) activity, caused by carbamate and organophosphate insecticides, represents a specific biomarker of exposure (Gil and Pla, 2001; Badawy *et al.,* 2015).

In terms of neonicotinoid exposure, the activities of AChE, carboxylesterase (CaE1, CaE2, CaE3), glutathione-S-transferase (GST), alkaline phosphatase (ALP) and catalase (CAT), have been previously investigated as potential bioindicators of thiamethoxam exposure in honeybees. No change however, was reported for AChE activity, while increased activity was found for the enzymes CaE2, GST and CAT and a decrease in CaE1 and CaE2 activity (Badiou-Bénéteau *et al.*, 2012). In addition Alburaki *et al.* (2017) also reports no differential transcript expression in AChE, CAT or GST when bees were exposed to imidacloprid, under laboratory conditions. However, a number of other studies suggest that AChE expression and activity, potentially represents a biomarker of pesticide exposure. For instance, Boily *et al.* (2013) report increased AChE activity in honeybees, whereas increased levels of AChE expression was reported in bumblebee workers, foraging in neonicotinoid (clothianidin and thiamethoxam) treated sites (Samson-Robert *et al.*, 2015). These contrasting findings indicate that AChE activity and expression although promising, may be unreliable as a neonicotinoid biomarker, and that alternative candidates need to be considered.

The yolk precursor protein vitellogenin has being proposed as a potential biomarker of general insecticide exposure, including exposure to pyrethroids, organophosphates and neonicotinoids (Christen *et al.*, 2019b). In regards to neonicotinoids, vitellogenin expression was found to be upregulated in the brains and fat bodies of honeys bees after a 24 hr exposure to 3 ng/bee of clothianidin (Christen *et al.*, 2019*)*.

Attempts have also been made to use antimicrobial responses as biomarkers of insecticide exposure. However, contradicting evidence on transcriptional responses in immune-related genes, in response to thiamethoxam and imidacloprid, suggest that they are not yet reliable indicators of pesticide impacts on bee health (Collison *et al.*, 2018).

Recently, Colgan *et al.* (2019) adopting a high throughput RNA sequencing approach to investigate genes and pathways affected by neonicotinoid exposure in bumblebees, highlighted a number of candidate biomarkers of pesticide toxicity. Their results suggest that mitochondria associated genes, such as alanine-glyoxilate aminotransferase and phosphoenolpyruvate carboxykinase, and genes associated with the regulation of lifespan, lipid metabolism, ion transport, developmental growth, neuron projection and regulation of the notch signalling pathways, may represent possible candidates. In addition, members of the CYP4, CYP6 and CYP9 family of cyt P450

detoxifying enzymes were also found to be differently expressed after clothianidin exposure.

Determining potential biomarkers of neonicotinoid exposure in bees is difficult but can reveal information about the environmental health of these important pollinators, in terms of responding to the anthropogenic factors giving rise to their decline. In addition, despite its obvious importance, neurotoxicity investigations seem to be progressing more slowly than other fields.

A number of biomarkers have been suggested for neurological dysfunction, including AChE activity (discussed above). Other markers that are spatially representative of the brain include Ca^{2+} influx, exocytosis and the localisation and activity of mono amine oxidases, and neuropathy target esterases, that can trigger axonal degeneration. However, the complexity of the nervous system makes the rapid identification of reliable biomarkers difficult (Gil and Pla, 2001). Therefore, in an attempt to identify new and reliable biomarkers of neonicotinoid pesticide exposure, the principal aim of this chapter, was to assess the proteomic datasets generated in this thesis, specifically the statistically differently abundant proteins (SSDA), identified from the quantitative experiments that evaluated the effects of exposure to the neonicotinoids clothianidin and imidacloprid on the *B. terrestris* brain (Chapters III and IV). For all candidate proteins, their reproducibility across datasets and changes in abundance, were evaluated, to determine their potential to represent biomarkers of neonicotinoid exposure.

5.2. Chapter aims and hypothesis

The principal aim of this chapter was to bioinformatically assess the sets of SSDA proteins identified after oral exposure to sub lethal acute and chronic doses of clothianidin and imidacloprid, identifying those proteins with similar expression profiles in each treatment, and timepoint, as possible biomarker candidates of exposure. This will be achieved through a number of specific aims including:

1. To compare all proteins identified in chapter III, to identify proteins with similar expression profile to acute exposure, generating a list of biomarker candidates to neonicotinoids, independent of chemical type.

2. To compare all proteins identified after acute or chronic clothianidin exposure, to generate a list of biomarker candidates independent of mode of exposure.

3. To compare all proteins identified in chapter III and IV, independent of neonicotinoid type and mode of exposure, to generate a list of possible biomarker candidates of general neonicotinoid exposure.

We hypothesise that as both neonicotinoids are defined as agonists of the nAChR in bees, we should expect that the same target cellular processes are affected under exposure, generating the expression of common protein biomarkers, independent of neonicotinoids, or mode of exposure, used in the experiments.

5.3. Experimental overview and methods

5.3.1. Filtering of proteins with potential to be biomarkers of neonicotinoid exposure in the brain of *Bombus terrestris*

All SSDA proteins identified in Chapters III and IV after clothianidin and imidacloprid exposure, independent of timepoint and mode of exposure, were considered in these analyses. Proteins were copied into an excel tab and assigned to their experiment of origin and concentration tested. In total 6 datasets of SSDA proteins were compared (Table 5.1). Those proteins that were present in all experiments, reproducibly, were retained as potential biomarkers. Venn diagrams were constructed using JVENN (Bardou *et al.*, 2014) and groupings of possible biomarker candidates were defined, in response to acute exposure, clothianidin exposure and general neonicotinoid exposure.

Table 5.1 General experimental parameters and results of the 6 used datasets.

Dataset	Neonicotinoid	Mode of Exposure	Identified proteins (3/3 replicates)	Non redundant identified (SSDA)	Range of fold differences	Experiment Duration	Estimated Final Dose
I	Clothianidin (2.5ppb)	Acute oral exposure	1,428	272	-10.8/+2.8	24h (3 timepoints)	0.087ng/bee
II	Clothianidin (10ppb)	Acute oral exposure	1,456	294	-3.36/+7.72	24h (3 timepoints)	0.35ng/bee
III	Imidacloprid (2.5ppb)	Acute oral exposure	2,356	259	-5.53/+7.36	24h (3 timepoints)	0.087ng/bee
IV	Imidacloprid (10ppb)	Acute oral exposure	2,379	582	-5.32/+8.47	24h (3 timepoints)	0.35ng/bee
V	Clothianidin	Chronic oral exposure	2,239	229	-3.2/2.4	120h (2 timepoints)	0.35ng/bee
VI	Clothianidin	Chronic free feeding oral exposure	2,301	178	-2.65/+5.5	120h (2 timepoints)	2.41µg/bee

5.4. Results

To identify possible biomarker candidates, the lists of SSDA proteins obtained in several quantitative experiments were reviewed and compared, using the unique protein identifier to match proteins across experiments. To identify biomarkers of acute neonicotinoid exposure, 4 groups were compared; the SSDA proteins identified from the brains of bees exposed to clothianidin or imidacloprid, at concentrations of 2.5 ng/ml or 10 ng/ml (I, II, III and IV, Table 5.1).

The two groups identified in chapter III after acute exposure to clothianidin were then compared with the list of SSDA proteins obtained post chronic low intensity and chronic free feeding exposure to 2 ppb of clothianidin, from chapter IV, in an attempt to identify protein biomarkers specific to clothianidin exposure.

Finally, a list of biomarkers for general neonicotinoid exposure was produced by comparing proteins in all 6 groups of SSDA proteins (Table 5.1).

5.4.1. Biomarkers of acute exposure

To identify biomarker candidates of acute neonicotinoid exposure, the SSDA proteins identified post exposure to 35 µl of 2.5 ng/ml (272 proteins) and 10 ng/ml (294 proteins) of clothianidin were compared with the SSDA proteins identified after exposure to same doses of imidacloprid (259 and 582 proteins). Ninety-three proteins were found to be shared between the clothianidin low and high doses, and ninety proteins were shared between the two doses of imidacloprid. For both neonicotinoids, the list of proteins affected by exposure were divergent in the low and the high dose. In addition, 38 proteins were found in at least 3 groups and only 1 protein, the myosin 2 essential light chain (XP_012170909.1) was found to react to pesticide exposure, independent of dose and neonicotinoid used (Figure 5.1).

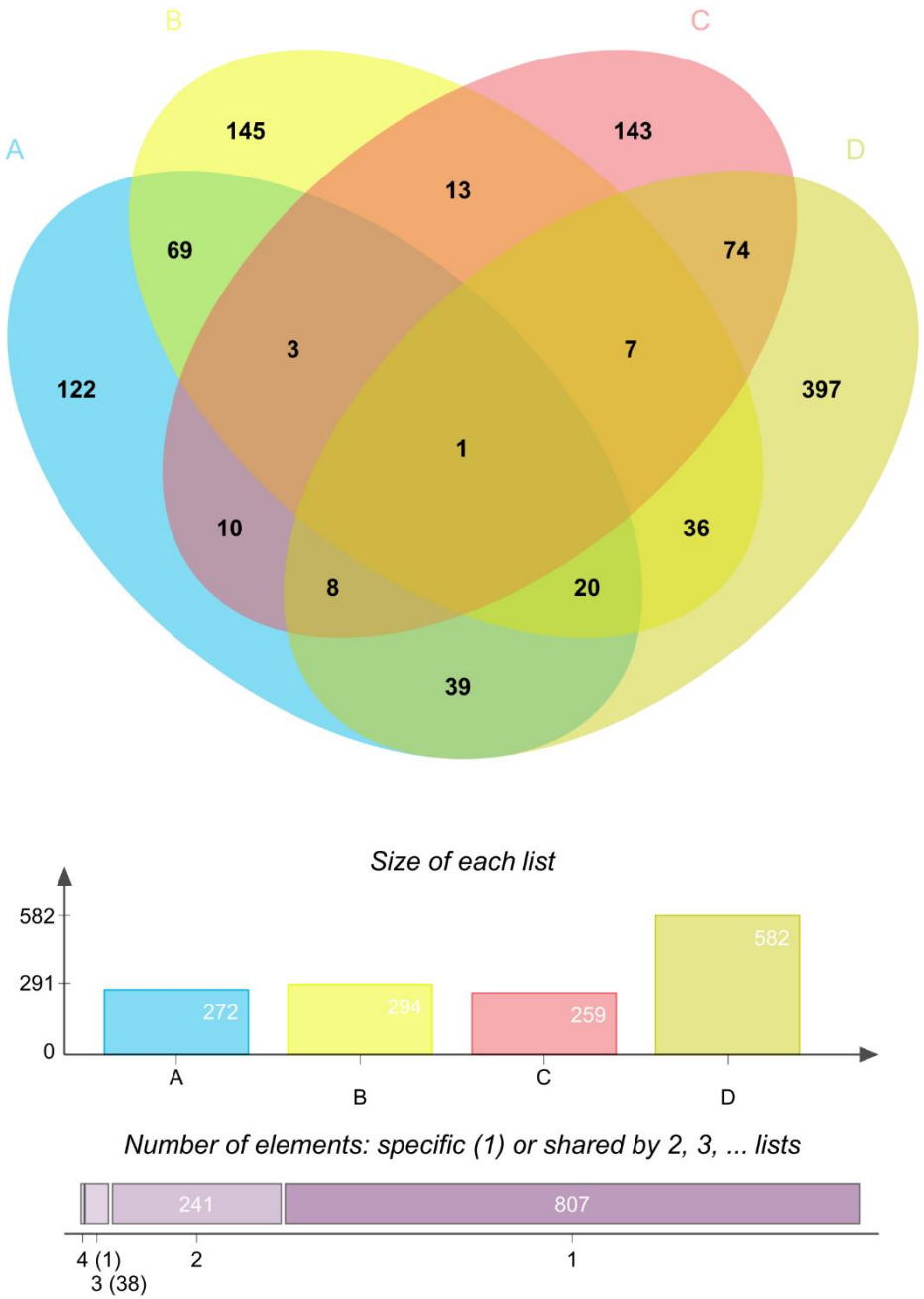


Figure 5.1 Venn diagram of the overlapping SSDA proteins among the four experimental groups acutely exposed to clothianidin and imidacloprid. Only one protein is common to all treatments. (A) Clothianidin 2.5 ng/ml, (B) Clothianidin 10 ng/ml, (C) Imidacloprid 2.5 ng/ml, (D) Imidacloprid 10 ng/ml).

5.4.2. Biomarkers of clothianidin exposure

As *B. terrestris* workers were acutely or chronically exposed to clothianidin in the experiments described in chapters III and IV, we then decided to compare the list of SSDA proteins identified in those experiments, to identify general biomarker candidates of clothianidin exposure, independent of dose and timepoint. The unique protein code for all SSDA proteins identified after acute exposure (described in chapter III; n=473) were

compared in a Venn diagram, with the SSDA proteins identified after chronic low intensity exposure (n=229), and chronic high intensity exposure (n=178, free feeding experiment), outlined in chapter IV. Fourteen proteins were found to be shared after chronic exposure (Figure 5.2B and C,) and the majority of the proteins identified in each experiment were not commonly found in any other set of SSDA proteins (750 proteins). Only 4 proteins, the protein phosphatase methylesterase (XP_003393428.1), transferrin (XP_012172140.1), endothelin-converting enzyme (XP_012171205.1) and the proteasome subunit beta (XP_003396794.1) were shared between all three groups assessed, representing the only possible protein biomarkers of clothianidin exposure (Figure 5.2).

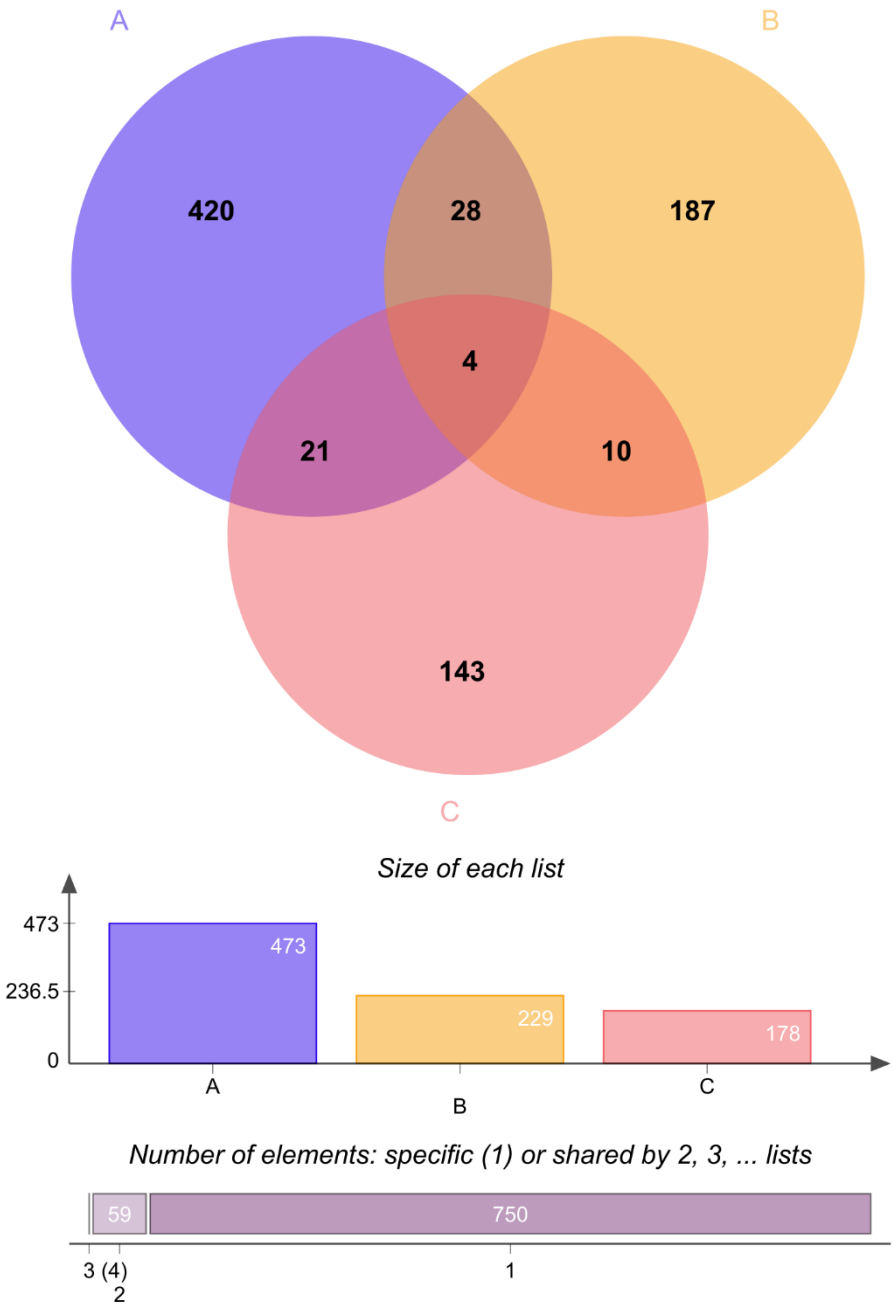


Figure 5.2 Venn diagram of the overlapping SSDA proteins among the three experimental groups from chapter III and IV exposed to clothianidin acute and chronically. Only four proteins are commonly found among all treatments. (A) Clothianidin acute, (B) Clothianidin chronic low intensity, (C) Clothianidin chronic free feeding or high intensity.

5.4.3. Common biomarkers to both pesticides and modes of exposure

The identification of biomarkers of pesticide exposure that were not restricted to neonicotinoid type, and mode of exposure, was carried out by directly comparing the SSDA proteins identified in each of the 6 individual experimental datasets. In total 1,814

proteins were considered, but no single protein was found to be shared between all experimental groups.

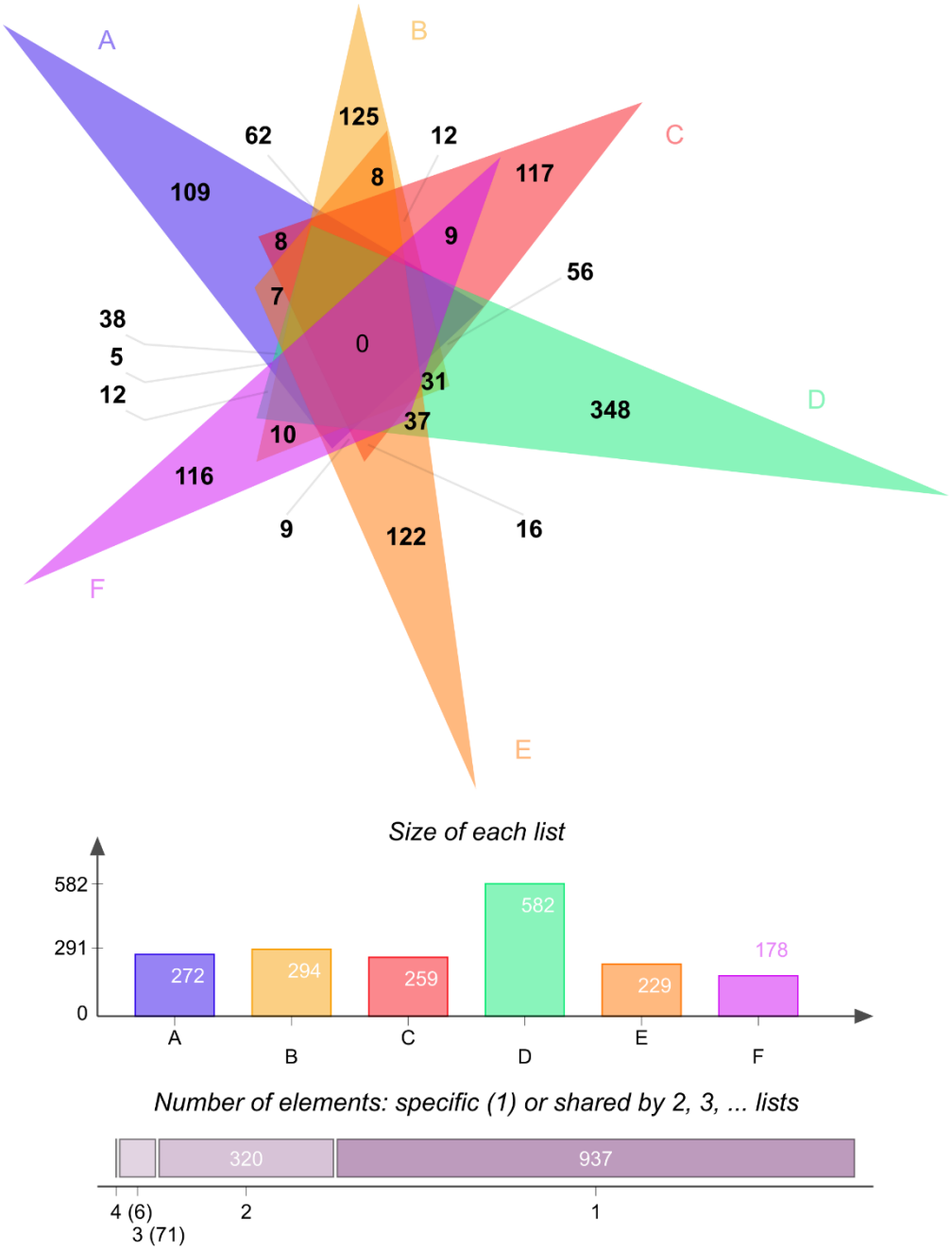


Figure 5.3 Venn diagram of the overlapping SSDA proteins among six experimental groups of neonicotinoid exposure experiments from chapter III and IV exposed to clothianidin acute and chronically and imidacloprid acutely. No protein is commonly found among all treatments. (A) Clothianidin 2.5 ng/ml, (B) Clothianidin 10 ng/ml, (C) Imidacloprid 2.5 ng/ml, (D) Imidacloprid 10 ng/ml), (E) Clothianidin chronic low intensity, (F) Clothianidin chronic free feeding or high intensity.

Given the lack of protein biomarkers identified when both neonicotinoids and modes of exposure are considered, the associated protein family (pfams) annotation was compared, rather than the individual protein representative. This approach identified 10 different protein families and domains, that show reproducible changes in abundance, in response to neonicotinoid exposure (Figure 5.4).

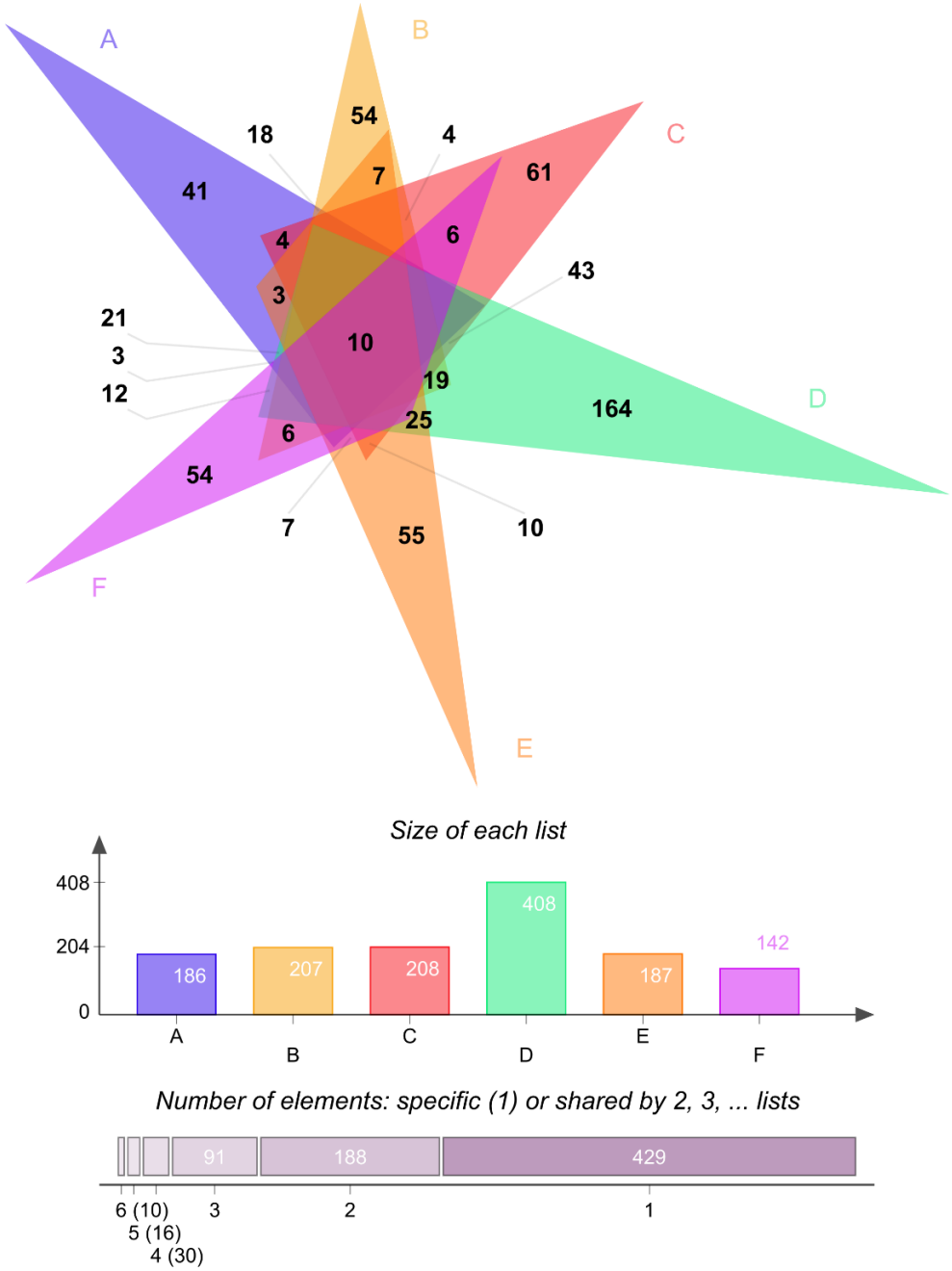


Figure 5.4 Venn diagram of the overlapping Pfams among six experimental groups of neonicotinoid exposure experiments from chapter III and IV exposed to clothianidin acute and chronically and imidacloprid acutely. Ten protein families or domains are commonly found among all treatments. Suggesting cohesive effect of neonicotinoid upon certain families of proteins (A) Clothianidin 2.5 ng/ml, (B) Clothianidin 10 ng/ml, (C) Imidacloprid 2.5 ng/ml, (D) Imidacloprid 10 ng/ml), (E) Clothianidin chronic low intensity, (F) Clothianidin chronic free feeding or high intensity.

The identified pfams (Table 5.2), demonstrate that neonicotinoids in general, affect catalytic activity including hydrolytic and kinase enzymes, mitochondrial carriers, calcium binding proteins, microfilament constituents and motors, signalling and transcription regulators (of the ras, WD40 and RRM_1) and lipid selective cell membrane domains. Therefore, no single protein in any dataset was deemed of sufficient potential to represent a biomarker of neonicotinoid exposure but the analysis of pfams appears promising.

Table 5.2 Protein families and domains commonly found in response to neonicotinoid exposure in the bumblebee brain

Common elements (Pfam) in A B C D E F:	Definition (source Pfam-EMBL-EBI)
Abhydrolase_6	Alpha/beta hydrolase superfamily of hydrolytic enzymes
Mito_carr	Mitochondrial carriers responsible for solute transfer across the membranes of the mitochondria
Pkinase	Protein kinase domain/ phosphorylation enzymes
EF-hand_6	Helix-loop-helix structural domain found in calcium-binding proteins
Actin	Family of microfilament constituents involved in muscle contraction, cell motility, cell division and cytokinesis, vesicle and organelle movement, cell signalling, and the establishment and maintenance of cell junctions and cell shape
Tubulin	Family of proteins involved in polymer formation
WD40	WD40 domain of proteins implicated in a variety of functions from signal transduction and transcription regulation to cell cycle control, autophagy and apoptosis
RRM_1	Putative RNA-binding domain RRM proteins bind heterogeneous nuclear ribonucleoproteins, regulate alternative splicing and RNA stability and translation
C2	Lipid selective protein structural domain involved in targeting proteins to cell membranes
Ras	Proteins called small GTPases involved in cellular signal transduction for cell growth, differentiation and survival.

5.5. Discussion

In clinical research, the identification of biomarkers can reflect disease spectrum and potential risk factors, providing early evidence to support and justify mitigation actions through pharmaceutical approaches (Mayeux, 2004). The identification of biomarkers of similar application for pesticide exposure, has long been an aim in the field of environmental toxicology, and their discovery, could stimulate the development of techniques that mitigate the risks of environmental stressors to populations of beneficial insects, including bees.

This is a difficult endeavour as by definition; biomarkers must be accurately and reproducibly identified to be able to serve as a diagnostic tool. In regards to beneficial insects, changes in the expression levels of AChE, CAT and GST have been previously associated with imidacloprid exposure, but most studies investigating effects with

detoxifying enzymes, have focused on establishing changes in activity for a variety of different compounds, both in bumblebees and honeybee species (Badiou-Bénéteau *et al.*, 2012; Boily *et al.*, 2013; Badawy, Nasr and Rabea, 2015; Alptekin *et al.*, 2016; Alburaki *et al.*, 2017). Generating problems where results are frequently contradictory and inconsistent, especially when effects triggered by different neonicotinoids are teased apart.

Our results demonstrate that when different types of chemicals and even modes of exposure are taken into consideration, the identification of one candidate protein biomarker is very difficult. Which corroborates with previous chapters and research that suggest neonicotinoid compound and dosage specific effects in the brains of bees (Cook, 2019). Through the comparison of the statistically significant differentially abundant proteins, derived from 6 datasets, no single protein was identified with a similar change in abundance, in response to general neonicotinoid exposure.

When only the acute exposure groups were analysed, although each dataset contained hundreds of statistically significant proteins, only one, the myosin 2 essential light chain protein (XP_012170909.1), an important regulator of structural plasticity and action potential generation, through its role in assembling axon initial segments in neurons (Berger *et al.*, 2018), was found to be consistently affected by imidacloprid and clothianidin. Similarly, after investigating the impact of imidacloprid and thiacloprid exposure on gene expression levels in mussels, and identifying that 97 and 80 genes responded to the exposure to each chemical individually and that 49 responded to the mixture of both. Dondero *et al.*, (2010) identified only two genes that were in common to the three conditions investigated. The same is seen in Colgan *et al.*, (2019)who demonstrated no common gene being affected in bumblebee worker brains after chronic exposure to imidacloprid and clothianidin, whereas 55 genes responded to clothianidin exposure only, and 1 gene responded to imidacloprid exposure. Such results demonstrate that compounds with the same hypothesised mode of action can give rise to different toxicodynamics, a finding that was replicated in our study. In addition, the same compound was also found to affect the organism differently depending on dose or timepoint assessed, as evidenced by the fact that only the proteins phosphatase methylesterase (XP_003393428.1), transferrin (XP_012172140.1), endothelin-converting enzyme (XP_012171205.1) and the proteasome subunit beta (XP_003396794.1) were shared between all clothianidin exposed groups. Reaffirming clothianidin effects upon neuronal cytoskeleton proteins, ion homeostasis and

neuritogenesis (Sontag *et al.*, 2010; Leitner and Connor, 2012).Determining the dose response to chemicals in toxicology, depends on a series of factors such as concentration, dose, mode of action, exposure duration, the endpoint being assessed and the environment of exposure itself (Short and Hannam, 2013). Therefore, it is not surprising that different degrees and ranges of molecular response emerge, from different neonicotinoids, exposure routes and experimental durations.

On the other hand, mortality is usually a linear effect of neonicotinoid exposure, increasing in accordance to increasing dose, until all individuals are dead, justifying its use when determining insecticide toxicity (Mommaerts *et al.*, 2010). However, when dealing with sub lethal effects, such as those observed through a characterisation of the proteome, we encounter difficulties in providing a clear and consistent set of results, to a variety of exposure scenarios. The complexity of the response observed at the proteome level, increases even further when we consider in such interpretations that neonicotinoid effects may represent a direct response, as a result of the stimulation of the targeted nAChR or an overcompensation stimulatory response, following damage caused by the neonicotinoids.

Even though we could not identify clear candidates to be further investigated, to verify their reproducibility and potential to represent biomarkers of general neonicotinoid exposure in bumblebees. We have, alternatively demonstrated the complexity of the nervous system, as suggested by Gil and Pla (2001) and the complications associated with trying to easily recognise candidates for biomarkers of neonicotinoid exposure.

Furthermore, we have successfully identified 10 protein families representing biochemical processes affected by neonicotinoids, independently of compound and exposure length. Some of these, represent general protein families such as alpha/beta hydrolases, protein kinases, WD40, ras and RRM_1, all proteins that are involved in crucial cellular signalling processes. Others, like the Mito_carr, EF hand 6, actin and tubulin proteins, and the C2 families, may explain some of the alterations in the mitochondria, calcium levels, membrane transport and maintenance of cell shape, currently reported in the literature (Rodrigues *et al.*, 2010; Oliveira *et al.*, 2014). In addition to effects on the mitochondria and energy metabolism, transcription, translation and oxidative phosphorylation that have also been reported as a result of neonicotinoid exposure at the molecular level in bees (Palmer *et al.*, 2013; Moffat *et al.*, 2015a; Shi *et al.*, 2017; Colgan *et al.*, 2019; Fotopoulou *et al.*, 2020).

We therefore conclude that our findings mirror the ambiguity associated with the classification of pesticides itself, particularly when their mode of action is considered. We suggest that when evaluating neonicotinoid exposure; the chemical type and mode of action must be considered carefully, along with the potential that different compounds can have the same critical effect or not, either by direct action on their molecular target or target tissue, by acting with the same pharmacological mechanism or even by sharing a common toxic intermediate (Mileson *et al.*, 1998). Determining these multi-level factors associated with pesticide action, provides a better understanding of their interaction with their possible target receptors, and helps resolve the cascade of molecular and biological upstream and downstream events of this interplay. It is impossible to ignore the various components of exposure, and we reiterate that they should be assessed prior to the comparison of results, obtained from different chemicals and exposure levels if successful candidates as biomarkers are to be resolved (Howd and Fan, 2008). In addition, the mass spectrometry based approach that we have used here provides insight into molecular effect dynamics, but is still limited by the amount of genetic information available for the studied organism and by the current mass spectrometry capabilities. In addition this chapter was constructed as a bioinformatic attempt to compare datasets obtained from independent mass spectrometry runs, and should therefore be interpreted with caution, as it aimed exclusively at enriching the debate surrounding the discovery of neonicotinoid biomarkers and not at providing the final word in such. Further experimental investigations regarding the accuracy and reproducibility of our finds is still required. With improved sensitivity and broader dynamic ranges, future mass spectrometers may identify a greater number of proteins and at lower quantities, which may then yield reproducible identification of biomarker proteins of neonicotinoid toxicityin bees and other insects.

Chapter VI

Insights into neonicotinoid insecticide metabolism and accumulation in *Bombus terrestris*

6.1. Introduction

Since their development in the 1990s, neonicotinoids became widely used around the globe, being extremely effective to combat phloem sucking pests of important commercial crops (Goulson, 2013). The presence of neonicotinoids in the environment, however, only began to be investigated much later. These new emerging data have demonstrated that neonicotinoid insecticides can accumulate in the soil (Bonmatin *et al.*, 2015), water (Sánchez-Bayo and Hyne, 2014) and plant components such as nectar and pollen in treated sites (Stewart *et al.*, 2014) and often in neighbouring wild plants (Botías *et al.*, 2016). Neonicotinoids are found in the field at concentrations ranging from 0.1 to 50 ppb in pollen and nectar and can reach ppm in guttation drops but the majority of samples have concentrations at the lower end of this range (Blacquière *et al.*, 2012; Bonmatin *et al.*, 2015), demonstrating their capacity to disperse and accumulate in the environment. This means that there are a variety of routes of exposure to beneficial insects in the wild, which poses danger and can be difficult to assess in a lab setting (Bonmatin *et al.*, 2015). Nevertheless, these studies have also provided a guide to the residue concentrations present in the field, which should be considered in toxicological experiments that attempt to simulate exposure to neonicotinoids. Many studies have also investigated the potential risk of insecticides to bees, by simulating sub lethal exposure by first determining the LD50 for the population tested and then, choosing concentrations that represented a proportion of that dose. In many cases, these exposure experiments were based on concentrations that are significantly higher than those in the field. Added to this, issues such as difficulties in making comparisons between studies and the lack of information provided, so that the final exposure levels can be determined, it is no wonder that governmental agencies are critical of much of the published literature (EFSA, 2012; EFSA, 2012b; Kyriakopoulou *et al.*, 2017).

It is true that estimating exposure levels in free feeding experiments is almost impossible, especially those carried out in the field with whole colonies (e.g. Cutler *et al.*, 2014) or those that directly exposed bees to treated crops (e.g. Larson *et al.*,2013). We understand that this is a challenge, meaning that it is important to improve our understanding of the pharmacokinetics and pharmacodynamics of these insecticides as they may guide interpretation of results.

When a bee is exposed to a specific amount of a toxic xenobiotic, very little is known about how the pesticide is distributed throughout an organism, although the presence of residues have been reported in whole honeybee bodies, in concentrations up to 5 ppb

(Blacquière *et al.*, 2012). Furthermore, divergences relating to their metabolism also exist in regards to the chemical type (Iwasa *et al.*, 2004). Therefore, it is essential to know how much of the parental or metabolite residues is delivered to the target organs, as a way of understanding the effects the neonicotinoid may trigger.

Characterising the chemistry, metabolism and potential distribution of these compounds can help us understand their toxicity, and potential accumulation in different segments of the body, which would be highly informative for future studies, that aim at characterising deleterious effects in insects.

The seven major commercial neonicotinoids are based on three component structures (Figure 6.1 A, B and C) and 12 unique substituents (Figure 6.1 bottom). The structural components consist of A, a heterocyclylmethyl moiety, B, a heterocyclic or acyclic spacer or C, an N-Nitroimine, Nitromethylene or N-Cyanoimine group. Registration of new chemicals requires that tests are carried out to determine their distribution, persistence and metabolic fate, in a way of understanding tolerance values in different organisms. It is the existence of these unique substituents, that determine the biodegradability and the possible metabolites formed by each neonicotinoid (Casida, 2011).

neonicotinoid	compound abbrev.	structural components
chloropyridinyl (CP)		
imidacloprid	IMI	A1 B1 C1
nitenpyram	NIT	A1 B2 C2
thiacloprid	THI	A1 B3 C3
acetamiprid	ACE	A1 B4 C3
clorothiazolyl (CT)		
clothianidin	CLO	A2 B5 C1
thiamethoxam	TMX	A2 B6 C1
tetrahydrofuryl (THF)		
dinotefuran	DIN	A3 B5 C1

A heterocyclylmethyl moiety
B heterocyclic or acyclic spacer
C $=NNO_2$, $=CHNO_2$ or =NCN tip

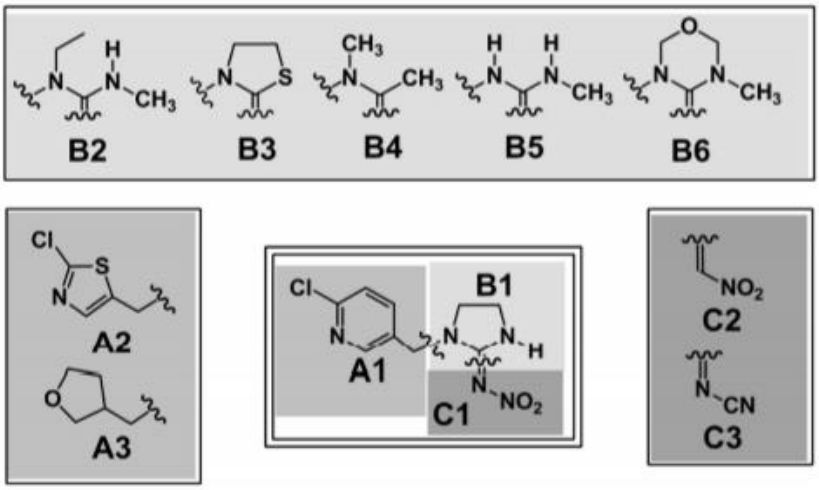


Figure 6.1 Seven commercial neonicotinoids consisting of 3 structural components (A, B, C) and 12 unique substituents (bottom). Image source Casida, (2011).

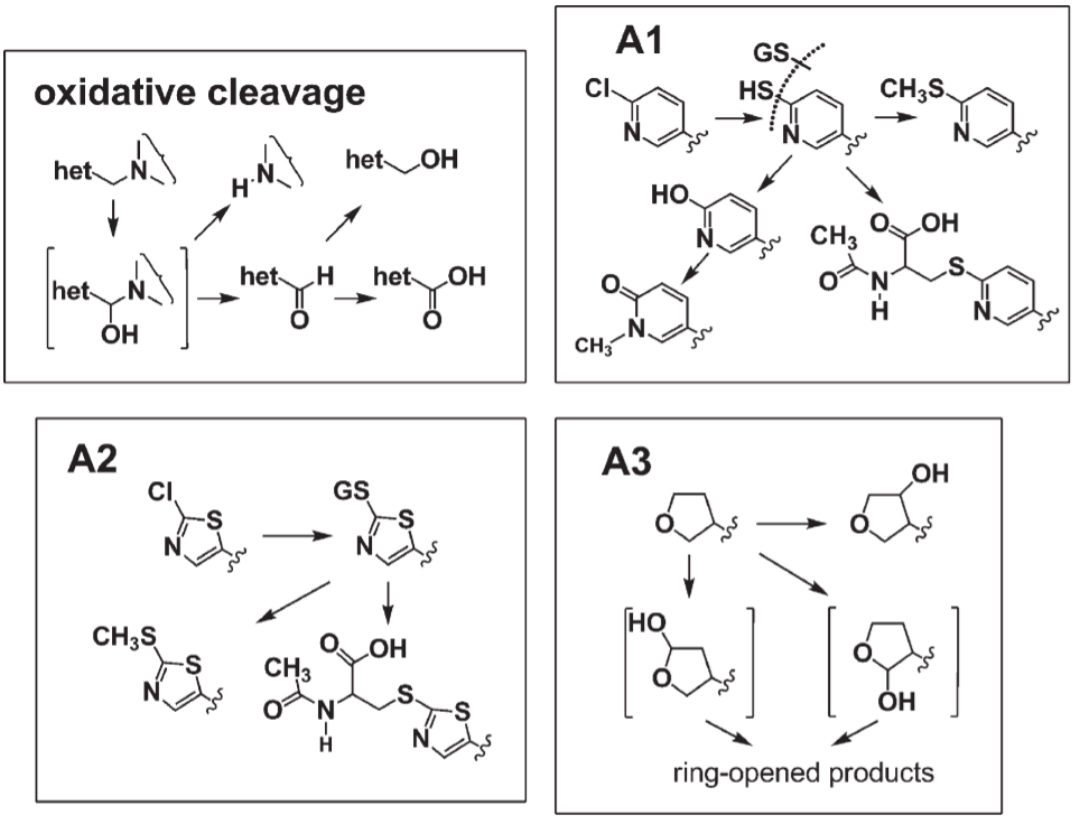


Figure 6.2 Common metabolic cleavage of the N-heterocyclylmethyl moiety (AB) and further modification of the chloropyridinyl (A1-CP), chlorothiazolyl (A2-CT), and tetrahydrofuryl (A3-THF) substituents. Image source Casida, (2011) .

A series of chemical reactions (Figure 6.2) can modify the structures of the neonicotinoids, generating different metabolites. Imidacloprid metabolites can be formed with the unchanged N-nitroguanidine functionality, including the 4-hydroxy, 4-O-glucuronide, 5-hydroxy, 4,5- dihydroxy, olefin, and desethano. Additional metabolites can be formed through the reduction of N-nitroguanidine to the N-nitrosoguanidine and the N-aminoguanidine, and the cleavage to the guanidine or hydrolysation to urea. Clothianidin cleavages (also observed with thiamethoxam) are primarily the result of N-demethylation (and remethylation) (Shi *et al.*, 2009; Casida, 2011). The substitution of four guanidines and an amine from the A-B cleavage are seen for clothianidin after common metabolism in mice and spinach. Casida, (2011) reports further examples of neonicotinoid metabolic pathways. The fact that many neonicotinoid metabolites have equal or greater toxicity than their parent compounds, highlights the importance of understanding how the original neonicotinoid is metabolised in different organisms.

6.1.1. Neonicotinoid metabolism

6.1.1.1. Detoxification

Neonicotinoids are agonists of nicotinic acetylcholine receptors (nAChRs) and their toxicity is generally related to their binding affinities to the relevant nAChRs subunits (Casida, 2011). For many years, there was an assumption that neonicotinoids posed no toxic risk to animals. However, this view has changed in light of a growing body of emerging evidence that demonstrates various toxic effects on vertebrates and invertebrates including; neurotoxicity, immunotoxicity, hepatotoxicity, nephrotoxicity, and reproductive cytotoxic (Iwasa *et al.*, 2004; Tomizawa and Casida, 2005; Wang *et al.*, 2018). The majority of our understanding of neonicotinoid metabolism is derived from imidacloprid, which has been documented to produce metabolites such as 4-hydroxy-IMI, 5-hydroxy-IMI, 6-hydroxy-IMI (6-hydroxy nicotinic acid), 6-chloronicotinic acid (6-CNA), 2-imidazolidone, olefin, guanidine (DNIMI), and urea derivatives in vertebrate animals (Ding and Peng, 2015).

The major enzyme super families responsible for toxin metabolism or detoxification, are the cytochrome P450 monooxygenases (P450s), glutathione transferases (GSTs) and the carboxylesterases (COEs) (Li et al., 2007). Detoxification is generally a two-phased process, involving detoxification followed by neutralisation. Phase I is part of the initial detoxification metabolic response that aims at making toxins less lipophilic, and forms bioactive molecules through oxidation, hydrolysis and reduction, and are generally mediated by the P450s. Phase II involves the formation of conjugates with endogenous compounds such as sugars, sulphate, phosphate, amino acids or glutathione for excretion or retention (Yu, 2014; Fortney *et al.*, 2017).

Glutathione-S-transferases are enzymes that catalyse the metabolism of pesticides by conjugation of reduced glutathione, via a sulfhydryl group, to an electrophilic centre on a wide variety of substrates. The P450s catalyse a range of reactions, including oxidation and demethylation, which may result in decreased activity and toxicity, and may involve the production of other active metabolites, as occurs with thiamethoxam which is converted to clothianidin in insects and plants (Jeschke and Nauen, 2007). Two imidacloprid metabolism pathways have been identified in humans involving the cytochrome P450 (CYP450) isozymes, one that acts via imidazolidine hydroxylation and desaturation to give 5-hydroxyimidacloprid and the olefins, respectively, and the other via nitroimine reduction and cleavage to yield the nitrosoimine (IMI-NO), DNIMI, and urea derivatives (Schulz-Jander and Casida, 2002).

The metabolism of thiamethoxam and clothianidin are closely related, probably involving CYP450 enzymes for the NCH_3, NCH_2, and OCH_2 hydroxylations and aldehyde oxidases or CYP450 enzymes for the NNO_2 reduction, and glutathione for dichlorination (Ford and Casida, 2006). Aldehyde oxidases are also involved in neonicotinoid metabolism in monkeys, humans, mouse, cows and rats but as yet, they have not been associated with neonicotinoid metabolism in insects (Hemingway *et al.*, 2000).

Although neonicotinoid conversion to their metabolites (e.g. imidacloprid to olefin or 5-hydroxy-imidacloprid; thiamethoxam to clothianidin) has been described in several insect species, further studies are required to elucidate the step by step process of detoxification pathways, and their participating enzymes (Yu, 2014; Nishiwakl *et al.*, 2004; Brunet *et al.*, 2005; Jeschke and Nauen, 2007). Most of what is known about neonicotinoid metabolism in insects is derived from a small number of model insect species including *Drosophila* and the honeybee *Apis mellifera.* Studies in *Drosophila melanogaster* have demonstrated a correlation in CYP450 6g1 activity with resistance to imidacloprid. Pesticide and their secondary metabolite detoxification mechanisms have been elucidated in honey bees, utilising *in vitro* metabolism assays (enzyme assays), toxicity bioassays in the presence or absence of enzyme inducers, or inhibitors (Mao *et al.*, 2009; Johnson *et al.*, 2012; Mao *et al.*, 2013). Interestingly, genomic analysis has revealed that honeybees have a 50% or greater reduction in the number of genes encoding for detoxification enzymes in comparison with other insects (Claudianos *et al.*, 2006). In addition, detoxification, immune and stress response genes associated with pesticide response have been determined using genome-wide transcriptome analysis of specific tissues such as the midgut or the abdomen (Mao *et al.*, 2013; Aufauvre *et al.*, 2014) in response to pesticide or xenobiotic exposure (Johnson *et al.*, 2012).

Although there is a basis for understanding pesticide detoxification in insects, there are limitations in terms of inferring mechanisms between different species. With respect to bumblebees, little is known about their tolerance and detoxification mechanisms to potentially toxic secondary metabolites and pesticides, and it is the aim of this study to improve this understanding. In addition, the majority of previous studies investigating pesticide exposure and detoxification mechanisms are based on mRNA expression, which provide very limited insight into biochemical level systems, as they generally correlate very poorly with final protein or enzyme amount or activity.

6.1.2. Toxicity and accumulation

Neonicotinoid and nicotinoid agonist action and toxicity are generally related to their binding affinities at the relevant nAChRs. Thus, nAChR potency is a good predictor of neonicotinoid toxicity, providing that the effects of metabolic activation or detoxification are minimised in the target organism (Casida, 2011). Their toxic effects on vertebrates and invertebrates are believed to be associated with oxidative stress through reactive oxygen species (ROS), and reactive nitrogen species (RNS), which can induce damage to lipids (lipid peroxidation), DNA, and proteins (Tomizawa and Casida, 2005). A general mode of neonicotinoid toxicity and detoxification has been proposed by Wang *et al.* (2018) which highlights the main molecular mechanisms involved in the pharmacokinetics of neonicotinoids in the cell and the process through which exposure increases oxidative damage, through the oxidation of lipids, proteins and DNA (Figure 6.3).

Oxidative stress occurs as a result of inadequate antioxidant defences, or overproduction of free radicals and plays important roles in many biological responses and cell signalling pathways. Enzymatic antioxidant defence systems play a critical role in protecting cells from ROS, such as hydrogen peroxide, superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), and glutathione S-transferase (GST) which act as the first-line indicators of the antioxidant state through oxidation/reduction processes. Interestingly, antioxidant enzymes display a threshold effect when animals are treated with neonicotinoids, and do not act in many cases until a high dose is applied (Wang *et al.*, 2018).

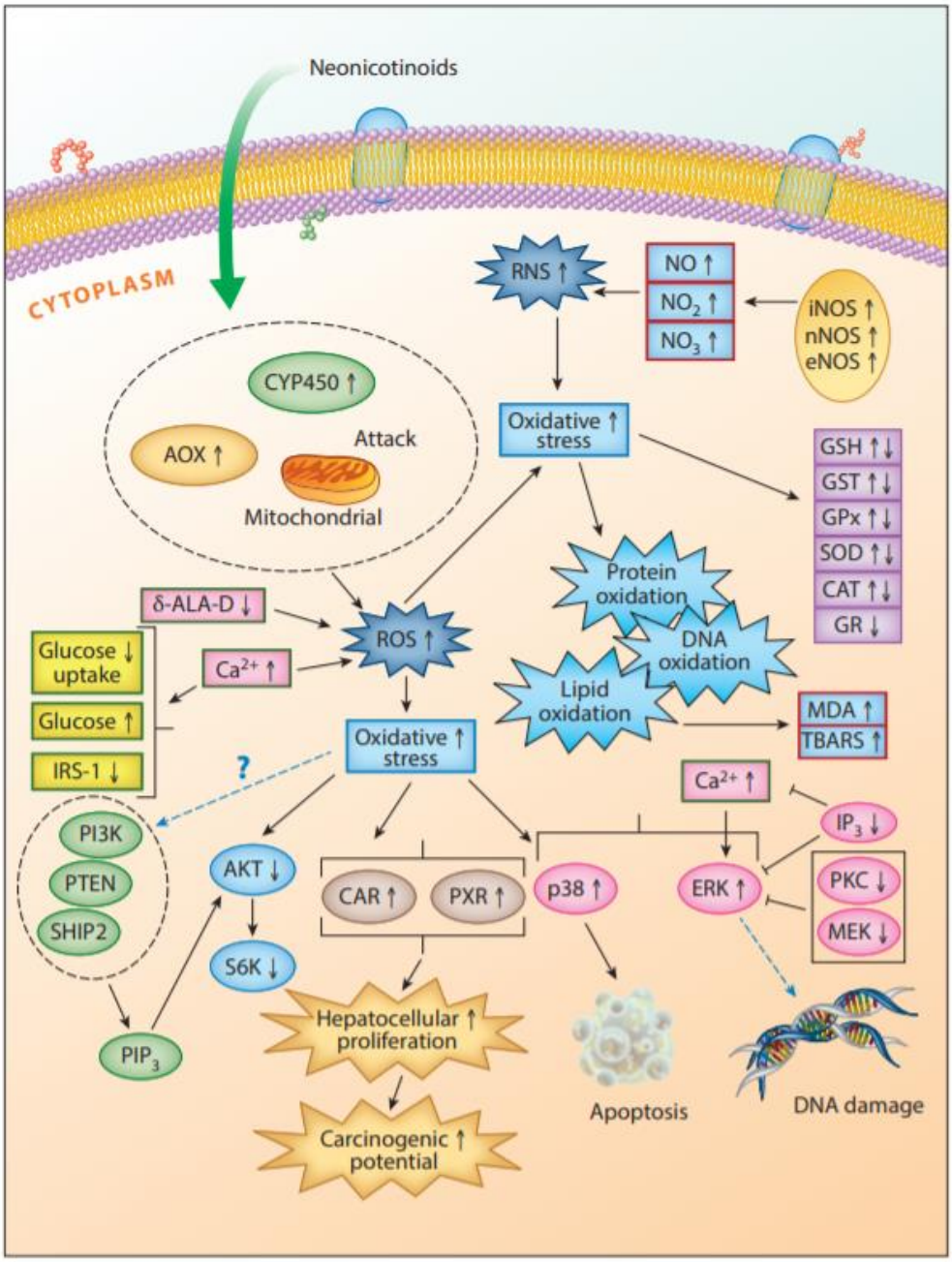


Figure 6.3 The major biochemical effects of neonicotinoids are mediated through their activity on nAChR. However, various signalling pathways also play a role in their toxicity, including their capacity to induce oxidative stress, ROS generation and Ca^{2+} levels. The figure above demonstrates the oxidative stress–mediated mode of action proposed for neonicotinoids, where increased generation of ROS and RNS, as well as an alteration in the antioxidant status, may induce lipid, protein, and DNA oxidation, leading to various toxicities and apoptosis via ERK, p38, AKT, Ca2+, and CAR/PXR pathways. Abbreviations: δ-ALA-D, δ-aminolevulinate dehydratase; AKT, protein kinase B; AOX, molybdo-flavoenzyme aldehyde oxidase; CAR, constitutive androstane receptor; CAT, catalase; CYP450, cytochrome P450; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal–regulated kinase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GST, glutathione S-transferase; iNOS, induced nitric oxide synthase; IP3, inositol 1,4,5-trisphosphate; IRS-1, insulin receptor substrate-1; MDA, malondialdehyde; MEK, MAPK/ERK; nNOS, neuronal nitric oxide synthase; PI3K, phosphoinositide 3-kinase; PIP3, phosphatidylinositol-3,4,5-triphosphate; PKC, protein kinase C; PTEN, phosphatase and tension homolog; PXR, pregnane X receptor; RNS, reactive nitrogen species; ROS, reactive oxygen species; S6K, ribosomal S6 kinase; SHIP2, SH2-containing inositol phosphatase 2; SOD, superoxide dismutase; TBARS, thiobarbituric acid reacting substances Image source: Wang *et al.* (2018)

Certain neonicotinoid metabolites have an equal or greater toxicity than their parent compounds, such as clothianidin, a metabolite of thiamethoxam (Simon-Delso *et al.*, 2015). Therefore, these initial metabolic detoxification steps generally do not reduce the risk to beneficial insects. Thus, it is necessary to understand not only the possible detoxification mechanisms but also the process by which neonicotinoids and their metabolites travel throughout organisms such as bees.

Detailed studies have identified the distribution and metabolic pathways of imidacloprid in honeybees, and suggest that it is readily metabolised (possibly by mixed function oxidases (Phase I metabolism)) to more water-soluble products with an elimination half-life of 5 hours (Suchail *et al.*, 2004; Suchail *et al*., 2004b). Six and 24 hours after imidacloprid ingestion at doses of 20 and 50 μg/kg per bee, no imidacloprid in its parental form was detected in bees, although the derivatives of imidacloprid were. Even though the midgut is assumed to represent the main detoxifying organ, reports on the distribution the pesticides and its metabolites throughout the insect body and variations in its kinetic profile, suggests that metabolism may also occur elsewhere (Magesh *et al.*, 2017). The main metabolites, urea and 6-chloronicotinic acid, were found in the mid gut and rectum, whereas 4/5-hydroxy-imidacloprid and olefin were predominantly found in the head, thorax and abdomens, all of which are nAChR rich tissues (Magesh *et al.*, 2017).

Studies on acetamiprid report a different metabolic profile to that of imidacloprid, with 50% of acetamiprid being completely metabolised after only 30 min of oral exposure to 100ppb of the neonicotinoid. The low toxicity of acetamiprid to honeybees is thus attributed to its rapid metabolism (half-life 25 min) and absence of insecticidal metabolites (Iwasa *et al.*, 2004). Brunet et al., (2005), also evaluated the distribution of acetamiprid and its metabolites in different compartments of the honeybee. Acetamiprid was found to be rapidly distributed in all compartments and metabolised, with its initial detection in the nAChR-rich tissues of the abdomen, head and thorax, with a distribution profile similar to that of imidacloprid (Suchail *et al.*, 2004). They described a cumulative distribution of the insecticide reaching levels of 33% of the ingested dose in the abdomens 30 min post exposure. The levels in the abdomen declined to 1% of the total ingested dose after 18 hours. Acetamiprid was also detected at 6.5% of the total ingested dose in the thorax, and 6% in the heads after 30 minutes, and then gradually declined. The distribution profile of acetamiprid described here, was similar to that of imidacloprid in the same body segments of honeybees (Suchail *et al.*, 2004). Both neonicotinoids presented the same kinetics of appearance and disappearance, although they present

different degrees of toxicity to insects (Iwasa *et al.*, 2004). Such differences of toxicity might be explained by a slightly weaker affinity of acetamiprid for nAChR when compared with imidacloprid, and or by an inability of either insecticide to induce detoxifying systems at low doses. In the case of neonicotinoids, the hypothesis that they could activate nAChRs via separate high and low affinity binding sites is more probable, and may explain their differences in toxicity in regards to their metabolism (Brunet *et al.*, 2005).

The distribution of clothianidin through the bee body has yet to be investigated comprehensively in relation to the dose ingested. However, the studies of Suchail *et al*., (2004) and Brunet *et al*., (2005) supports the possibility that clothianidin kinetics obeys the same patterns as acetamiprid and imidacloprid, by persisting in its parental or metabolised form in nAChR-rich tissues, such as the head and the thorax. It is the main aim of this chapter to further investigate whether this is the case or not.

6.2. Chapter aims and hypotheses

The principal aim of this chapter was to determine the accumulation and distribution patterns of clothianidin through different bumblebee body segments, by measuring the xenobiotic in the tissues from bees chronically exposed to 10 ng/ml of clothianidin.

This was achieved through the:

1. Quantification of clothianidin, and the presence of its metabolites in the heads, thorax and abdomens of bumblebees exposed continuously to contaminated sucrose solutions, for the duration of 4 or 48 hours, 5 days and 10 days.
2. Determination of clothianidin accumulation levels in the different segments of the bumblebee body, and production of a refence map that could be used in future toxicological assessment studies.

We hypothesise that clothianidin and its metabolites should be detected at time dependent increasing levels throughout the body of bumblebees, in concentrations directly proportional to the body mass of each segment analysed.

6.3.Experimental outline and methods

6.3.1. Bumblebee microcolony maintenance and exposure

Commercial bumblebee workers were obtained from colonies purchased from Biobest (Belgium), and acclimatised to a microcolony environment as described previously in sections 2.3.1, 2.3.2, 3.3.1, 4.3.1 and 4.3.2 of chapter II, III and IV. Continuous clothianidin exposure through oral ingestion of contaminated sucrose solution 50% w/v, was carried out similarly to the protocol described in section 4.3.2.3 in chapter IV. Bees were given fresh feeders with the contaminated solutions at the concentration of 10 ng/ml daily throughout the duration of the experiment. The concentration was chosen based on a tolerance pilot experiment of exposure to 35 ng/ml clothianidin that resulted in significative death of the majority of bees tested (described in chapter IV). Four boxes were set up for each timepoint with 10 bees in each box (n=40). Consumption volume was monitored, and body segment samples were collected after 4 and 48hr and 5 and 10 days of continuous exposure. A control group was also monitored for 10 days for comparison of accumulation results, totalling 5 experimental groups.

6.3.2. Sample preparation

Sample preparation was carried out on the dissection of heads, thorax and abdomens of bees, belonging to each experimental group, at their respective timepoints. Ten heads, thorax or abdomens were dissected, pooled together, and weighted making one biological replicate, to be used for pesticide residue detection. Four biological replicates per timepoint and body segment, were used in the analysis, totalizing 60 samples originating from 200 bees (200 bees, 600 body parts, 10 used in each replicate). Three spiked biological replicates per body segment were obtained in the same way and used to calculate recovery and validate the method adopted in this experiment. Legs and wings were not used = and body segments were snap frozen with liquid nitrogen and stored in a -70°C freezer, until pesticide extraction took place.

6.3.3. Pesticide residue extraction and LC/MS analysis

Once samples were dissected, divided into replicates and weighed, they were kept in the freezer until a scaled down adapted version of the Quick Easy Cheap Effective Rugged and Safe (QuEChERS) method proposed by Anastassiades *et al.*, (2003) was performed for clothianidin residue extraction. Each sample was homogenised in 5 ml of acetonitrile. The supernatant was transferred to fresh 15 ml falcon tubes containing 2g of

$MgSO_4$ and 0.5g of NaCl, tubes were vigorously vortexed for 1 min and centrifuged at 3000g for another minute. The supernatant was again transferred to a fresh 15ml falcon tube and samples were left in the freezer for 1 hour. Centrifugation was repeated at 1500g for 3 minutes and supernatant was again, transferred to new tubes. Samples were dried to pellet using a SpeedyVac concentrator at low temperature (Thermo Scientific Savant DNA120) and pellets were reconstituted with 100 (heads and thorax) or 200 µl (abdomens) of acetonitrile.

The samples were analysed in a Sciex 6500 Q-Trap LC-MS/MS system, residues were separated by 5-100% gradient of 0.315 g/L ammonium formate solution in methanol/ ultra-pure water on a Kinetix C18 150 x 2.1 mm, 2.5 µm (100Ă pore size LC column), using a 20 minutes' reverse-phase gradient at a flow rate of 400 µl/min.

6.3.4. Data analysis

Pesticide residue concentrations were detected for four biological replicates per timepoint and body part. Concentration values were plotted for each body segment and comparisons were visualized through box plot analysis. Residue concentrations were compared across body segments and normalised based on tissue weight present in each sample. Analysis were carried out using Prism GraphPad v.5 (one-way ANOVA with a post hoc Tukey's test).

Obtained clothianidin quantitation values were compared with expected results, estimated based on total volume of contaminated solution ingested by bees, in each experimental box, throughout the experiment duration. Recovery values were calculated by dividing obtained concentrations by amount of spike added and multiplying the result by 100.

Mortality was assessed for each experimental group and survival curve analysis was performed using Prism GraphPad v.5 with percentage survivability reported.

6.4.Results

6.4.1. Total pesticide residue accumulation

After exposure to a 50% sucrose solution (w/v) contaminated with 10 ng/ml of clothianidin for 10 days. Heads, thorax and abdomens were collected and processed for pesticide residue analysis after 4h, 48 h and 5 and 10 days using a Sciex 6500 Q-Trap LC-MS system. Pesticides residues were extracted from pooled samples of 10 bees according to an adapted version of the QuEChERs method (Anastassiades *et al.*, 2003).

For each body segment, 4 biological replicates containing 10 body parts each were analysed.

Clothianidin was found to accumulate in average to concentrations of 0.65 ng/ml, 2.5 ng/ml and 6.6 ng/ml within 4h of exposure in the heads, thorax and abdomens respectively (Table 6.1). The concentrations of the insecticide rose to 0.98 ng/ml in the heads, 2.9 ng/ml in the thorax and 6.3 ng/ml in the abdomens after 48h of exposure. From that point onwards no statistically significant increase was detected. Residue accumulated to values of 1ppb (1 ng/ml), 3.5ppb (3.5 ng/ml) and 6ppb (6 ng/ml) after 5 and 10 days for heads, thorax and abdomens respectively.

Greater accumulation of the parental form of clothianidin was also found in abdomens in comparison to the other two body segments assessed. Clothianidin travels through the bee body accumulating at concentrations around 1ppb in the heads and 2-4 ppb in the thorax (Table 6.1 and Figure 6.4). Clothianidin concentrations were relatively high at 4h, after the first exposure, but do not increase with time, remaining at a constant low level during the 10 days of the experiment (Figure 6.4).

Table 6.1 Pesticide residue measurements for each body segment and replicate (lcl = lowest calibrated level).

Samples	Concentration of clothianidin (ng/ml)	Average (ng/ml)
Control Heads	0 < lcl	
Control Heads	0 < lcl	
Control Heads	0 < lcl	< lcl
Control Heads	0 < lcl	
Heads 4h 1	0.234	
Heads 4h 2	0.982	
Heads 4h 3	0.616	0.64975
Heads 4h 4	0.767	
Heads 48h 1	0.525	
Heads 48h 2	0.708	
Heads 48h 3	1.090	0.98325
Heads 48h 4	1.610	
Heads 5 days 1	0.945	
Heads 5 days 2	1.060	
Heads 5 days 3	1.160	1.033
Heads 5 days 4	0.967	
Heads 10 days 1	1.080	
Heads 10 days 1	0.963	
Heads 10 days 1	1.030	0.97475
Heads 10 days 1	0.826	
Control Thorax 1	0.0715 < lcl	
Control Thorax 2	0.0266 < lcl	
Control Thorax 3	0 < lcl	
Control Thorax 4	0 < lcl	< lcl
Thorax 4h 1	1.700	
Thorax 4h 2	3.640	
Thorax 4h 3	1.810	2.5025
Thorax 4h 4	2.860	
Thorax 48h 1	2.630	
Thorax 48h 2	2.790	
Thorax 48h 3	3.320	2.92
Thorax 48h 4	2.940	
Thorax 5 days 1	3.310	
Thorax 5 days 2	3.740	
Thorax 5 days 3	3.530	3.4125
Thorax 5 days 4	3.070	
Thorax 10 days 1	3.700	
Thorax 10 days 2	3.410	
Thorax 10 days 3	3.330	3.4825
Thorax 10 days 4	3.490	
Control Abdm	0.086 < lcl	
Control Abdm	0 < lcl	
Control Abdm	0 < lcl	< lcl
Control Abdm	0 < lcl	
Abdm 4 h 1	3.710	
Abdm 4 h 2	10.500	
Abdm 4 h 3	4.820	6.68
Abdm 4 h 4	7.690	
Abdm 48 h 1	6.500	
Abdm 48 h 2	4.590	
Abdm 48 h 3	8.240	6.315
Abdm 48 h 4	5.930	
Abdm 5 days 1	4.850	
Abdm 5 days 2	7.590	
Abdm 5 days 3	5.460	6.6725
Abdm 5 days 4	8.790	
Abdm 10 days 1	3.850	
Abdm 10 days 2	5.460	
Abdm 10 days 3	4.730	5.5525
Abdm 10 days 4	8.170	

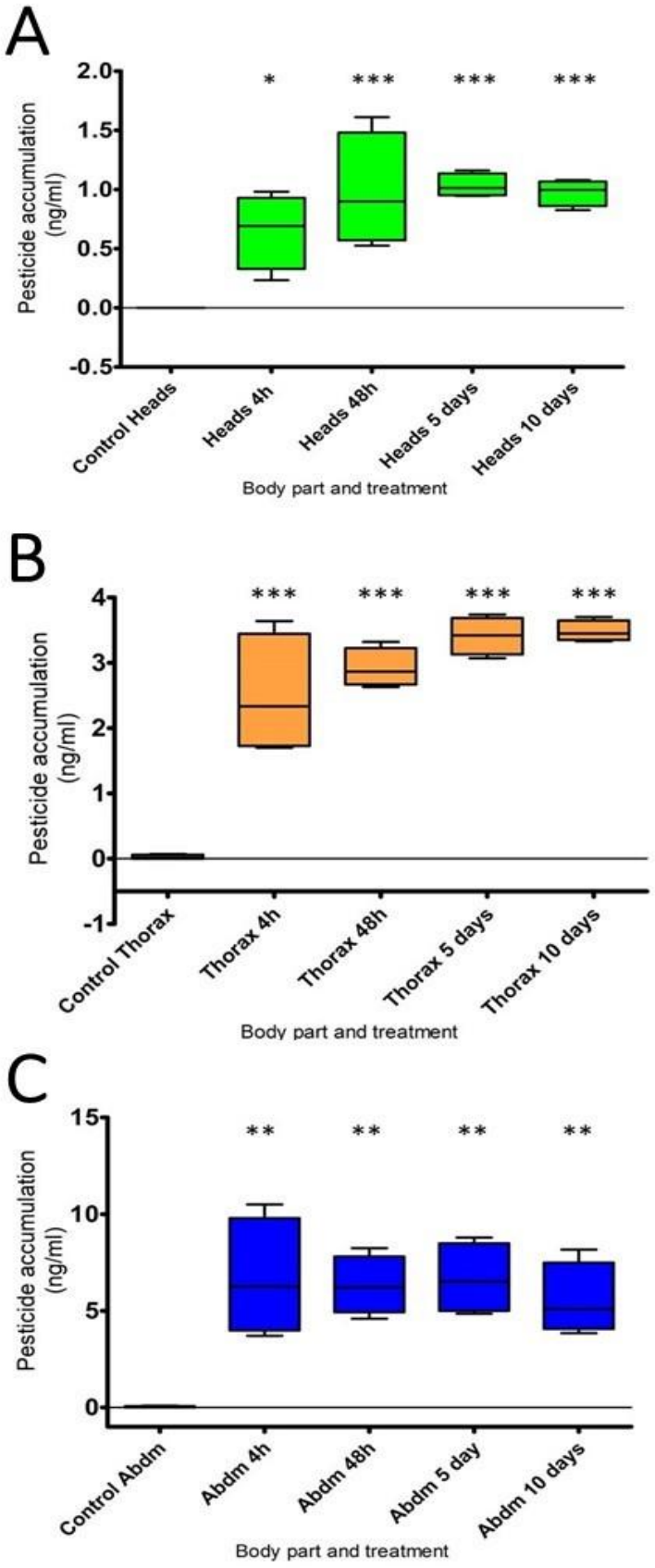


Figure 6.4 Pesticide residue measuments after chronic exposure to 10 ng/ml clothianidin for (A) heads, (B) thorax, and (C) abdomens. Clothianidin levels significantly accumulated in all three body segments in concentrations ranging from 0.64 to 6.6 ng/ml (ppb) in average. (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$ Student's t-tests (Prism GraphPad v.5 one-way ANOVA with a post hoc Tukey's test).

Recovery values were calculated as a way of validating the protocol and were found to be around 114,113 and 88.3% for heads, thorax and abdomens respectively (Table 6.2).

Table 6.2 Pesticide residue measurements of spiked samples for recovery calculation.

Spike Results				
Spike	**Result (ppb)**	**Recovery (%)**	**Expected (ppb)**	**Recovery average (%)**
Heads 1	61.7	123.4	50	114.2
Heads 2	52.9	105.8	50	
Heads 3	56.7	113.4	50	
Thorax 1	59.8	119.6	50	113.8
Thorax 2	55.4	110.8	50	
Thorax 3	55.6	111.2	50	
Abdm 1	15.3	61	50	88.3
Abdm 2	31	124	50	
Abdm 3	20.2	80	50	

6.4.2. Consumption and mortality

The total consumption of contaminated solution was also estimated on the last day for each experimental timepoint. As time passed the total volume of the solution consumed on average, by each replicate of 10 bees, increased (Figure 6.5). During the first 4 hours it was estimated that a group of 10 bees consumed in average 675µl of the clothianidin contaminated solution. This amount increased to 8.5 mls, 12.9 mls and 21.75 ml for 48 h, 5 days and 10 days respectively.

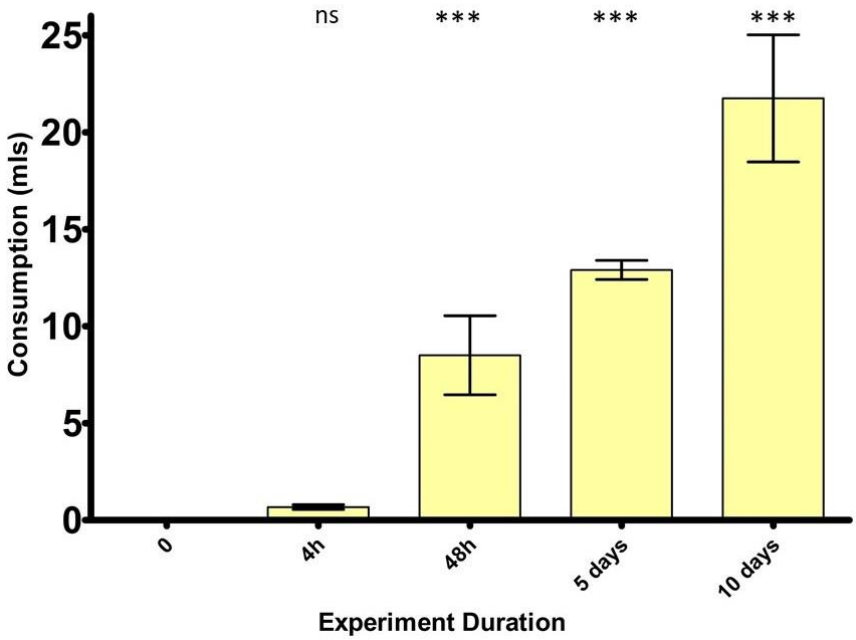


Figure 6.5 Average volume of 50% w/v contaminated sucrose solution consumed by 10 bees during the total length of the experiment in each treatment group. Consumption increases with duration of exposure as it is to be expected. In 4 hours, 10 bees consumed in average a total of 675 µl of clothianidin contaminated solution (10 ng/ml). During the other timepoints, the average consumption per replicate (10 bees) was of 8.5 mls, 12.9 mls and 21.75 ml for 48h, 5 days and 10 days respectively. ns = not significant, (***) $p \leq 0.001$ Student's t-tests.

The consumption values were used to estimate overall dosage and exposure to clothianidin, the results of which demonstrate that at 4h, 10 bees consumed 6.75 ng of clothianidin, which then increased to 85 ng in 48 h, 129 ng in 5 days and 217.5 ng after 10 days of exposure to the contaminated solution (10 ng/ml), on average (Table 6.2). Based on these values our results show that after 4 h, only about 24 % of clothianidin in its parental form could be detected in the bumblebee body, and that the percentage dropped to less than 2 % of the ingested clothianidin at 48 h and 5 days, and to less than 1 % after 10 days of exposure. These values were calculated based on the estimated final dosage of clothianidin but do not reflect the fact that clothianidin was steadily found at similar concentrations in each body segment after 4 h (Figure 6.4 and Table 6.3, % distribution of clothianidin).

Table 6.3 Mean respective values for pesticide residue accumulation, consumption and body weight of bumblebees (n=10 in 4 replicates) exposed to 10 ng/ml of clothianidin.

	4h	**48h**	**5 days**	**10 days**
Estimated average consumption (ml)	0.68	8.50	12.90	21.75
Estimated average exposed dose (ng)	6.75	85.00	129.00	217.50
Quantity of residue detected in average	1.65	1.65	1.78	1.56
% of parental form of clothianidin detected in relation to expected	24.46	1.95	1.38	0.72
Presence of detected of clothianidin in the heads (%)	3.94	5.94	5.80	6.26
Presence of detected clothianidin in the thorax (%)	15.13	17.66	19.18	22.38
Presence of detected clothianidin in the abdomens (%)	80.90	76.39	75.00	71.36

When the quantity of pesticide residues detected was compared with the body mass of the segments analysed no statistically significant difference in accumulation between body segments was evident (Figure 6.6). In average, clothianidin accumulated in the heads of bumblebees to up to 2.5 ng per gram of tissue, within 4h of exposure. The normalised averages for the other groups were of 3.9 ng/g, 4 ng/g and 4.5 ng/g at 48 h, 5 days and 10 days respectively. While normalised accumulation in the thorax was of 3.5 ng/g, 3.8 ng/g, 4.6 ng/g and 4.7 ng/g at 4 h, 48 h, 5 days and 10 days respectively. The average accumulation in abdomens was of 4.8 ng/g, 5.1 ng/g, 5.2 ng/g and 4.5 ng/g again at 4 h, 48h, 5 days and 10 days respectively. The quantity of residues found in each body segment across timepoints was similar, suggesting an even distribution of clothianidin in its parental form throughout bumblebee bodies (Table 6.4and Appendix 6).

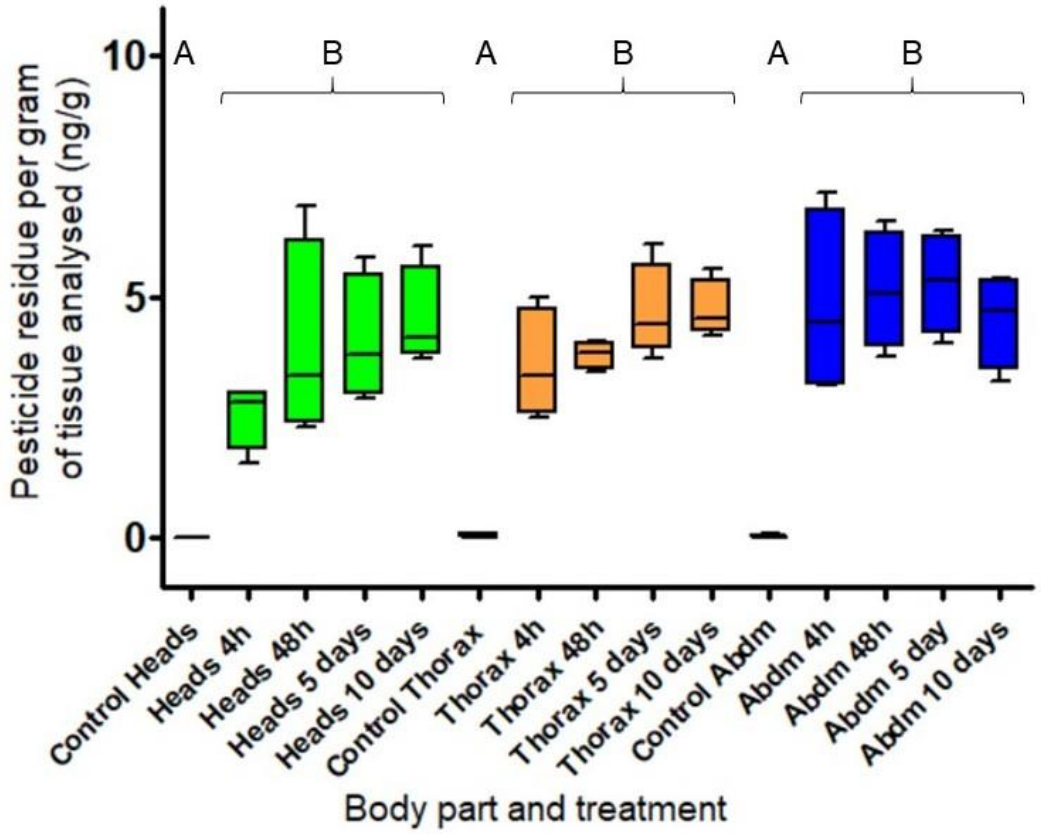


Figure 6.6 Normalised pesticide residue measuments after chronic exposure to 10 ng/ml clothianidin for 10 days (green) heads, (orange) thorax, and (blue) abdomens. Clothianidin levels significantly accumulated in all three body segments in comparison with control but not statistically significant differences were found between body segments (Prism GraphPad v.5 two-way ANOVA).

Table 6.4 Normalised pesticide residue measurements for each body segment and replicate.

Samples	Weight (g)	Concentration ng/g	Normalised value (ng of pesticide per g of tissue)	Average
Control Heads	0.2	0.0	0.0	
Control Heads	0.1	0.0	0.0	
Control Heads	0.2	0.0	0.0	
Control Heads	0.1	0.0	0.0	0.0
Heads 4h 1	0.2	0.2	1.6	
Heads 4h 2	0.3	1.0	3.0	
Heads 4h 3	0.2	0.6	2.7	
Heads 4h 4	0.3	0.8	3.0	2.6
Heads 48h 1	0.2	0.5	2.7	
Heads 48h 2	0.3	0.7	2.3	
Heads 48h 3	0.3	1.1	4.1	
Heads 48h 4	0.2	1.6	6.9	4.0
Heads 5 days 1	0.2	0.9	4.4	
Heads 5 days 2	0.3	1.1	3.2	
Heads 5 days 3	0.2	1.2	5.8	
Heads 5 days 4	0.3	1.0	2.9	4.1
Heads 10 days 1	0.3	1.1	3.7	
Heads 10 days 1	0.2	1.0	4.0	
Heads 10 days 1	0.2	1.0	6.1	
Heads 10 days 1	0.2	0.8	4.3	4.5
Control Thorax 1	0.7	0.1	0.1	
Control Thorax 2	0.7	0.0	0.0	
Control Thorax 3	0.7	0.0	0.0	
Control Thorax 4	0.7	0.0	0.0	0.0
Thorax 4h 1	0.7	1.7	2.5	
Thorax 4h 2	0.7	3.6	5.0	
Thorax 4h 3	0.7	1.8	2.8	
Thorax 4h 4	0.7	2.9	4.0	3.6
Thorax 48h 1	0.8	2.6	3.4	
Thorax 48h 2	0.8	2.8	3.7	
Thorax 48h 3	0.8	3.3	4.1	
Thorax 48h 4	0.7	2.9	4.0	3.8
Thorax 5 days 1	0.7	3.3	4.4	
Thorax 5 days 2	0.6	3.7	6.1	
Thorax 5 days 3	0.8	3.5	4.4	
Thorax 5 days 4	0.8	3.1	3.7	4.7
Thorax 10 days 1	0.7	3.7	5.6	
Thorax 10 days 2	0.7	3.4	4.6	
Thorax 10 days 3	0.8	3.3	4.2	
Thorax 10 days 4	0.8	3.5	4.5	4.7
Control Abdm	1.1	0.1	0.1	
Control Abdm	1.1	0.0	0.0	
Control Abdm	1.2	0.0	0.0	
Control Abdm	1.1	0.0	0.0	0.0
Abdm 4 h 1	1.2	3.7	3.2	
Abdm 4 h 2	1.5	10.5	7.1	
Abdm 4 h 3	1.5	4.8	3.3	
Abdm 4 h 4	1.3	7.7	5.7	4.8
Abdm 48 h 1	1.4	6.5	4.6	
Abdm 48 h 2	1.2	4.6	3.8	
Abdm 48 h 3	1.3	8.2	6.6	
Abdm 48 h 4	1.1	5.9	5.5	5.1
Abdm 5 days 1	1.2	4.9	4.0	
Abdm 5 days 2	1.3	7.6	5.9	
Abdm 5 days 3	1.1	5.5	4.9	
Abdm 5 days 4	1.4	8.8	6.4	5.3
Abdm 10 days 1	1.2	3.9	3.2	
Abdm 10 days 2	1.1	5.5	5.2	
Abdm 10 days 3	1.1	4.7	4.3	
Abdm 10 days 4	1.5	8.2	5.4	4.5

The survivability of bees was also monitored, and mortality was detected only in the groups of bees exposed to clothianidin for 5 and 10 days. At around 48 h the first bee was found dead, but mortality became more apparent after day 5 (Figure 6.7). In total 10 bees died during the 10 days exposure experiment and 5 bees died during the first 5 days. No dead bees were used for the pesticide residue analysis. Once dead, they were replaced by bees from a spare experimental box of the same conditions.

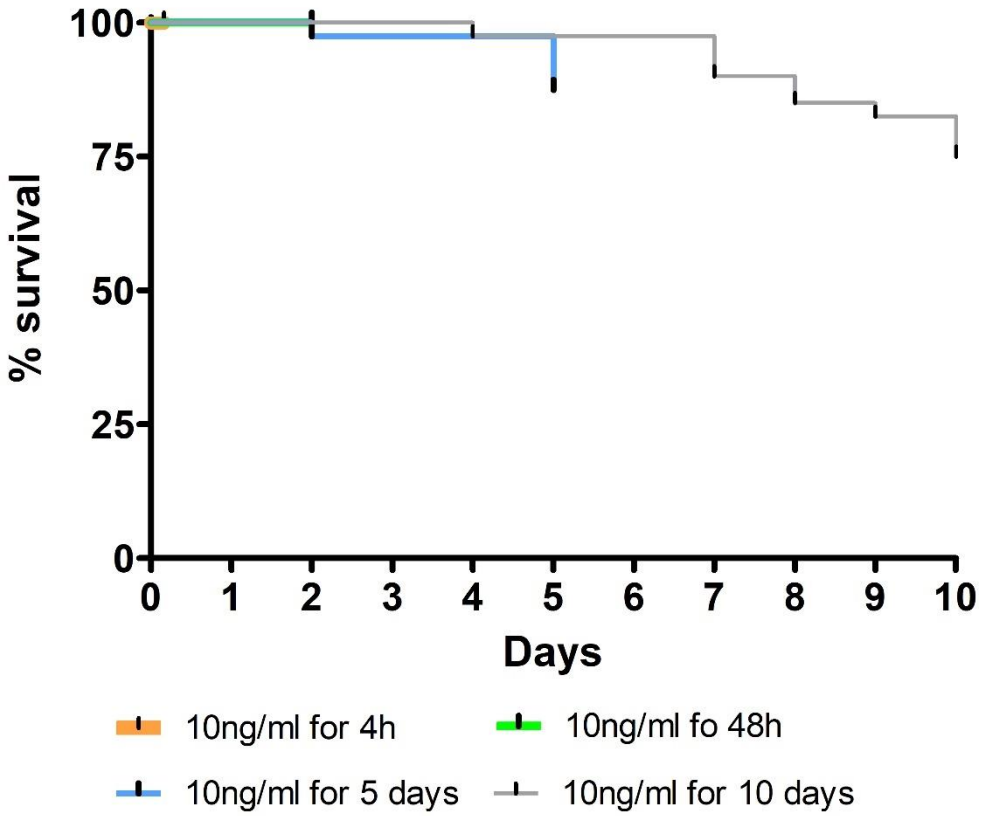


Figure 6.7 Survivability curves based on the number of dead bees found per timepoint and treatment. Mortality started to be detected at around 48h with 1 dead bee found in the group exposed for 10 days but is higher after day 5. In total 10 bees died during the 10 days exposure experiment and 5 bees died during the first 5 days. No mortality was recorded in the groups exposed for 4 and 48 hr.

Due to the lack of standards of metabolites of interest the quantitation analysis of clothianidin metabolites could not be undertaken.

6.5.Discussion

The results of this study demonstrate that the majority of clothianidin in its parental form is possibly excreted or metabolised in bumblebees. However, after continuous exposure, a relatively stable concentration of clothianidin was detected throughout the body parts of the bees that were exposed. The concentration of residues of the neonicotinoid did not increase significantly with time, as it could be expected, considering that consumption did, but also was not reduced.

It is not clear if the detoxification of clothianidin in bumblebees can only be triggered above certain concentrations, as suggested by Wang *et al.*, (2018) in respect to the mode of action of certain antioxidant enzymes, or if the detoxifying process is just insufficient to completely eliminate the insecticide from the organism.

Brunet *et al.,* (2005) described a 33% accumulation in the total ingested dose of acetamiprid in abdomens of honeybees, and a 6.5 and 6% accumulation in the thorax and heads after half an hour of consumption, totalling 45.5% of detection of the ingested dose of acetamiprid in its parental form, after 30 min. Acetamiprid is known to be rapidly metabolised (Iwasa *et al.*, 2004), thus it is possible that if not excreted, the remaining percentage of the dose ingested could be found as a metabolite in every segment of the body. Our results from the 4h timepoint, demonstrate that 24.46% of the ingested clothianidin dose were still detected in its parental form in the bumblebee segments. This possibly suggests a longer half-life of the compound in comparison to acetamiprid but shorter in relation to imidacloprid, that was shown to have a half-life of 4-5h in bees (Suchail *et al.*, 2004; Brunet *et al.*, 2005).

Furthermore, as in previously published acetamiprid and imidacloprid studies (Iwasa *et al.*, 2004; Suchail *et al.*, 2004), the presence of clothianidin in relation to the dose ingested was drastically decreased in all body segments assessed, with the passing time, but never reached zero. Although the presence of clothianidin metabolites and the rates of excretion were not investigated in our experiment, our results demonstrate that the majority of clothianidin is either excreted or metabolised within the first 4 hours post exposure, possibly generating novel compounds that can then negatively impact the bee. It may also be worth investigating if the constant concentrations of clothianidin in our samples can be in any way related to the percentage of the neonicotinoid's molecules bound to their target receptors and if when bound they are inaccessible to detoxifying agents. Clothianidin shows a high affinity and super agonistic characteristics to nAChR (Ihara *et al.*, 2004; Brown *et al.*, 2006) but information regarding its dissociation time is lacking.

With a constant presence of the parental form of clothianidin throughout the body and its potential to generate insecticidal metabolites, with direct and indirect consequences, deleterious effects resulting from chronic long term activation of nAChR are certain to occur, which can lead to sublethal and lethal effects as seen during the 5- and 10-day duration of our experiment, where mortality had reached levels of 12.5 and 25% respectively, by the end of the experiment.

Additionally, it was interesting to observe that although distribution of neonicotinoids in the organism seems to correlate with tissues exhibiting high numbers of nAChR (Brunet *et al.,* 2005), our data demonstrate that such a distribution is also correlated with the proportion of tissue analysed with the majority of parental clothianidin being found in the abdomens and with the accumulation values being statistically significant in relation to their respective controls in all body parts but not when compared among timepoints.

Accuracy and precision of the analytical method was evaluated through recovery calculations, following guidelines available on the Guidance document on analytical control and validation procedures for pesticide residues, developed by the European Commission. Obtained values for all samples ranged from 61 to 124% for clothianidin with averages per group ranging from 88.3 to 114.2 % falling within the satisfactory range of 70-120% as determined by the SANCO/12571/2013 document. We used matrix matched calibration standards to account for any compensating effects arising from matrix interference that could change the ionisation efficiency of the analyte triggering signal suppression or enhancement, which could have a direct effect leading to poor recoveries. Abdominal samples had the lowest reproducibility with highest variability of recoveries, which may suggest matrix interference due to high lipid content of the samples. An increase in replicates and further efforts to remove lipids prior to analysis may therefore increase reproducibility of obtained results.

We recommend that further studies are conducted that investigate the distribution of clothianidin metabolites through the bumblebee body. Once such information is known, the results generated in this study can be better interpreted, and may provide guidance to future toxicological experiments, informing the scientific community of which body components are more likely to feel the force of off target neonicotinoid effects.

Chapter VII

Synthesis

7.1. Synthesis

7.1.1. General Discussion

The role of insecticides, and among them the neonicotinoids, in the decline of pollinators remains a controversial and as yet, unresolved issue. Neonicotinoids, which are generally characterised as agonists of the nAChR in insects (Simon-Delso *et al.*, 2015) are undoubtedly an important tool in the combat of crop pests and sustainable food production. However, their deleterious effects on beneficial pollinating insects, such as bees, has the potential to offset overall agricultural output, through the losses in the production of crops that rely on pollination.

In regards to bees, extensive research has been carried out that demonstrates that neonicotinoids negatively affect colony establishment, survival and ecological performance of honey bees, bumblebees and other native species (Larson *et al.*, 2013; Cutler *et al.*, 2014; Sanchez-Bayo and Goka, 2014; Sánchez-Bayo *et al.*, 2016). Correlations (both negative and positive) have been shown to exist between pesticide use and deficient colony growth (Gill *et al.*, 2012a; Whitehorn *et al.*, 2012), brood production (Laycock and Cresswell, 2013), pollinating performance, homing ability, motor activities (Morandin and Winston, 2003; Gill and Raine, 2014), efficient functioning of the immune system (Christen, Joho, *et al.*, 2019), learning and memory (Gauthier *et al.*, 2002; LaLone *et al.*, 2017), oxidative stress, mitochondrial dysfunction (Moffat *et al.*, 2015b), energy metabolism and many other cellular functions (Wang *et al.*, 2018).

It must be acknowledged, however, that pesticide effects can be dependent on geographical context (Woodcock *et al.*, 2017) and association with a series of other biotic and abiotic factors (Sanchez-Bayo and Goka, 2014), and that in the field a great divergence of effects can occur due to the complexity associated with pesticide exposure in the environment. For example, Osterman *et al.*, (2019) describe the absence of negative effects on robust honeybee colonies, in what the author refers to as "a real-world agricultural landscape". In addition, when some studies were assessed by governmental agencies, concerns were raised regarding the complexity of methods and the justification for the exposure levels, and context chosen for the laboratory and semi field experiments. Most research carried out to date was found to be non-representative of a field relevant exposure scenario, either because studies did not take into consideration realistic exposure levels, time and rate, or the utilised protocols did not produce comparable results to those that were previously published (EFSA, 2012). In this thesis we tried to keep that in mind when designing protocols, so that they represented realistic rates of neonicotinoid exposure that take into consideration, volume, concentration and time of assessment, in

relation to foraging activity patterns of workers. Apart from the research that explores the deleterious effects of neonicotinoids on bees at the colony and individual level, very few molecular studies exist that investigate the molecular basis of the physiological and organismal effects reported in the literature.

To contribute to the debate regarding potential negative effects of neonicotinoids on bumblebees and improve our understanding of the cellular and molecular processes involved, the research outlined in this thesis was conducted. We specifically aimed to investigate neonicotinoid effects on the brain of *B. terrestris* workers and provide new insight into the molecular mechanisms that mediate the interactions between sublethal levels of the insecticide, and the bumblebee neurological system.

To begin, Chapter II involved the comprehensive proteomic characterisation of the bumblebee brain, the most important organ of the central nervous system. The principal aims of this study were to i) characterise the pathways, processes and proteins that typify the bumblebee brain, therefore providing insights into brain morphology, function and physiology at the molecular level, ii) determine how the bumblebee brain proteome changes as the bee ages and iii) use this information to inform the design of future experiments, particularly in the choice of age profile for bees, to be used in quantitative experiments. Our study highlighted the utility of LFQ proteomics as an efficient way to assess molecular changes associated with aging and maturation in bumblebees. We demonstrated that the brain is a highly catalytic organ, represented by processes of energy production and structural reorganisation, all of which are associated with the maintenance of synaptic activity. The brain proteome is abundantly populated by elements of oxidation reduction processes and driven by transport and macromolecule localisation. In regards, to organelles, vesicles and members of the respiratory complex, including the mitochondria, are extremely abundant.

During development, the adult bee brain goes through a restructuration of neurological processes, that form the adult neurological connections and processes, thereby transforming it into a highly active organ, capable of regulating complex behaviours. This transformation probably results from changes that start to occur at the pupal stage and is mostly likely concluded within the first 5 days of life of the worker, as suggested by our data. The fact that the 5 day old brain differs very little from that of the 20 day old bee, as reported in chapter II, provides evidence of the rapid maturing of bumblebee brains, which in relation to honeybees, corroborates the hypothesis that bumblebees undergo varied social specialisation of labour, in accordance to colony necessity, and not age, as is the case with honeybees.

After clarifying aspects of the biochemistry and functioning of the bumblebee brain using proteomics, we then investigated the impact of two neonicotinoid stressors, clothianidin and imidacloprid, on the neurophysiology of this organ. The neonicotinoids are classified as agonists of the cholinergic system, based on studies of a model of the nAChR formed by α4 and β2 subunits (Morales-Perez *et al.*, 2016). Bumblebees possess 11 genes coding for nAChR subunits(Ben M. Sadd *et al.*, 2015), but how these subunits organise to form different receptor subtypes in bees, has still to be fully resolved. This information along with the fact that no functional invertebrate receptor has been successfully expressed in heterologous systems (Thany and Tricoire-Leignel, 2011) led us to question the assumption that all neonicotinoids have the same mode of action. Therefore, Chapter III involved a comparison of responses to acute clothianidin and imidacloprid exposure at the behavioural, cellular and molecular level in bumblebees using assays, confocal microscopy and LC-MS/MS. The acute mode of exposure was based on the guideline for oral exposure to insecticides by the OECD, (1998), but we adapted the volume and delivery method to a less stressful approach, that allowed us to assess complete consumption of the neonicotinoid consumed, and final exposed dose with certainty. We then delineated the molecular effects of neonicotinoid exposure to each insecticide over the course of 24 hours, assessing the development of the response, and the negative effects over time. The results obtained indicate that clothianidin exhibits a higher toxicity to bumblebees than imidacloprid, as evidenced by the survivability assays. In addition, imidacloprid results in an inhibitory effect on behavioural activity, while clothianidin triggers hyper excitability in bumblebee workers. Both neonicotinoids affect neuronal cellular communication, but specificities were observed for each insecticide. Imidacloprid increases the level of the synaptic functioning associated protein synapsin in specific neuropils, and altered energy metabolism, through effects on purine and pyrimidine metabolism, ion binding, cell junction and adherence, and some elements of cytoskeleton organisation. Clothianidin appeared to increase the pressure upon the mitochondria and other members of the energy generation complex, increasing oxidative stress and affecting intracellular expression of several elements associated with transport of vesicles, organelles and cytoskeleton organisation.

One of the most illuminating findings of this research was that the temporal response to neonicotinoid pesticide is a highly complex, dynamic and rarely linear situation, particularly at the molecular level. For instance, different doses and timepoints resulted in different cellular and physiological responses being observed. The responses to neonicotinoid seem to be highly dynamic, and dose dependant, and our proteomic

approach merely allows us to take a temporal and spatial snapshot of that response, which in no way can be assumed to represent an end-point. Capturing a multifactorial response, while it is still changing, that involves different processes and pathways, responding in different ways and levels, makes it extremely difficult to disentangle correlation and causation and highlights the importance of caution, when trying to interpret the results of such exposure-response type experiments. However, what is clear, is that our study provides unquestionable evidence that these two pesticides, at field relevance sub-lethal doses cause molecular reorganisation and responses in the bumblebee brain, a result that would undoubtedly still be challenged by the companies that sell them.

Furthermore, our results indicate that acute exposure results in more detrimental sub lethal effects in comparison to chronic exposure which, led us to hypothesise that acute exposure in the field will result in negative effects to bees while foraging, including memory formation and homing capacity and makes them more susceptible to predators, factors that have been discussed by other ecological studies (Blacquière *et al.*, 2012; Sanchez-Bayo and Goka, 2014; Stanley and Raine, 2016). We are not certain why, but there is the possibility that lower continuous doses in the field relevant level, allow bees to prime and respond better to chronic exposure, resulting in an improved biochemical protection.

Chapter IV involved three experiments that varied the length of exposure to field relevant doses of clothianidin, measuring changes caused on the brain proteome and expression of pre-determined genes of interest. Based on the proteomic data, it seems that the effects of chronic exposure did not worsen in relation to length of exposure. In addition, there was very little correlation between the protein and mRNA (as determined by RT-qPCR) amounts and levels of change in the genes selected for study. The behavioural study reported in chapter IV, also demonstrated that chronic exposure has the potential to reduce bumblebee lifespan and that a threshold potentially exists, that if exceeded leads to substantial mortality. In our experiments, exposure to 10 ng/ml of clothianidin for 5 days did not cause high levels of mortality but 35 ng/ml led to the death of over 50% of bees tested. Through the setting up of the behavioural experiments, we developed a new method for assessing memory and learning that is comprised of presenting bees with a drop of contaminated sugar solution in a neutral environment, monitoring the time taken for the bees to find the drop each day, and ensuring that each bee consumed a controlled dose of pesticide of known amount.

From a molecular and cellular perspective, chronic exposure to clothianidin results in alteration of numerous processes including vacuolar transport, endocytosis, ion binding

and cellular homeostasis, energy metabolism, transport between the endoplasmic reticulum and the golgi complex, and a range of other processes, all of which were discussed in depth in Chapter IV.

The results of Chapter IV presented an interesting question in terms of general responses in bumblebees to insecticide exposure, which would be of general interest to researchers in the field, particularly those attempting to identify symptoms that can diagnose or indicate insecticide exposure. In an attempt to address this further a comparison of the statistically significant differently abundant proteins identified from all experiments presented in chapters III and IV, in an attempt to identify potential biomarker candidates of neonicotinoid pesticide exposure was developed in chapter V. Unfortunately, no individual protein was identified as having a common expression profile, in terms of the response to both pesticides tested. The diversity of exposure scenarios observed in the wild, that are attempted to be replicated in laboratory experiments (oral or contact exposure, acute or chronic and a diversity of assessment temporal endpoints and techniques used), seem to be reflected in the complexity of the molecular results obtained in our experiments. Another issue that seems to inhibit the identification of potential candidates as biomarkers of pesticide exposure, is the limited coverage of identified proteins that can be identified using currently available mass spectrometers. Although individual protein biomarkers could not be identified, a number of protein families were found to respond consistently to neonicotinoid exposure, highlighting that it may be possible to identify biomarker families of general exposure. According to the pfam characterisation, the neonicotinoid insecticides, imidacloprid and clothianidin, both affect catalytic activity in the brains, including a series of hydrolytic and kinase enzymes, mitochondrial carriers, calcium binding proteins, microfilament constituents and motor, signalling and transcription regulators, and lipid selective cell membrane domains.

After finalising the interpretation of molecular non target effects of neonicotinoids in bumblebee brains we decided in Chapter VI, to examine how clothianidin is distributed through different body segments of bumblebee workers. LC-MS was used to quantify clothianidin residues in its parental form, in samples of 4 different timepoints after continuous exposure to 10 ng/ml of clothianidin. We observed that clothianidin seems to be readily metabolised or eliminated from the body, as only about 25 % of the ingested dose was detected in the samples 4 hours after the exposure began. The percentage of detected residue decreased even more in the other timepoints, where the parental form of clothianidin did not account for not even 2 % of the estimated exposed dose. Interestingly,

the levels of clothianidin were consistently found throughout the whole experiment to accumulate at low levels in all body segments. In addition, the distribution of clothianidin in its parental form seem directly proportional to the mass of the body segment analysed. Unfortunately, we were not able to monitor the accumulation of metabolite residues, which would have been very informative to help explain lethal and sublethal effects caused by the insecticide.

Taken together the results presented in this thesis can serve as a reference for future research, particularly in terms of studies that aim to characterise the effects of insecticides on beneficial arthropods such as bees. We propose a visual guide to cellular processes suggested to be affected by our data, which are deserving of further investigation (Figure 7.1).

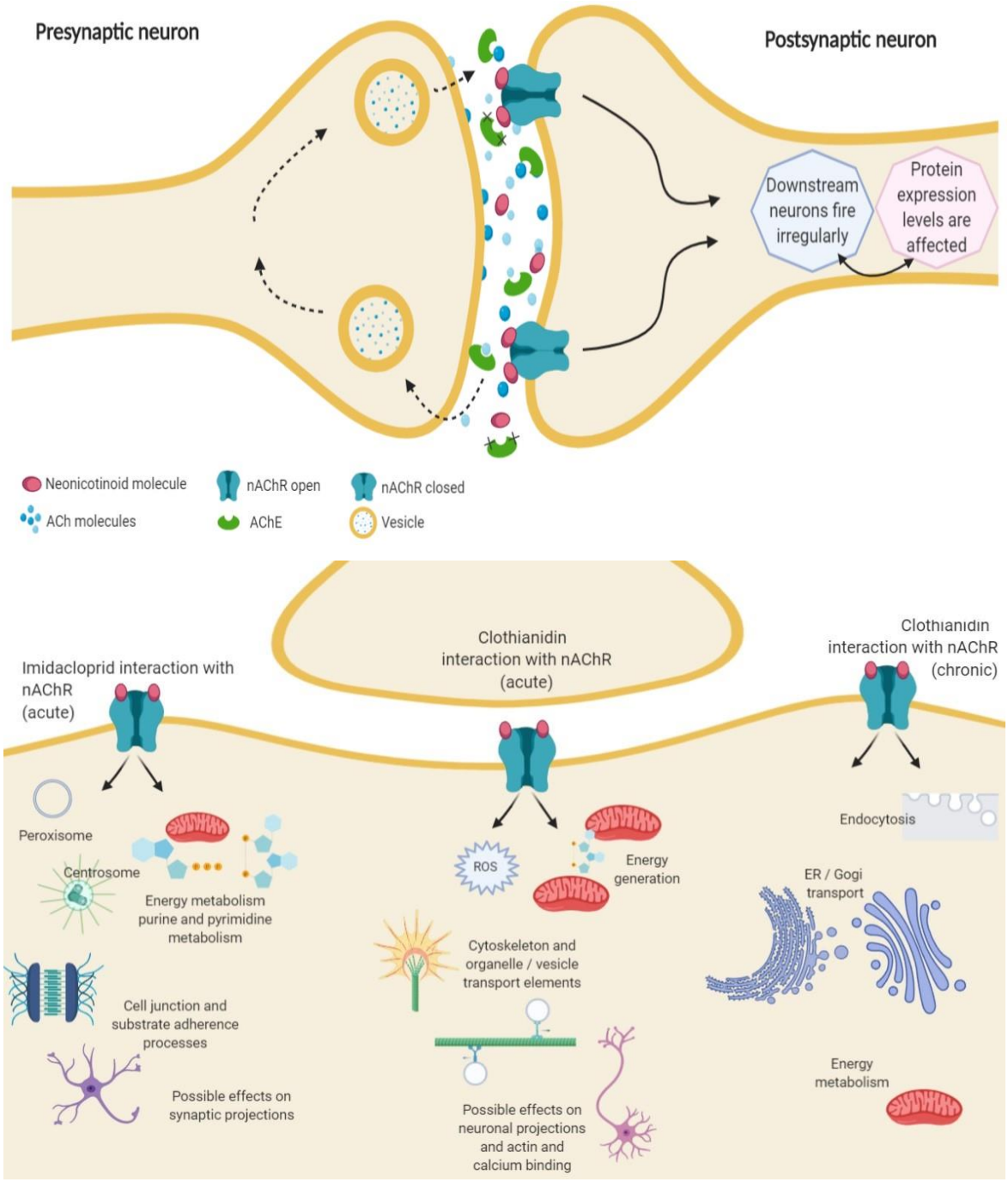


Figure 7.1 Neonicotinoid action and neurotoxic effects on bumblebee brains. Imidacloprid and clothianidin bind irreversibly to nAChRs and are not broken down by AChE, generating either blockage or super activation of receptors, which cause downstream neurons to fire irregularly, influencing gene and protein expression at sublethal levels (Top). In the image, molecules of Ach (blue) compete with those of neonicotinoids (red). Ach is broken down by AChE in the synaptic cleft and recycled in the presynaptic neuron. The same does not occur with neonicotinoid molecules which trigger a range of cellular effects under different exposure scenarios (bottom) due to direct interaction with receptors or non-target systems before being either metabolised intracellularly or eliminated. A detailed description of each exposure scenario and results are available in chapter III and IV.

It is clear from our results that is essential that the mode of action of insecticides is carefully considered, and that assumptions on the proposed interaction between pesticides and target receptors are not taken for granted, as different neonicotinoids demonstrate specific effects at the molecular level and the literature regarding their mode of action may be misleading.

We would also like to express the warning that proteomic analysis of bumblebee brains constitutes a highly complex endeavour, underpinned by the high degree of variability and individuality in the brains of different bees. Environmental factors that include interactions with other bees in a microcolony, can have a huge influence on the expression of proteins in the highly active and responsive brain, which inhibits the clear resolution of the differences in responses that are usually discernible in the remainder of the proteome. Furthermore, the use of Omics-based techniques on non-model organisms also represents a challenge, as biological information about identified proteins is often assumed and gene ontology annotations may be still incomplete. Many of the conclusions about potential function of identified proteins remains for the most part based on similarities to proteins in well characterised model organisms, which does mean that certain conclusions must be regarded with caution, until functional studies can be conducted.

In this thesis we used STRING Gene ontology analysis to obtain functional information of the sequence similarity comparison of identified bumblebee proteins with the *Drosophila melanogaster* database. The resulting information obtained for enriched ontology terms was used to rank proteins based on the coverage of identified terms, and total number of terms, as a way of obtaining clear and non-biased information about protein biological function, and the overall biochemical pathways and processes affected by neonicotinoids. This methodology was chosen after issues with the enrichment processes and treatment of redundancy with the softwares cytoscape, WeGo and Gorilla. These platforms provide enrichments generally keeping parental GO terms that in some cases eliminate objective information, by keeping general or non-informative information (lacks specificity).

Although we are cautious about the interpretation of the proteomics results presented in this thesis, the advances in high-throughput proteomics and associated computational tools, have made our attempt to use LFQ proteomics to study the brains of this pollinating insect a highly successful endeavour, despite some limitations. We have tried to adopt an experimental design that minimised stress to bees, other than the stress we wished to assess and considered field relevant exposure in all our experiments. The

protocols developed and adapted for the continuity of this thesis will highly benefit future PhD students in my lab, who will be able to build upon what I have developed increasing our capacity to expose and analyse effects of agricultural contaminants in beneficial insects.

In essence, we have for the first time provided an in-depth proteomic analysis of the bumblebee brain in response to neonicotinoid exposure and with the experience obtained throughout, we invite those involved in similar studies to consider the following:

1. When considering molecular endpoints of analysis in toxicological studies, a complexity of results is to be expected, as responses to xenobiotics at the molecular level may not be linear, as may be the case with behavioural and mortality studies.
2. When investigating toxicological effects of pesticides, obtaining a molecular perspective may represent the best way to completely understand the dynamic nature of the effects, and to uncover a way to mitigate potential deleterious effects.
3. Major knowledge gaps regarding the interaction of chemical components with biological systems still remain, (chemicals interaction with the target receptor and non target receptors).Thus, more research is necessary to really elucidate the mechanisms involved in responding to specific insecticides, as diverging effects are frequently reported, and this complicates the identification of effects, especially when new pesticide formulations are so often regulated, even before these interactions have been fully elucidated.
4. Follow on studies that monitored calcium levels within different brain neuropils, offer the possibility to assess neuronal activity under neonicotinoid exposure. Such studies, associated with measurements of oxidative phosphorylation and monitoring of neuronal dendritic and axonal projections in cell culture, could produce critical data that associated with more targeted proteomics studies and perhaps metabolomics could corroborate with the hypothesis of effects raised within this work. We suggest that timepoints and exposure concentrations are chosen accordingly to what was demonstrated in Chapter III and IV due to the dynamic nature found within the bee`s neurological processes, which could generate variability of results.

7.1.2. Concluding remarks

Future work surrounding the identification of the insecticidal effects on beneficial arthropods should involve further structural analysis of the targeted receptors, type of stimulation, and characteristics of the target system found *in vivo*. Differences between species are to be expected as little is known about organisation and composition of receptor subtypes found in each species. Toxicological studies should consider foraging activity of bees to calculate the risk and degree of exposure to contaminants in the environment, and as such, use this information to design the best exposure methodology. Standardisation of exposure methods is still necessary especially in relation to nutritional status of bees, as unhealthy bees respond differently to stressors. Specialists should make use of biological knowledge of their tested organism, to guide their experiments, considering age and behaviour characteristics, to choose the experimental approach that best suits the biological history and exposure reality to that species.

I also believe that exposure levels should be based on studies that calculate the persistence of residues of respective chemical *in situ*, eliminating studies that used high concentrations, which are not field relevant when attempting to determine pesticide effects. In addition, I believe it to be necessary to estimate the final exposure/dose and to design experiments that allow for such estimative, as it favours better data comparison and interpretation between different studies.

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