

Systemic impacts of low dose insecticide exposures in *Drosophila*:
a mechanism centred on oxidative stress

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*“The plain fact is that the planet does not need more successful people.
But it does desperately need more peacemakers,
healers, restorers, storytellers, and lovers of every kind.
It needs people who live well in their places.
It needs people of moral courage willing to join the fight
to make the world habitable and humane.
And these qualities have little to do with success as we have defined it.”*

David W. Orr

Abstract

The plight of insect populations around the world has gained increasing attention. A recent meta-analysis published in *Science* reported an average decline of terrestrial insect abundance by ~9% per decade since 1925 (van Klink et al. 2020). While this is a lower rate of decline than reported in earlier meta-analyses (Sánchez-Bayo and Wyckhuys 2019) it still suggests that many terrestrial insect species are under threat. The extinction of terrestrial insect species would severely affect agriculture and ecosystems due to the vital role that many species play in pollination, the recycling of organic matter, pest control and other ecosystem services. Insecticide exposure has been proposed to be one of the significant contributing factors for population declines of non-pest species. Insecticide contamination in biomes, resulting from intensive usage on agricultural crops, is likely to lead to exposures for many non-pest insect species. Low doses of insecticides are known to impact the fitness and behaviour of various insect species, but the underlying molecular, cellular, and physiological impacts of such doses in insects are not well defined. The absence of a mechanism that explains how low doses affect insects is an obstacle to ascertaining the extent to which insecticides may contribute to the demise of populations. The aim of this study was to scrutinize the impacts of low insecticide doses on the metabolism and physiology of the model organism *Drosophila melanogaster* in order to propose a mechanism to explain the impact of such doses on insect biology. Two insecticides were investigated in detail. The first of these is the synthetic neonicotinoid imidacloprid. Having been banned in the EU due to some evidence of a role in collapse in honeybee colonies, imidacloprid remains one of the most widely used insecticides in the world. The second insecticide is spinosad. Composed of two structurally similar natural fermentation products from the soil bacterium *Saccharopolyspora spinosa*, this insecticide is classified as organic and considered to be less harmful to beneficial insects. Both insecticides target evolutionarily conserved nicotinic acetylcholine receptors (nAChRs) in the Central Nervous System (CNS) of insects. nAChRs are pentameric ligand gated ion channels. Activation by the natural ligand, acetylcholine, leads to a flux of Ca^{2+} , Na^{+} or K^{+} ions into neurons, regulating a myriad of responses in the insect brain. The *Drosophila* genome encodes 10 nAChRs subunits ($\text{D}\alpha 1$ to $\text{D}\alpha 7$ and $\text{D}\beta 1$ to $\text{D}\beta 3$), meaning that there is a vast number of subunit combinations that could assemble to form functionally distinct receptor subtypes. Imidacloprid targets the $\text{D}\alpha 1$, $\text{D}\alpha 2$, $\text{D}\beta 1$ and $\text{D}\beta 2$, subunits, whilst spinosad targets the $\text{D}\alpha 6$ subunit. Acute exposure to imidacloprid, at

doses that do not kill *Drosophila* larvae, rapidly increased in the levels of reactive oxygen species (ROS) in the brain, most likely due to the sustained Ca^{2+} flux into neurons caused by the interaction between the insecticide and its nAChR targets. This led to oxidative stress marked by mitochondrial dysfunction that in turn led to a significant decrease in energy (ATP) levels. While this process was initiated in the brain, lipid storage in the metabolic tissues (fat body, Malpighian tubules, and midgut) was affected. Transcriptomic analysis of the larval brain and fat body revealed a significant perturbation in the expression of genes involved in metabolism, oxidative stress, and immune response. Using genetic manipulations to elevate ROS levels exclusively in the brain, lipid storage was shown to be perturbed in the metabolic tissues, indicating that a ROS signal initiated in the brain radiates to other tissues. Severe damage to glial cells and neurons (i.e. neurodegeneration) was observed in the visual system of adults subjected to chronic low-dose exposure to imidacloprid. This precipitated a progressive loss of vision. Spinosad showed a different mode of action, blocking nAChRs and preventing Ca^{2+} influx. The blocked receptors were shown to be recycled from the neuronal membranes through endocytosis. This mechanism led to an increase in the number and size of lysosomes in the CNS, characteristic of lysosomal storage diseases, which precipitates elevated generation of ROS by impairing mitochondrial activity and neurodegeneration. The high levels of ROS measured in the CNS after spinosad exposure, were associated with a cascade of phenotypes in metabolic tissues similar to the ones observed after imidacloprid exposure. Experiments examining the lipid environment in *Dα6* knockout mutants (resistant to spinosad) indicated that impacts observed in the metabolic tissues of spinosad-exposed larvae are due to the interaction between *Dα6* and spinosad. These data corroborate the hypothesis that impairments observed in metabolic tissues are triggered by a chemical signal from the brain, suggested to be a peroxidized lipid. Although there were some differences in the responses observed for the two insecticides (e.g. in transcriptomes and lipidomes), a similar cascade of processes was observed to be initiated following the elevation of ROS levels in the brain. A potent antioxidant, N-Acetylcysteine amide, strongly suppressed a range of phenotypes observed in both larvae and adults, indicating a causal role for ROS and oxidative stress. As the nAChR targets of these insecticides are conserved among insects, it is likely that similar impacts would be precipitated by exposures in other non-pest species, albeit at different doses. As insecticides from a wide range of chemical classes create markers of oxidative damage, the low dose mechanism of action observed for imidacloprid and spinosad may apply

more broadly. This requires investigation. Considered together, the low dose impacts of imidacloprid and spinosad severely impair insect biology, without necessarily killing. These impairments could render insect species more vulnerable to the other major threats proposed to contribute to the decline of populations: climate change, habitat loss, pathogens, and parasites.

Declaration

This is to certify that:

- i. The thesis comprises only my original work towards this Ph.D. except where indicated in the Preface;
- ii. Due acknowledgement has been made in the text to all other material used;
- iii. The thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies, and appendices.

Felipe Martelli Soares da Silva

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Preface

I acknowledge the valuable contributions of the following people and companies:

Chapter Two

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Table of Contents

Chapter One (Introduction)	1
1.1 Why insects?	2
1.2 A worldwide decrease of entomofauna and its drivers	3
1.3 Drosophila – the experimental organism for insecticide research	5
1.4 A brief overview on the mode of action of neurotoxic insecticides	6
1.5 Insecticides impact on metabolism	11
1.6 Lipid metabolism in insects	14
1.6.1 Lipids and lipid metabolism	14
1.6.2 Regulation of lipid metabolism in insects	18
1.6.3 Fat body – the central metabolic organ	20
1.6.4 Lipid droplets – the lipid reservoir of cells	22
1.6.5 Lipid metabolism genes in Drosophila	23
1.7 Different insecticide classes produce oxidative stress	25
1.8 Oxidative stress, lipids, and neurodegeneration	27
1.9 Study aims and hypothesis	28
Chapter Two (Imidacloprid)	30
2.1 Introduction	31
2.2 Material & Methods	33
2.2.1 Fly strains and rearing	33
2.2.2 Collecting third instar larvae	34
2.2.3 Food media recipes	34
2.2.4 Insecticide and hydrogen peroxide dilution and exposure	34
2.2.5 Larvae movement assay	35
2.2.6 Larvae viability and adult survival tests	35
2.2.7 Tissue dissection	36
2.2.8 GCaMP assay	36
2.2.9 Expression of $\alpha 6$ nAChRs in larval brains	37
2.2.10 Evaluation of mitochondrial turnover	37
2.2.11 Systemic mitochondrial aconitase activity	38
2.2.12 Systemic ATP levels	38
2.2.13 Measurement of superoxide (O_2^-) levels	38
2.2.14 Generating oxidative stress in brains	39
2.2.15 Nile red staining of adult retinas	39
2.2.16 Evaluation of lipid environment of metabolic tissues in larvae	40

2.2.17 Lipid quantification in larvae hemolymph	40
2.2.18 Antioxidant treatment	41
2.2.19 Evaluation of JNK and GstD1 activity	41
2.2.20 Lipid extraction for LC-MS.....	42
2.2.21 Lipid analysis using LC-MS.....	43
2.2.22 RNA isolation and transcriptomics analysis	44
2.2.23 Gene Ontology and KEGG pathway analysis	45
2.2.24 Electrophysiology of the retina	45
2.2.25 Adult brain histology (Hematoxylin & Eosin staining)	45
2.2.26 Transmission Electron Microscopy (TEM).....	45
2.2.27 Bang Sensitivity	46
2.2.28 Climbing assay	46
2.2.29 Graphs and Statistical analysis	46
2.3 Results & Discussion	47
2.3.1 Low imidacloprid doses reduce larvae motility without affecting survival	47
2.3.2 Low dose of imidacloprid elicits a low but enduring Ca^{2+} flux into nAChR neurons ..	48
2.3.3 Low exposures impact mitochondria and give rise to oxidative stress	50
2.3.4 Oxidative stress generated in the brain precipitates changes in the lipid environment of metabolic tissues	53
2.3.5 Oxidative stress generate by imidacloprid impacts the metabolic tissues and larvae motility	55
2.3.6 Hydrogen peroxide exposure induces similar disturbances on the lipid environment	61
2.3.7 Knockout of GstD1 increases imidacloprid susceptibility	62
2.3.8 The existence of multiple nAChR targets to imidacloprid hinders the rescue of the LD exposure phenotype	64
2.3.9 Low dose exposure to imidacloprid induces the activation of major stress response pathways	65
2.3.10 Imidacloprid triggers major alterations in the lipidome.....	67
2.3.11 Low acute exposures to imidacloprid perturbs gene expression in the brain and fat body.....	69
2.3.12 Adult flies chronically exposed to imidacloprid show signs of neurodegeneration.	79
2.3.13 The visual system shows severe lesions and neurodegeneration in response to chronic exposures	82
2.3.14 Impacts of low chronic exposures to imidacloprid may not be noticed if not investigated in depth.....	89
2.3.15 A model for imidacloprid low dose exposure impacts on insects	90
Chapter Three (Spinosad).....	92

3.1 Introduction.....	93
3.2 Material & Methods	95
3.2.1 Fly strains and rearing.....	95
3.2.2 Collecting third instar larvae	95
3.2.3 Insecticide dilution and exposure.....	95
3.2.4 Larvae movement assay.....	96
3.2.5 Larvae viability and adult survival tests	96
3.2.6 Tissue dissection.....	96
3.2.7 Measurement of superoxide (O_2^-) levels	97
3.2.8 Evaluation of mitochondrial turnover	97
3.2.9 Systemic mitochondrial aconitase activity.....	97
3.2.10 Systemic ATP levels.....	97
3.2.11 Evaluation of lipid environment of metabolic tissues in larvae	97
3.2.12 Lipid quantification in larvae hemolymph	97
3.2.13 Antioxidant treatment	98
3.2.14 Evaluation of JNK and GstD1 activity	98
3.2.15 Lipid extraction and analysis for LC-MS	98
3.2.16 RNA isolation, transcriptomics analysis, GO and KEGG pathway analysis.....	98
3.2.17 GCaMP assay	98
3.2.18 Investigating impacts on lysosomes	98
3.2.19 Nile red staining of adult retinas	99
3.2.20 Electrophysiology of the retina	99
3.2.21 Spinosad crystals stain with Nile red	99
3.2.22 Expression of $\alpha 6$ nAChRs in adult brains	100
3.2.23 Transmission Electron Microscopy (TEM).....	100
3.2.24 Bang Sensitivity	100
3.2.25 Climbing assay	100
3.2.26 Adult brain histology (Hematoxylin & Eosin staining)	100
3.2.27 Graphs and Statistical analysis	101
3.3 Results & Discussion	102
3.3.1 Low doses of spinosad affect larvae motility and survival.....	102
3.3.2 Spinosad creates oxidative stress and impairs mitochondria	104
3.3.3 Knockout of GstD1 increases spinosad susceptibility.....	106
3.3.4 Oxidative stress created by spinosad affects the lipid environment, motility, and survival.....	107
3.3.5 Spinosad doses that do not affect survival also impair the lipid environment.....	110

3.3.6 A brain signal triggers the impacts of spinosad on metabolic tissues.....	113
3.3.7 Spinosad induces the activation of major stress response pathways	116
3.3.8 Spinosad triggers major alterations in the lipidome	116
3.3.9 Acute exposures to spinosad cause major perturbations in gene expression.....	119
3.3.10 Spinosad is not a nAChR agonist	129
3.3.11 Determining a spinosad dose for chronic exposure in adults	135
3.3.12 Chronic exposures to spinosad cause a diffuse lipid accumulation in fly retinas, progressive loss of vision, and neurodegeneration	136
3.3.13 nAChR Dα6 knockout mutant flies have defective ERGs and photoreceptors	143
3.3.14 Flies chronically exposed also show behavioural and histological signs of neurodegeneration	146
3.3.15 A model for spinosad low dose exposure impacts on flies	149
Chapter Four (Discussion).....	151
4.1 The insecticides	152
4.2 The agonistic action of imidacloprid on nAChRs.....	153
4.3 The antagonistic action of spinosad on nAChRs	154
4.4 Both insecticides trigger a cascade of damage by generating ROS in the brain.....	157
4.5 The effects of acute insecticide exposures on metabolic tissues	159
4.6 The effects of acute insecticide exposures on lipidomics profiles.....	161
4.7 The effects of acute insecticide exposures on transcriptomics profiles.....	162
4.8 The impacts of chronic insecticide exposures on insect visual system	165
4.9 The onset of neurodegeneration	167
4.10 Insecticide exposures and the decline of insect populations	168
4.11 Conclusions	171
References	175

List of Figures

Chapter One

Figure 1.1 Schematic representation of insect nicotinic acetylcholine receptors.....	9
Figure 1.2. Distribution of nAChR subunits in the insect phylogenetic tree.	10
Figure 1.3. Overview of lipid metabolism and transport in insects	18
Figure 1.4. Model for the mechanism of lactate and lipid transfer and accumulation in glia and neurons	28

Chapter Two

Figure 2.1. Imidacloprid chemical structure.....	32
Figure 2.2. Toxicology and viability assays with larvae exposed to different doses of imidacloprid for 2 hr.....	488
Figure 2.3. nAChR subunit Dα6 expression in <i>Drosophila</i> larval brain.....	49
Figure 2.4. Imidacloprid elicits Ca ²⁺ flux into neurons and impairs cholinergic response.....	50
Figure 2.5. Imidacloprid induces the accumulation of ROS in brains and midguts	51
Figure 2.6. Mitochondrial turnover is increased in larvae exposed to 2.5 ppm imidacloprid for 2 hr.....	52
Figure 2.7. Imidacloprid reduces systemic levels of mitochondrial aconitase activity and ATP ..	53
Figure 2.8. Inducing ROS in the brain impacts the lipid environment of metabolic tissues.....	55
Figure 2.9. Imidacloprid increases lipid storages in fat body and antioxidant treatment reduces this accumulation.....	56
Figure 2.10. Antioxidant treatment improves the movement of imidacloprid exposed larvae..	57
Figure 2.11. Impacts on adult emergence after larval exposure to imidacloprid	58
Figure 2.12. Higher doses of imidacloprid cause greater accumulation of lipid storage in fat body	59
Figure 2.13. Imidacloprid reduces lipid stocks in Malpighian tubules and midguts.....	60
Figure 2.14. Imidacloprid reduces lipid levels in hemolymph	61
Figure 2.15. Hydrogen peroxide (H ₂ O ₂) induces lipid accumulation in larval fat body and in hemolymph after 2 hr exposure	62
Figure 2.16. Knockout of an antioxidant enzyme increases sensitivity to imidacloprid	63
Figure 2.17 Larvae resistant to imidacloprid (Dα1 knockout) show lipid accumulation in fat body when exposed to this insecticide.....	65

Figure 2.18. Imidacloprid increases activation of GstD1 and JNK in anterior midgut	66
Figure 2.19. Imidacloprid disturbs lipid profiles of exposed larvae	69
Figure 2.20. Transcriptome analysis of brains and fat bodies of larvae acutely exposed to imidacloprid.....	70
Figure 2.21. Adult chronic exposures to imidacloprid	80
Figure 2.22. Chronic exposure to imidacloprid increases bang sensitivity	80
Figure 2.23. Chronic exposure to imidacloprid reduces climbing and antioxidant treatment rescues the phenotype.....	81
Figure 2.24. Antioxidant treatment rescues survival.....	82
Figure 2.25. Representation of a transverse section of the adult fly ommatidium.....	83
Figure 2.26. Representation of a longitudinal section through the Drosophila adult visual system	84
Figure 2.27. Chronic exposure to 4 ppm of imidacloprid causes accumulation of lipids in retinas of flies.....	85
Figure 2.28. Chronic exposure to imidacloprid creates defects in retina	86
Figure 2.29. Representation of a transverse section of the adult fly laminar cartridge.....	87
Figure 2.30. Chronic exposure to imidacloprid creates defects in the lamina.....	88
Figure 2.31. Chronic exposure to imidacloprid impairs vision	89
Figure 2.32. Chronic exposure to imidacloprid does not cause obvious impacts in brain anatomy	90
Figure 2.33. Proposed mechanism of action for low dose exposure to imidacloprid	91

Chapter Three

Figure 3.1. Spinosyn A and D chemical structures.....	94
Figure 3.2. Toxicology and viability assays with larvae exposed to different doses of spinosad for 2 hr	103
Figure 3.3. One day old pupae appearance of larvae exposed to spinosad.....	103
Figure 3.4. Spinosad induces the accumulation of ROS in the brain and midgut.....	104
Figure 3.5. Mitochondrial turnover is increased in larvae exposed to 2.5 ppm of spinosad for 2 hr	105
Figure 3.6. Spinosad impairs ATP levels and reduces mitochondrial aconitase activity.....	106
Figure 3.7. Knockout of the antioxidant enzyme GSTD1 increases sensitivity to spinosad..	107
Figure 3.8. Spinosad increases lipid storage in fat body and antioxidant pre-treatment reduces this accumulation.....	108

Figure 3.9. Antioxidant pre-treatment improves the movement of spinosad exposed larvae.	109
Figure 3.10. Antioxidant pre-treatment improves survival of larvae exposed to spinosad.....	109
Figure 3.11. Impacts on adult emergence after larval exposure to different spinosad doses..	110
Figure 3.12. Higher doses of spinosad cause greater accumulation of lipid storage in fat body	111
Figure 3.13. Spinosad reduces lipid levels in the Malpighian tubules and midgut.....	112
Figure 3.14. Spinosad increases lipid levels in the hemolymph.	113
Figure 3.15. Dα6 knockout (KO) resistant larvae show a decreased impact on lipid environment in response to spinosad exposures.....	115
Figure 3.16. Spinosad increases the expression of GstD1 and the JNK pathway in the anterior midgut.....	116
Figure 3.17. Spinosad disturbs the lipid profile of exposed larvae.....	118
Figure 3.18. Transcriptomic analysis of the brain and fat body of larvae acutely exposed to spinosad	120
Figure 3.19. Spinosad blocks Ca ²⁺ flux into neurons and impairs the cholinergic response....	129
Figure 3.20. Proposed mechanism for internalization of spinosad after binding to the D α 6 nAChR target.....	131
Figure 3.21. Spinosad leads to the accumulation of lysosomes in brain	132
Figure 3.22. The accumulation of lysosomes in brain is time dependent.....	132
Figure 3.23. nAChRs are removed from cell membrane and transferred to lysosomes in response to spinosad exposure	133
Figure 3.24. KEGG pathway analysis reveals perturbation of lysosome dynamics in brains	134
Figure 3.25. KEGG pathway analysis reveals perturbation of sphingolipids metabolism in brains	135
Figure 3.26. Adult chronic exposures to spinosad.....	136
Figure 3.27. Chronic exposure to 0.2 ppm spinosad causes the accumulation of lipids in the retina	137
Figure 3.28. Spinosad crystals are Nile red positive	138
Figure 3.29 nAChR subunit Dα6 expression in Drosophila adult brain	138
Figure 3.30. Chronic exposure to spinosad increases the number of mitochondria in retina..	140
Figure 3.31. Chronic exposure to spinosad creates defects in the lamina.....	141
Figure 3.32. Chronic exposure to spinosad impairs vision	142
Figure 3.33. nAChR Dα6 knockout mutants show impaired vision	144
Figure 3.34. nAChR Dα6 knockout mutants show defective photoreceptors and lipid accumulation in retinas.....	145

Figure 3.35. CantonS D α 6 KO mutants show no impacts in the lamina	146
Figure 3.36. Chronic exposure to spinosad increases bang sensitivity and reduces climbing ability	147
Figure 3.37. Chronic exposure to spinosad causes vacuolation of the adult brain	148
Figure 3.38. Adult Drosophila brain morpho-functional areas.....	148
Figure 3.39. Proposed mechanism of action for low doses of spinosad.....	150

Chapter Four

Figure 4.1. Spinosad leads to accumulation of larger lysosomes in the fat body	156
Figure 4.2. Comparison of lipidomic profiles of larvae exposed to imidacloprid or spinosad ..	162
Figure 4.3. Comparison of transcriptomic profiles of larvae exposed to imidacloprid or spinosad	164
Figure 4.4. KEGG pathway analysis reveals perturbation of proteasome pathway in brains of larvae exposed to spinosad	165

List of Tables

Chapter One

Table 1.1. Most relevant genes involved on lipid metabolism in <i>Drosophila</i>	23
--	----

Chapter Two

Table 2.1. Imidacloprid perturbs the expression of P450s and GSTs.....	73
Table 2.2. Imidacloprid affects the expression of genes involved in lipid metabolism, neurodegeneration, and oxidative stress	76
Table 2.3. Imidacloprid affects the expression of genes involved in immune response	78

Chapter Three

Table 3.1. Spinosad perturbs the expression of P450s and GTs	123
Table 3.2. Spinosad affects the expression of genes involved in lipid metabolism, neurodegeneration, and oxidative stress	126
Table 3.3. Spinosad affects the expression of genes involved in immune response.....	128

Chapter Four

Table 4.1. The cascade of impacts precipitated by small doses of imidacloprid and spinosad on <i>Drosophila melanogaster</i>	172
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Chapter One

Introduction

1.1 Why insects?

“Hence I have very little doubt, that if the whole genus of humble-bees became extinct or very rare in England, the heartsease and red clover would become very rare, or wholly disappear” (Darwin 1859). This excerpt from *On the Origin of Species by means of Natural Selection* written by Charles Darwin reveals the significance that the great naturalist accredited to bees for guaranteeing the delicate maintenance of ecosystems. But the father of the theory of evolution was neither the first nor the last one to emphasize the importance of bees for pollination. Bees are indeed truly fascinating creatures; there are over 20,000 species (Bystriakova et al. 2018), a number five times greater than the total number of mammal species. However, bees themselves represent a modest fraction of the Class *Insecta*. There are currently over 1 million insect species described. Recent estimates suggest the total number of insect species alive on the planet right now could be around 5.5 million (Stork 2018). This means that 80% or more of insect species are unknown to humans, so their biology and ecological roles are not understood (Stork 2018).

Insects are thus the most diverse and, arguably, the most evolutionarily successful group of animals on the planet. Considering only the 1 million of insect species described, they represent over 50% of all known eukaryote species. Insects are champions not only in number, but also in weight. It is estimated that the total biomass of insects is 3-fold greater than that of humans and that they may represent 10% of total animal biomass of the planet (Bar-On et al. 2018). Most of the insect megadiversity is found in hot spots such as the tropical rainforests, but they occupy niches in virtually every region of the planet, from deserts to thermal springs, from sea level to mountain tops (Stork 2018).

Insects are of paramount significance for the structure and maintenance of life on the planet, forming complex networks that exert a pivotal driving force of species coevolution, ecological interactions, and community stability (Cardoso et al. 2020). A small number of species are parasites of plants or animals, leading to substantial losses for agriculture and livestock, and some even parasitoids of other insect species, representing an effective pest control tool used in plant crops (Zhi-zhi et al. 2019). A fraction of insect species is also vector of human diseases, but most species are important nutritional sources in food webs for birds, mammals, and reptiles. The consumption of

decaying matter, mostly by cockroaches and beetles, is fundamental for nutrient recycling and the droppings, full of nitrogen, fertilize soil favouring plant growth. Different insect species also promote seed dispersion, soil aeration, and eliminate pests, boosting floral diversity. Some species also provide pharmaceutical and industrial products and around the world over 2000 insect species are used as food for human consumption (Cardoso et al. 2020).

Returning to bees, they fulfill what probably is the most well-known role attributed to insects: pollination. It is estimated that 87.5% of flowering plants rely on animal pollination, most of which is performed by bees (Ollerton et al. 2011). It is estimated that worldwide, 75% of the crops depend to some extent on insect pollination (Klein et al. 2007). Animal pollination, mostly performed by bees, qualitatively and quantitatively improve fruit and seed production by 70 % for 1330 tropical crops and 85 % for 264 European crops (Aizen et al. 2009). The yield and diversity of agriculture rely immensely on biotic pollination, particularly on the service provided by honeybees (*Apis mellifera*), the single most important pollinator species (Aizen et al. 2009).

1.2 A worldwide decrease of entomofauna and its drivers

The biological roles performed by insects is of such value that, in their absence, the flora and the fauna would collapse; life, as we know it, would drastically change. While the extinction of all insect species is unlikely, recent scientific findings have been pointing to a rapid decline in size for many insect populations. Hallmann et al. (2017) investigating insect populations in German showed that in the last 27 years the total biomass of flying insects declined by over 75%. Lister and Garcia (2018), studying the rainforests of Puerto Rico reported an annual average loss of ground-foraging and canopy-dwelling arthropod biomass of 2.5% over the last 36 years, representing a shocking 78% to 98% loss of biomass over the entire period. In a review, Sánchez-Bayo and Wyckhuys (2019) identified extremely large declines in the numbers for many species. In terrestrial ecosystems butterflies, bees and dung beetles are among the most affected groups, whilst in aquatic environments dragonflies, mayflies, stoneflies, and caddisflies are suffering extensive losses. The authors suggest that if these trends continue around 40% of insect species could face extinction in the next few decades (Sánchez-Bayo and Wyckhuys 2019). However van Klink et al. (2020) in a large metanalysis study

involving 166 long-term surveys of insect assemblages across 1676 locations spanning from 1925 to 2018 found an average decline of terrestrial insect abundance by ~9% per decade and an ~11% increase in freshwater insect abundance. While authors disagree in the exact numbers, there is an unequivocal consensus that terrestrial insect populations are in decline and that the reduction of pollination services in agriculture and ecosystems must be addressed urgently (Montgomery et al. 2020).

The discussion surrounding the decline of pollinators has also gained visibility in the non-scientific media; the New York Times dubbed the problem “Insect Apocalypse” (Jarvis 2018). For over a decade scientists have been alerting the world to the alarming, rapid decline of honeybee colonies, a phenomenon referred to as Colony Collapse Disorder (Aizen et al. 2009; Steinhauer et al. 2018). The losses in honeybee colonies, which many countries are experiencing, raise great concern that the so-called pollination crisis might threaten agricultural production (Aizen et al. 2009; Steinhauer et al. 2018). The conservation of insect diversity is vital for future generations, given ecosystem and human dependency on insects (Cardoso et al. 2020). The decline in population numbers, observed by many different authors (Montgomery et al. 2020; Sánchez-Bayo and Wyckhuys 2019) demand immediate action to prevent extinctions (Cardoso et al. 2020; Montgomery et al. 2020).

It is becoming very clear that the demise of insect populations is a multifactorial phenomenon. Recent publications have extensively reviewed the drivers of the “Insect Apocalypse” (Cardoso et al. 2020; Sánchez-Bayo and Wyckhuys 2019). Among these drivers the most critical contributors seem to be: (1) habitat loss caused by large-scale urbanization and agriculture; (2) climate change affecting insect resilience and fitness; (3) new pathogens and parasites that seem to be an important factor in the colony collapse disorder; and (4) pollution, where collateral damage caused by insecticides used for pest control is under scrutiny (Cardoso et al. 2020; Sánchez-Bayo and Wyckhuys 2019). These drivers may act in combination but, in the first instance, each of them requires focused research.

1.3 *Drosophila* – the experimental organism for insecticide research

To investigate the impact of insecticides on insect population, the vinegar fly, *Drosophila melanogaster* was used as an experimental organism. *Drosophila* is non-pest insect that is a widely used model in insecticide research and it is exposed to insecticides in the field (Perry and Batterham 2018). *Drosophila* is a well-studied organism with detailed information available on its ontogeny and physiology. It is a species of small fly (Order Diptera, Family Drosophilidae) that has been used since the early 1900s as a scientific model organism to study numerous biological processes such as genetics, development, aging and behavior (Jennings 2011). A short generation time (10 to 12 days at room temperature), the ease of maintenance and genetic manipulation and the ready access to a vast array of genetic resources make *Drosophila* the organism of choice (Balakrishnan et al. 2015; Liu et al. 2017).

The *Drosophila* genome was one of the first to be fully sequenced (Adams et al. 2000) and comparisons with the genomes of other species reveal the conservation of a core set of proteins with other invertebrates and mammals (Balakrishnan et al. 2015; Jennings 2011). The *Drosophila* genome is 17 times smaller than human genome and has approximately half the number of genes. This lower number is in part explained by genome duplication in the human lineage during evolution. The *Drosophila* genome contains orthologs for almost 75% of genes implicated in human diseases (Balakrishnan et al. 2015). Given that so many genes and processes are conserved, *Drosophila* has been adopted as a model of choice for the rapid discovery of genes responsible for a wide range of human monogenic diseases (Liu et al. 2015, 2017).

The use of *Drosophila* has recently emerged as a formidable experimental model to study the core aspects of metabolism. For example, the vital need for lipid and carbohydrate usage and storage across animal species has led to an evolutionary conservation of metabolic pathways that are more easily investigated in *Drosophila* than in complex organisms such as mammals (Padmanabha and Baker 2014). This is of fundamental importance in the context of this study, since the scientific literature indicates that insecticides can have detrimental impacts on metabolism (**Chapter One - 1.5**). Over the last 6 decades, *Drosophila* has been the model to investigate the genetic basis of insecticide resistance, which is facilitated by the ability to create resistant and

susceptible mutants (Perry and Batterham 2018). Furthermore, even though vinegar flies are not the primary targets of the insecticides used in agriculture, they are exposed and have evolved resistance to many classes of insecticides (Daborn et al. 2002; Ffrench-Constant et al. 1993; Green et al. 2019; Pyke et al. 2004; Sun et al. 2019).

1.4 A brief overview on the mode of action of neurotoxic insecticides

Insecticides are among the most important weapons used for the control of agricultural pests and insects that vector pathogens (Lasram et al. 2014; Scott and Buchon 2019; Yadav et al. 2015). The use of insecticides in agriculture is vital for improving the quality and quantity of crops produced. Crop yields are increased by 6 to 79% by killing and/or preventing insects from engaging in destructive behaviors (Duzguner and Erdogan 2010; Scott and Buchon 2019). In a world where there is a need to produce more food to feed a growing global population from less arable land, insecticides are making a huge contribution. The great diversity in the mode of action of insecticides can be grouped based on their main or primary physiological target: nerve & muscle; growth & development; respiration; midgut; unknown or non-specific (IRAC - Insecticide Resistance Action Committee; <https://irac-online.org/>). However, although insecticide usage is intensive, knowledge of their mode of action is limited. While the protein targets of most neurotoxic insecticides are known (Perry and Batterham 2018; Perry et al. 2011; Scott and Buchon 2019), there is minimal knowledge of what molecular events occur downstream of the insecticide:target interaction. These events may differ between insecticides given the diversity of chemicals that are being used for insect pest control (Sparks and Nauen 2015). Neurotoxic insecticides can be synthetic, such as neonicotinoids, or naturally occurring (organic) such as the spinosyns.

From a didactic point of view, the action of a given insecticide can be divided in 5 steps: (1) uptake - orally or through the cuticle; (2) movement into/between tissues; (3) metabolism; (4) interaction with target proteins; and (5) excretion of insecticide/metabolites (Scott and Buchon 2019). It is important to notice however, that even such a logical model contains flaws. For instance, steps 3, 4 and 5 can occur concomitantly or in a different order, with the chemical reaching its molecular target before being metabolized, since these tasks functions reside in different tissues. As the fly has an open circulatory system, insecticides that penetrate the cuticle are able to reach targets in the

brain without passing through metabolic tissues. Finally, insecticide formulation and, most importantly, dosage can have a great influence on its mode of action, as the study here will demonstrate.

Steps 3 and 4 are also the most thoroughly investigated (Scott and Buchon 2019). Enzymes from a number of large multigene families (e.g. cytochrome P450s, carboxylesterases, glutathione-S-transferases and UDP-glucuronosyltransferases) are involved in insecticide metabolism (Scott and Buchon 2019). The genes encoding these enzymes are rapidly evolving. Therefore, the understanding of metabolism gained in one species is not readily transferable to other species. Metabolic resistance often affords a relatively low level of protection for insecticides, but may evolve more readily than target site resistance due to lower fitness cost (Bass et al. 2015; Somers et al. 2018). An understanding of the target provides a layer of information about the mode of action of the insecticide. Target site mutations often confer very high levels of resistance that threaten the viability of the insecticide as a control agent, thus attracting significant research interest. As the genes encoding targets are highly conserved among insect species, lessons learned in studying the target in one species is readily transferable to others (Perry and Batterham 2018). The neurotoxic insecticides that are in current use target a limited number of proteins (Sparks and Nauen 2015). Some examples of these targets include the voltage sensitive sodium channel for pyrethroids and DDT; acetylcholinesterase enzyme for carbamates and organophosphates; and nicotinic acetylcholine receptors (nAChRs) for imidacloprid and spinosad (Perry and Batterham 2018; Scott and Buchon 2019).

Here the focus is on nAChRs, since these receptors are targeted by imidacloprid and spinosad, the neurotoxic insecticides investigated in this study. nAChRs are the archetype members in the family of ligand-gated ion channels, responding to endogenous neurotransmitters and triggering fast intercellular communication (Barrantes 2004). The nAChRs are involved in the cholinergic response, a vital excitatory synaptic transmission pathway widely distributed throughout the nervous system of both vertebrates and invertebrates (Thany 2010).

In the nervous system and at neuromuscular junctions nAChRs mediate quick response to acetylcholine (ACh). They promote or prevent ion influx into cells upon

binding or cleavage of agonists such as ACh. The nAChRs are composed of five subunits, arranged pseudo-symmetrically around a central axis, which forms as a cation channel (Baenziger et al. 2015). Each subunit has four transmembrane domains (M1-4) and an extracellular N-terminal domain with a Cys-loop motif (two cysteines separated by 13 amino acid residues forming a disulphide bond) (**Figure 1.1**). The binding site for ACh is found at the interface between two adjacent subunits. It is formed by six distinct regions present in the extracellular N-terminal domain: loops A, B and C present in an α subunit; and loops D, E and F present in either an α or non- α subunit. Subunits possessing two adjacent cysteine residues in loop C are defined as α subunits while subunits lacking them are classified as non- α (β , δ , ϵ or γ). nAChRs diversity is thus generated by multiple subunits encoded by numerous genes. The subunit composition of a given nAChR determines its function and physicochemical properties (Barrantes 2004; Dupuis et al. 2012; Jones and Sattelle 2010; Miyazawa, Fujiyoshi, and Unwin 2003).

nAChRs dwell in three allosteric states: resting, open and desensitized. The ion channel is closed and, thus non-conducting in the resting and desensitized states. In the open state there is conduction of Na^+ , K^+ or Ca^{2+} , leading to depolarization of the postsynaptic membrane. The change in allosteric states is a response to the binding of the neurotransmitter acetylcholine (ACh), which rapidly shifts nAChRs from resting to open state. Under normal physiological circumstances, after being released, ACh is removed from the synaptic cleft within milliseconds either by diffusion or by acetylcholinesterase, which hydrolyses ACh to form choline and acetate. The removal of ACh shifts nAChRs back to the resting state. Upon longer neurotransmitter exposure nAChRs shift to the desensitized state, which even though non-conducting shows greater affinity for ACh than in the resting state (Bradshaw and Dennis 2003).

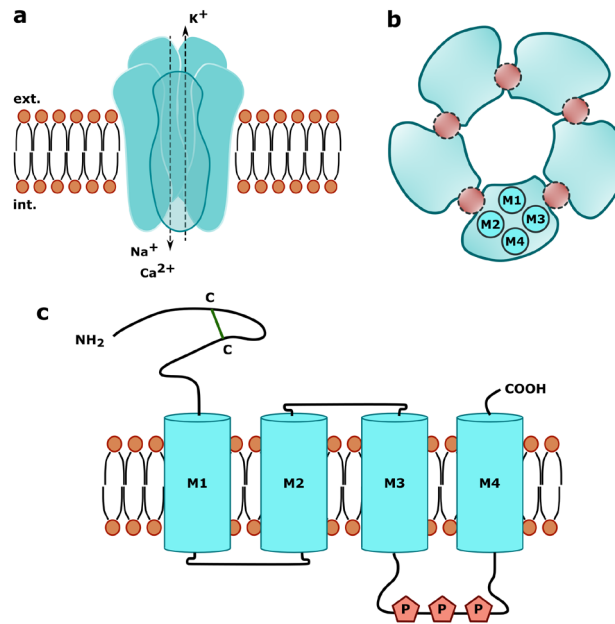


Figure 1.1 Schematic representation of insect nicotinic acetylcholine receptors. a. Homomeric nAChR (five identical subunits) showing the direction of cation flux under the open state. **b.** Bird's eye view of the nAChR showing the binding sites of ACh (brown dotted-line circles) lying between two adjacent subunits. **c.** Representation of a single nAChR subunit showing the four transmembrane domains (M1–4), the intracellular loop between M3 and M4 harbouring phosphorylation sites (brown pentagons) and the extracellular N-terminal domain containing a Cys-loop motif. Modified from (Dupuis et al. 2012).

ACh is synthesized by choline acetyltransferase (ChAT), which combines Acetyl coenzyme A produced in mitochondria with choline, which is packed into synaptic vesicles by the vesicular acetylcholine transporter (VACHT). An ATP-dependent proton pump, present in the vesicle membrane, acidifies the vesicle lumen creating a pH gradient between vesicle and cytoplasm, which is used by VACHT to exchange ACh for protons (Chatzidaki and Millar 2015; Erickson et al. 1994; Hurst, Rollema, and Bertrand 2013; Naser and Kuner 2018; Szutowicz et al. 1999). Loaded synaptic vesicles fuse to the cell membrane releasing ACh into the synaptic cleft, which then binds to nAChRs, usually located on post-synaptic cells. After exerting their response, ACh molecules are then hydrolysed by acetylcholinesterase (AChE) and the resulting choline transported back into the presynaptic neuron by choline transporter (HACHT, or ChT), becoming available for new ACh synthesis (Chatzidaki and Millar 2015; Erickson et al. 1994; Hurst et al. 2013; Naser and Kuner 2018; Szutowicz et al. 1999). Choline is obtained from diet and through cleavage of phosphatidylcholine lipid species by phospholipase D (PLD-1). The phospholipid phosphatidylcholine is thus the main form of choline obtained in diet

(Chatzidaki and Millar 2015; Erickson et al. 1994; Hurst et al. 2013; Koc et al. 2002; Naser and Kuner 2018; Szutowicz et al. 1999).

nAChRs show a high level of sequence homology among different insect species despite a divergence time of over 300 million years back to the last common ancestor (**Figure 1.2**). This conservation is likely due to the vital roles that nAChRs play in nervous system function. A lower sequence homology is found when comparing insect and vertebrate subunits (Jones and Sattelle 2010; Thany 2010). Genome comparisons show that the nAChR family consists of 10 to 12 genes in insects - 10 *D. melanogaster* and *A. gambiae*; 11 *A. mellifera*; 12 *B. mori* and *T. castaneum* (Jones and Sattelle 2010; Thany 2010). In the *Drosophila* genome, 7 genes encode α subunits (Da1-7) and 3 genes encode β subunits (D β 1-3) (Perry et al. 2008).

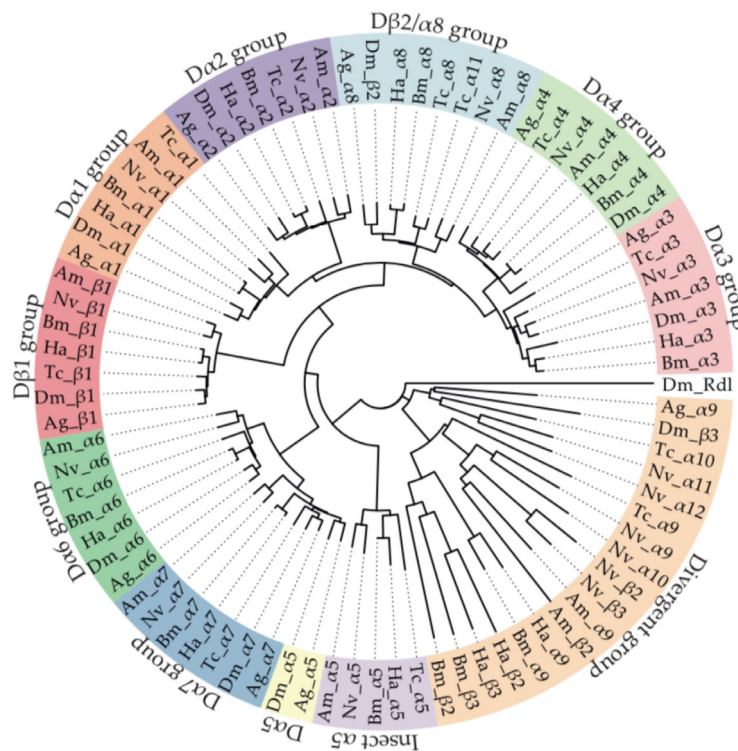


Figure 1.2. Distribution of nAChR subunits in the insect phylogenetic tree. Groups are named according to their *D. melanogaster* representatives. Species: Ag, *A. gambiae*; Am, *A. mellifera*; Bm, *B. mori*; Dm, *D. melanogaster*; Ha, *H. armigera*; Nv, *N. vitripennis*; Tc, *T. castaneum*. *D. melanogaster* Rdl was used as outgroup. Figure adapted from Somers (2015).

The nAChRs play an important role in neuroprotection and pathological consequences may arise from factors interfering in the magnitude and duration of ACh response and the efficiency of synaptic transmission. Numerous neurological and psychiatric disorders, such as Parkinson's disease, Tourette's syndrome, schizophrenia and Alzheimer's disease present some level of nAChRs dysfunction (Barrantes et al. 2010; Gotti and Clementi 2004; Quirk et al. 2007). Cholinergic abnormalities in these diseases can include reduction in acetylcholine release and choline acetyltransferase activity, loss of receptor expression, perturbations in choline uptake and/or deposit of toxic metabolites in the central nervous system. Such abnormalities may provoke decline of cognitive abilities and behavioural disturbances (Barrantes et al. 2010).

Several classes of insecticides exert their neurotoxic effects by affecting nAChR function. Carbamates and organophosphorus, for instance, act as acetylcholinesterase (AChE) inhibitors, thus exhibiting cholinergic toxicity. Phosphorylating or carbamylating AChE diminishes its capacity to hydrolyse ACh, which accumulates in the synaptic cleft, causing hyperstimulation followed by desensitization of nAChRs. In humans and other mammal models, the inhibition of AChE activity induces seizures, convulsions, severe muscle paralysis, respiratory failure and even death (Milatovic et al. 2006). Neonicotinoids, such as imidacloprid, and spinosad also exhibit a high degree of selectivity towards insect nAChRs and high doses of these chemicals will cause receptor malfunction leading to insect death (**Chapters Two & Three**).

1.5 Insecticides impact on metabolism

Many studies have focused on how insect metabolism reduces the toxicity of insecticides, which is essential for the understanding of the evolution of insecticide resistance. These studies emphasise the importance of cytochrome P450s (CYPs), esterases, and glutathione *S*-transferases (GSTs) and UDP-glucuronosyltransferases, which can convert insecticides into more hydrophilic compounds, facilitating excretion (Fusetto et al. 2017; Perry and Batterham 2018; Scott and Buchon 2019). Less attention has been given to the other side of the coin, i.e. how insecticides can impact the metabolism of insects. These impacts may be of less significance and interest on pests exposed to high doses that kill rapidly. But, on insect species that are not targeted by insecticides, but are exposed to lower doses, it is possible that metabolic impacts could

have downstream consequences in terms of health, reproductive output, longevity, and behaviour. Most of the available evidence about insecticide impacts on metabolism come from evidence of impairments in mammalian models.

Many insecticides are lipophilic compounds and are thus capable of binding to fatty components of breast milk, blood, and fatty tissues. Such insecticides are therefore bio-accumulative compounds, present in food chains. As a result, humans are exposed to these chemicals by eating foods that have been insecticide-treated, grown in contaminated soil or with contaminated water (Yadav et al. 2015). The hydrophobicity of some insecticides can, thus, drive the impact of these chemicals on the lipid environment and lipid metabolism. Understanding this is likely to be a key to understanding low dose toxicity of many insecticides to insects but, to date, research on this has been largely neglected. The hydrophobicity of insecticides allows them to bind to plasma proteins for transport in biological fluids, distribution, and metabolism. In *Helicoverpa zea* larvae, for instance, the hemolymph lipid transporters lipophorin and arylophorin have been shown to bind the insecticide dieldrin (Haunerland and Bowers 1986; Shapiro 1991). In the spider *Polybetes pythagoricus*, the organophosphorous insecticide fenitrothion has been shown to penetrate high density lipoproteins HDL1 and HDL2, altering the lipid dynamics in their hydrophobic cores and hydrophilic outer regions (Cunningham et al. 2002).

Insecticides like organophosphates and organochlorines are also capable of interfering with liposome membrane permeability, disturbing the interaction of cholesterol with other membrane lipids (Antunes-Madeira and Madeira 1979). The composition and physicochemical properties of the biological membranes are important for insecticide mechanism of action (Cunningham et al. 2002; Levitan et al. 2010). For example, it is well established that the functional properties of nAChRs are affected by the surrounding lipid environment in membranes (Barrantes 2004). Studies on humans accidentally exposed to organophosphates showed neurotoxicity effects caused by the degeneration of axons and myelin in both the central and peripheral nervous systems (Rezg et al. 2010). These are specific effects of insecticides in the lipid membranes of nervous system.

The lipid composition of membranes in which nAChRs are embedded affects their allosteric transitions (channel opening and closing), as well as the kinetics of desensitization (Baenziger et al. 2015; Barrantes 2004; Barrantes et al. 2010). Cell membrane lipids such as sphingolipids, ceramides and cholesterol affect many aspects of nAChR biology - trafficking from the production site to the plasma membrane, distribution and organization on the membrane and the rate of endocytosis (Baenziger et al. 2015; Barrantes et al. 2010). The highly lipid-exposed α -helix M4 domain of nAChR subunits is proposed to act as a lipid sensor that interacts with the surrounding lipid components of the cell membrane to stabilize the receptor resting conformation (daCosta and Baenziger 2009).

Using metabolite profiling, Brinzer et al. (2015) showed that exposing *Drosophila* larvae to the pyrethroid insecticide permethrin causes an increase in the levels of free fatty acids and dicarboxylate products and a weak trend of depletion of lysolipids that are derived from choline. Insecticide exposure can also alter metabolism within the fat body. In *Bombyx mori* organophosphate exposure induces the fat body to mobilize trehalose and glucose to hemolymph (Nath 2002) and increases the expression levels of cytochrome P450s and glutathione-S-transferases (Gu et al. 2015), events related to the suppression of lipolytic activity and detoxification, respectively. Imidacloprid was shown to disturb the fat body expression of genes related to antimicrobial peptides (AMPs), significantly reducing antimicrobial activity in the hemolymph of *Apis mellifera* (Brandt et al. 2016). Moreover, exposure to organochlorines or organophosphates has been linked to excessive weight gain and an increase in the severity of diabetes in humans and animal models like rats and mice (Karami-Mohajeri and Abdollahi 2011; Rezg et al. 2010; Slotkin 2011; Thayer et al. 2012). One study showed that low concentrations of the organochlorines DDT and DDE increased lipid accumulation and adipocyte differentiation in a 3T3-L1 adipocyte model (mouse) after 8 days of exposure (Kim et al. 2016). Several transcription factors and genes related to lipid metabolism such as fatty acid synthase (FAS), adipose triglyceride lipase (ATGL), CCAAT/enhancer-binding protein α (C/EBP α), peroxisome-proliferator activated receptor- γ (PPAR γ), have been shown to be activated in response to insecticides (Kim et al. 2016). DDT and DDE also decreased phosphorylated forms of AMP-activated protein kinase- α (AMPK α), and acetyl-CoA carboxylase (ACC) (Kim et al. 2016). All these events are related to lipid production and accumulation. The activation of AMPK is likely to inhibit adipogenesis, since it could reduce the expression of

transcription factors PPAR γ and C/EBP α that regulate lipid metabolism and lipid anabolism enzymes such as FAS and ACC (Habinowski and Witters 2001). Regulation of ACC is the key step in the anabolism and catabolism of fatty acids; it catalyzes the synthesis of the substrate for fatty acid production, malonyl-CoA. AMPK-mediated phosphorylation of ACC is a molecular mechanism for the inhibition of lipogenesis (Daval et al. 2005). Park et al. (2013) also showed that imidacloprid triggers lipid accumulation by increasing adipocyte differentiation and lipid stores. A similar study pointed to the same effects when 3T3-L1 adipocytes were exposed to fipronil (Sun et al. 2016).

In mammalian models, organochlorines were shown to accumulate in adipose tissues and brain, elevating levels of triacylglycerols (TAG), fatty acids and fasting glucose. It also increases the oxidation of fatty acids within peroxisomes and endothelial reticulum, leading to exaggerated production of Reactive Oxygen Species (ROS). Oxidative stress, the imbalance between ROS generation and degradation, can decrease mitochondrial ATP production by disrupting the respiratory chain and Krebs cycle enzymes. These phenomena elevate energy demand activating gluconeogenesis and PKC, which regulates the secretion of glucagon and insulin (Karami-Mohajeri and Abdollahi 2011). Oxidative stress could be a factor in insecticide toxicity and needs to be carefully considered.

1.6 Lipid metabolism in insects

Considering the evidence for insecticide impacts on lipids and lipid metabolism and how this may contribute to insecticide toxicity, a review on the most relevant aspects of insect lipid metabolism is provided here.

1.6.1 Lipids and lipid metabolism

Lipids are a highly diverse group of compounds present in all living organisms which have a key role in numerous biological processes, serving not only as energy storage but also structural components of cells and as signaling molecules (Fahy et al. 2011; Liu and Huang 2013). Lipids represent a broad range of molecules with complex structures due to transformations which occur during their biosynthesis, reflecting their

functional diversity. The elements of structural diversity include variations in chain length, biochemical changes such as oxidation, reduction, substitution and formation of rings as well as modifications with functional groups such as sugar residues (Fahy et al. 2011).

Lipids are hydrophobic or amphipathic molecules formed by carbanion-based condensations of ketoacyl thioesters and/or by carbocation-based condensations of isoprene units. Due to the difficulties in elucidating their chemical structures, there are no reliable estimates of the number of different lipids in nature, but based on possible permutations of glycan groups and acyl/alkyl chain over 200,000 different lipid species could exist (Fahy et al. 2011). They are divided into eight major categories: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, polyketides (derived from condensation of ketoacyl subunits), sterol lipids and prenol lipids (derived from condensation of isoprene subunits) (Fahy et al. 2011). A lipidome, the collection of lipids in a given sample, may include over 1000 different lipid species for a single cell (Meer et al 2008; Wenk 2010) and 10,000 to 100,000 species for tissues or organisms (Yetukuri et al. 2008). Lipidome studies have contributed to the understanding of physiology and metabolism in different organisms. That is because lipids not only exhibit fundamental biological functions, but also display collective behaviors that are not displayed by isolated molecules (Fahy et al. 2011).

The main classes of lipids found in eukaryotic cell membranes are glycerophospholipids (GPL), which are key components of biological membranes and are also involved in metabolism and cell signaling; sphingolipids (SPL), a family of compounds that share a sphingoid base backbone (synthesized *de novo* from the amino acid serine and a long-chain fatty acyl CoA) which play important roles in signal transmission and cell recognition, especially in central nervous system; and sterols (ST) derived from the same fused four-ring core structure, which have different biological roles as hormones and signaling molecules (Meer et al. 2008).

In *Drosophila* third instar larvae, approximately 90% of membrane lipids are GPL, with phosphatidylethanolamine (PE) being the majority of it (65%), followed by phosphatidylcholine (PC) (Guan et al. 2013). *Drosophila* GLP do not contain fatty acids with acyl chains longer than 18 carbons. These are common in mammals. SPL, based on

a tetradeca-4-sphingenine (C14), are the second major class of lipids in *Drosophila* membranes. SPL play critical roles in many cellular and developmental processes being key regulators of cell metabolism, cell cycle/apoptosis and growth in vertebrates. In *Drosophila* SPL biosynthesis has been implicated in the regulation of energy homeostasis and fat metabolism. They are always saturated and shorter than in mammals, with arachidic acid (20:0) being the most abundant. No sphingomyelins (group of SPL common in the central nervous system of vertebrates) are present in *Drosophila*, but ceramide phosphoethanolamines are found instead (Raghu et al. 2012).

Regarding the dietary requirements, insects are able to synthesize the common C6 and C8 saturated and monounsaturated fatty acids from non-lipid precursors or pre-existing fatty acids. It is suggested, however, that they are unable to add a second or third double bond into a fatty acid, and consequently require polyunsaturated fatty acids in diet, such as linoleic and linolenic acids (Beenackers et al. 1985). All insects also require ST in their diets and cholesterol is the major one found in them. Cholesterol serves not only as a structural component of cell membranes but also as a substrate for the production of ecdysteroids, the molting hormones. Numerous insect species have thus evolved metabolic pathways to convert dietary STs into cholesterol (Canavoso et al. 2001).

In *Drosophila*, the dietary lipids undergo digestion and absorption in the midgut, where enterocytes produce lipases that hydrolyze dietary triacylglycerol (TAG), forming monoacylglycerols and free fatty acids. Glycolipids are also hydrolyzed releasing sugar and diacylglycerol (DAG). Cholesterol ester are directly absorbed in the midguts, while free cholesterol requires first intracellular esterification. After digestion, enterocytes absorb the sugar, free fatty acids, DAG and partial acylglycerols and convert them into DAG, TAG, and phospholipids. Conversion within enterocytes involves acylation of 2-monoacylglycerol or the *de novo* pathway that involves acylation of sn-glycerol-3-phosphate. After conversion, enterocytes export DAG to the hemolymph and most of it ends up stored in the fat body after being first transformed into TAG. Under energetic need TAG stores in the fat body can be once again converted into DAG and exported to the hemolymph for transport to tissues to produce energy by β -oxidation (Canavoso et al. 2001). Altogether, this mechanism guarantees the absorption of fatty acids from the midgut lumen while preventing an increase in the intracellular concentration of both free

fatty acids and DAG, whose high concentrations can exert cellular toxicity (Canavoso et al. 2001).

Lipids released by enterocytes are dispersed in hemolymph through an open circulatory system, enhanced by the pumping action of a tubular heart. Most of the lipid in hemolymph is associated with a lipoprotein particle, generically called lipophorin (Lpp), a member of the apolipoprotein B (ApoB) family. Lpp is crucial for fat storage and peripheral tissue membrane homeostasis. Under most physiological conditions, Lpp exists as high-density lipophorin (HDLp). Every HDLp particle contains one molecule each of two apolipoproteins: apolipophorin-I and apolipophorin-II. A third apolipoprotein (apolipophorin-III), also exists in *Drosophila*, but it is found associated with low-density lipophorin (LDLp) or free in the hemolymph (Canavoso et al. 2001). Lpp contains a phospholipid-protein surface and a neutral lipid core composed of DAG and TAG (factor of 15:1, respectively). Small amounts of sterols, free fatty acids, carotenoids, and monoacylglycerols are also transported. Lpp and the transported lipids are packaged together to form a soluble lipoprotein particle, in a process called lipidation, leading to the formation of very-low-density lipoproteins (VLDL) (Canavoso et al. 2001). Lpps transfer lipid molecules to receptors present in the membrane of target cells, without being internalized in the process, thus making the transfer more efficient. Two other components are involved in this transfer: lipophorin receptor (a candidate is HDLp-binding protein) and the lipid transfer particle (LTP) (Padmanabha and Baker 2014). LTP is a very-high-density lipoprotein (VHDL) which redistributes lipids between Lpps and between Lpp and the lipophorin receptors (Canavoso et al. 2001). An overview of this process and the major components of lipid metabolism in insects is shown in **Figure 1.3**.

In insects, the majority of stored lipids are found in the fat body, with more than 90% of this being TAG (Azeez et al. 2014). *Drosophila* has several enzymes involved in TAG *de novo* synthesis from fatty acids, including glycerol-3-phosphate acyltransferase, acylglycerol phosphate acyltransferase, phosphatidate phosphatase (Lipin), and diacylglycerol acyltransferase (DGAT). DGAT produces TAG by esterification of DAG in a reaction that uses fatty-acyl-CoA (Arrese and Soulages 2010; Liu and Huang 2013). In *Drosophila*, the transcription factor dSREBP regulates fatty acid synthesis and their incorporation into PE and PC, being inhibited by excessive PE production. dSREBP controls the activity of genes like *mdy*, which encodes *Drosophila* DGAT (Liu and Huang

2013). Fat body TAG can also result from de novo lipid synthesis from carbohydrates or amino acids (Canavoso et al. 2001). Amino acids can be deaminated, forming acetyl CoA and urea, whilst pyruvate and lactate (as well as fatty acid and glycerol from fat digestion) can also be converted to acetyl CoA. Acetyl CoA is then converted to malonyl CoA and used to synthesize TAG (Azeez et al. 2014).

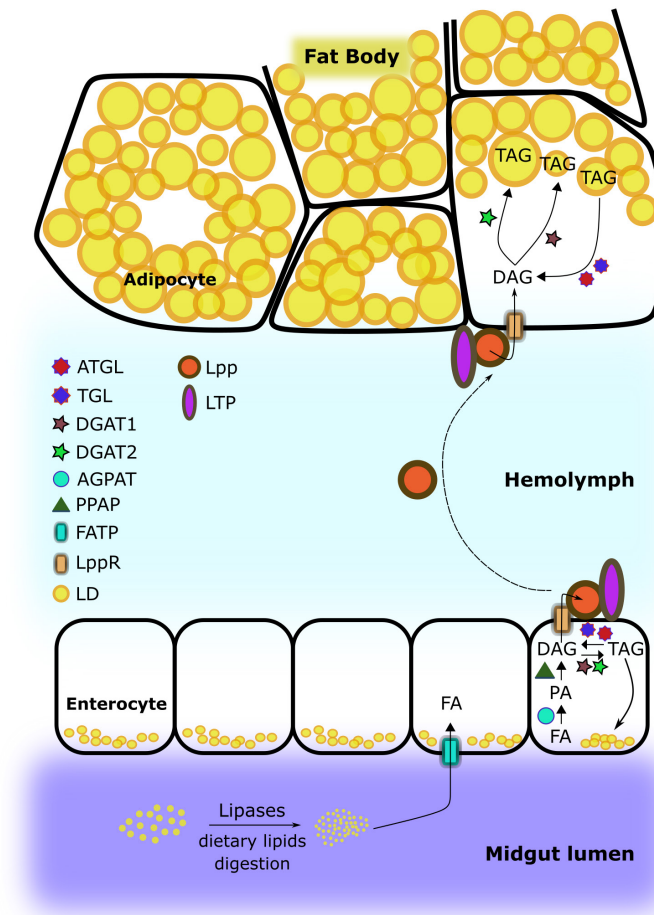


Figure 1.3. Overview of lipid metabolism and transport in insects. FA - Fatty acid; PA - Phosphatidic acid; DAG - Diacylglycerol; TAG - Triacylglycerol; ATGL - Adipose Triglyceride Lipase; TGL - Triglyceride Lipase; DGAT1 - Diacylglycerol Acyltransferase 1; DGAT2 - Diacylglycerol Acyltransferase 2; AGPAT - 1-acylglycerol-3-phosphate O-acyltransferase; PPAP - Phosphatidic Acid Phosphatase; FATP - Fatty Acid Transporter; Lpp - Lipophorin; LTP - Lipid Transfer Particle; LppR - Lipophorin Receptor; LD - Lipid Droplet.

1.6.2 Regulation of lipid metabolism in insects

The basic metabolic and signaling pathways involved in lipid metabolism are evolutionarily and functionally conserved between *Drosophila* and mammals (Liu and

Huang 2013). In *Drosophila* the maintenance of lipid homeostasis is critical to maintain growth (Ugrankar et al. 2011), lifespan (Piper et al. 2005), control of movement and reproduction (Gutierrez et al. 2007). In insects, lipid mobilization from the fat body is induced by two types of hormones: glucagon-like adipokinetic hormone (AKH) and octopamine (Canavoso et al. 2001). Lipids are mobilized as DAG, not as free fatty acids in mammals. In *Drosophila* the *corpora cardiaca* cells located in the larval ring gland can secrete AKH and behave like pancreatic α -cells (Liu and Huang 2013). AKH stimulates the formation of DAG and its release in hemolymph, probably by activating fat body lipases to catalyze TAG hydrolysis (Canavoso et al. 2001). Two lipases expressed in the fat body are known: Brummer lipase (insect adipose triglyceride lipase - ATGL) and triglyceride lipase (TGL) (Arrese and Soulages 2010).

In insects, AKH release from the *corpora cardiaca* is regulated by the neurotransmitter octopamine, an analog of the vertebrate catecholamine noradrenaline. Octopamine also acts as a neurohormone, directly mobilizing energy stores. The *Drosophila* genome also encodes seven insulin-like peptides (DILPs), master regulators of metabolism, mainly produced by the insulin-producing cells (IPCs) in the insect central nervous system (analogous to the vertebrate pancreatic β -cells). Fat body adipocytes can also express some level of DILPs (Liu and Huang 2013; Padmanabha and Baker 2014). The insulin/ insulin like growth factor 1 (IIS) pathway, and the protein kinase TOR (target of rapamycin) pathway interconnect energetic metabolism, growth and nutrients uptake (Baker and Thummel 2007; Conn and Qian 2013; Edgar 2006; Partridge, Gems, and Withers 2005). After feeding organisms face a transient state of hyperglycemia and hyperlipidemia which promotes IIS/TOR activation and insulin release into the circulatory system, culminating in cellular uptake of glucose and lipids. Insulin stimulates glycolytic metabolism, which produces energy and promotes lipogenesis. Lipogenesis lead to TAG and phospholipids production and stocking. During a fasting state, AKH in insects and glucagon in vertebrates, promotes glucose release in circulatory system through glycogenolysis (breaking glycogen) and gluconeogenesis (producing glucose). Prolonged fasting induces lipolysis, the breaking of fatty acids through β -oxidation to produce energy (Rui 2014).

1.6.3 Fat body – the central metabolic organ

Fat body is the main biosynthetic organ of insects, analogous to vertebrate liver and white adipose tissue (Arrese and Soulages 2010; Azeez et al. 2014; Padmanabha and Baker 2014; Roma et al. 2010). It is the main organ involved in energy metabolism, mobilizing energy reserves when necessary, besides responding to its own metabolic requirements. It integrates multiple hormonal (neurohormone octopamine, juvenile hormone, ecdysone) and nutritional signals coordinating the appropriate metabolic and survival responses, regulating development and metamorphosis. It is also the major storage site for glycogen, proteins and especially lipids which represent 70% of its dry mass. It maintains communication with hemocytes and other cells in the hemolymph via cytokines, regulating hematopoiesis and clotting proteins. It also expresses miR-8, responsible for the production and regulation of AMPs (antimicrobial peptides) such as drosomycin and diptericin that regulate the innate immune response, besides being involved in the acute stress response (Azeez et al. 2014; Liu and Huang 2013; Roma et al. 2010).

The balance between energy storage and utilization, as well as the determination of where energy is required, is performed in the fat body by Mef2 (myocytes enhancer factor 2). Mef2 is a transcription factor present in eukaryotes from yeast to humans. It is expressed in muscles, neurons, and immune cells, where it regulates the activation of lipogenic and glycogenic enzymes. In numerous cell types Mef2 has a key role as a regulator of cell differentiation, antimicrobial peptide expression, proliferation, morphogenesis, survival, and apoptosis. In the fat body, Mef2 acts as a genetic switch coordinating the energetic investment in either immune response or metabolism. In healthy flies, the phosphorylation of Mef2 at a conserved site promotes the expression of lipogenic and glycogenic enzymes. Such phosphorylation is lost upon infection resulting in the expression of antimicrobial peptides (Azeez et al. 2014).

The fat body also has a role in the storage and metabolism of non-utilized substances, which can be released in hemolymph when body demand increases. It also acts in the process of molt, supplying energy and hydrocarbons, besides being the site of synthesis for proteins such as vitellogenin that are secreted in oocytes, (Roma et al. 2010). The fat body also synthesizes most of the hemolymph proteins and circulating

metabolites, storage proteins and lipophorins. Most of the insect's intermediary metabolism also takes place in the fat body, such as the metabolism of lipids, carbohydrates, proteins, amino acids, and other nitrogen compounds. To perform these many metabolic functions, the fat body integrates and interprets signals from other organs. The *Drosophila* fat body specifically expresses the amino acid transporters Slif that functions as a nutrient sensor and regulates TOR activity (Azeez et al. 2014).

Regarding its structure, fat body is a large, loosely organized, and dispersed tissue. It exists as a weak aggregate of a mass of whitish cells, present in thin layers that are 1-2 cells thick and distributed throughout the insect body, functioning as connective tissue between organs, filling the cavities of the head, thorax and abdomen (Padmanabha and Baker 2014; Roma et al. 2010). This structure provides maximal exposure to nutrients, proteins, and hormones that are transported via the hemolymph and is thus vital for the organism to adjust appropriately to metabolic demands (Arrese and Soulages 2010; Padmanabha and Baker 2014; Roma et al. 2010). The fat body can be divided into the perivisceral layer, located around various organs; and the parietal layer, located adjacent to the integument. Studies suggest that in Diptera, the parietal fat body would be mainly involved in the synthesis and the storage of lipids, while the perivisceral fat body would function in the storage of proteins (Roma et al. 2010). Fat body is a mesodermal tissue formed mainly by trophocytes. Apart from the oenocytes that have an ectodermal origin, other fat body cells are differentiated from trophocytes to accomplish their specific functions. These cells are the mycetocytes, chromotocytes, urate cells and hemocytes (Roma et al. 2010).

The trophocytes are rounded or polygonal cells, possessing a large irregular nucleus and many nucleoli, characterized by the presence of numerous lipid droplets and other substances stored in their cytoplasm. Trophocytes synthesize, absorb and store materials, especially those coming from the hemolymph (Roma et al. 2010). Oenocytes are involved in the synthesis of lipids or lipoproteic complexes that are deposited in the insect cuticle to form an impermeable barrier (Gutierrez et al. 2007; Padmanabha and Baker 2014; Roma et al. 2010).

Lipids in the *Drosophila* larval fat body comprise 6% the total body weight in newly hatched first instar larvae, increasing to 15% in third instar larvae. After

metamorphosis lipid reserves are depleted to 6.5% of body weight. This change in lipid content corresponds to a shift in fat body function from directing body growth and TAG storage during larval stages to maintaining energy homeostasis in the adult (Baker and Thummel 2007). Besides having large amounts of TAG, fat body is also enriched in PE compared with other tissues. Fat body has higher proportions of shorter and more saturated fatty acids and phospholipids than other tissues and has a lower proportion of PS, which contains longer and more unsaturated fatty acids than other phospholipid classes. Fat body also contains relatively lower levels of lipids known to be enriched in plasma membranes such as sterols and phosphatidylethanolamine ceramides (PECer) (Carvalho et al. 2012).

1.6.4 Lipid droplets – the lipid reservoir of cells

The main intracellular storage site of TAG is a specialized cytoplasmic compartment called the lipid droplet (LD). Almost all tissues can synthesize and store TAG in lipid droplets, but fat body cells are specialized for this function. LDs remove potentially toxic lipids and store them safely away from other cellular compartments. They guarantee a relatively easy cellular access to lipids for producing energy, membrane components, and signaling molecules. Evidence shows that LDs are dynamic organelles serving a central role in fat and energy metabolism. They are highly motile, moving along microtubules using the molecular motors kinesin-1 and cytoplasmic dynein. LDs consist of a core of neutral lipids (TAG and cholesterol esters) surrounded by a monolayer of phospholipids and cholesterol, into which specific proteins are embedded or peripherally associated (Arrese and Soulages 2010; Liu and Huang 2013).

LDs are functionally complex, containing dozens, or hundreds, of different lipids and proteins, that differ between cell types. They show functional and physical interactions with endoplasmic reticulum, mitochondria, and phagosomes, and their composition, size, and intracellular distribution are actively regulated. Vinegar flies express two enzymes catalyzing the conversion of DAG and fatty acyl-CoA into TAG. These enzymes, DGAT1 and DGAT2, are expressed throughout development and mediate LD size control. The DGAT1 pathway generates small droplets, and the DGAT2 pathway larger droplets (Welte 2015).

The surface composition of the LDs also provides another regulatory layer to lipolysis, since the lipolytic enzymes that digest the highly nonpolar TAGs would have to first cross a barrier of polar phospholipids. This process is regulated by proteins from the PAT family, which are evolutionarily conserved and localized at the surface of LD. Insect genomes encode two PAT proteins, Lsd-1 and Lsd-2. Lsd-2 is suggested to promote lipid accumulation, whereas Lsd-1 promotes lipolysis. In the moth *Manduca sexta* Lsd-1 is phosphorylated in response to AKH, activating TGL lipase and controlling the lipolytic response (Arrese and Soulages 2010).

Using Lsd-2::GFP reporters and multi-isotope imaging mass spectrometry Bailey et al. (2015), showed that oxidative stress induces glial LDs accumulation in the *Drosophila* nervous system. Such induction is even stronger when diet is supplemented with linoleic acid due to the preference for oxidative species to attack the carbon double bonds in polyunsaturated fat acids, leading to lipid peroxidation (Negre-Salvayre et al. 2008). Oxidative stress causes a bulk redistribution of polyunsaturated fatty acids from cell membranes to LDs where they are incorporated as TAGs, turning such molecules less susceptible to peroxidation (Bailey et al. 2015).

1.6.5 Lipid metabolism genes in *Drosophila*

Based on this literature review, the most important genes involved in lipid metabolism in *Drosophila* are listed in **Table 1.1**. These genes are further investigated in the context of insecticide exposure in this study.

Table 1.1. Most relevant genes involved on lipid metabolism in *Drosophila*. Gene ID (FBgn) and summary of biological function retrieved from the online database for *Drosophila* genetics and molecular biology – FlyBase (Thurmond et al. 2019).

Gene	Function
<i>ACC</i> FBgn0033246	<u>Acetyl-CoA carboxylase</u> : Catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, the rate-limiting substrate for fatty acid synthesis.
<i>Agpat2</i> FBgn0026718	<u>1-Acylglycerol-3-phosphate O-acyltransferase 2</u> : Phosphatidic acid biosynthetic process.
<i>APOE</i> FBgn0283686	<u>Apolipoprotein E</u> : Assembles LTP to transport lipids on hemolymph.
<i>apolpp</i> FBgn0087002	<u>Apolipoporphin</u> (Lpp, ApoLII): Assembles the major hemolymph lipoprotein transfer particles (LTP - ApoB family). Transports lipids between tissues.
<i>Apoltp</i> FBgn0032136	<u>Apolipoprotein lipid transfer particle</u> : Forms high density LTP. Transfers lipids from gut to circulating lipophorins, and from them to peripheral tissues.

<i>Bmm</i> FBgn0036449	<u>Brummer</u> : Triglyceride lipase involved in glycerol-lipid metabolism, regulation of lipid storage and triglyceride homeostasis
<i>Dgat2</i> FBgn0033215	<u>Diacylglycerol acyltransferase</u> : triglycerides synthesis. Involved on the formation of large lipid droplets.
<i>Dhap-at</i> FBgn0040212	<u>Dihydroxyacetone phosphate acyltransferase</u> : Fatty acid metabolism, phospholipid biosynthesis.
<i>Eip75B</i> FBgn0000568	<u>Ecdysone-induced protein 75B</u> : Nuclear receptor that regulates feeding behavior, fat deposition, and neuronal remodelling (PPAR γ).
<i>Fabp</i> FBgn0037913	<u>Fatty acid binding protein</u> : Involved in lipid binding and long-term memory.
<i>FASN1</i> FBgn0283427	<u>Fatty acid synthase 1</u> : Fatty acid synthase involved in glycogen metabolism and triglyceride biosynthesis.
<i>FASN2</i> FBgn0042627	<u>Fatty acid synthase 2</u> : Required for the synthesis of branched/methylated long chain fatty acids that are the precursors of methylated cuticular hydrocarbons.
<i>FASN3</i> FBgn0287184	<u>Fatty acid synthase 3</u> : Fatty acid synthase activity.
<i>Gpat4</i> FBgn0034971	<u>Glycerol-3-phosphate acyltransferase 4</u> : <i>de novo</i> synthase of lysophosphatidic acid. Lipid droplet organization.
<i>Hr96</i> FBgn0015240	<u>Hormone receptor-like in 96</u> : Protein that binds cholesterol as a ligand and is required to maintain cholesterol and lipid homeostasis.
<i>Ldsdh1</i> FBgn0029994	<u>Lipid droplet subset dehydrogenase 1</u> : Lipid homeostasis of lipid droplets.
<i>Lipin</i> FBgn0263593	<u>Lipin</u> (Phosphatidate phosphatase): Central role in fat body function and energy metabolism. Required for insulin pathway signaling. Triglyceride biosynthesis.
<i>Lpr1</i> FBgn0066101	<u>Lipophorin receptor 1</u> : Transmembrane receptor. Cellular uptake of neutral lipids from the circulating protein encoded by <i>apolpp</i> . Regulates immune response.
<i>Lpr2</i> FBgn0051092	<u>Lipophorin receptor 2</u> : Transmembrane receptor. Cellular uptake of neutral lipids from the circulating protein encoded by <i>apolpp</i> .
<i>LRP1</i> FBgn0053087	<u>LDL receptor protein 1</u> : Cell receptor for low-density lipoprotein transported on hemolymph.
<i>Lsd-1</i> FBgn0039114	<u>Lipid storage droplet-1</u> : Lipid droplet membrane associated protein (PAT family). Control lipolysis and lipid storage in lipid droplets.
<i>Lsd-2</i> FBgn0030608	<u>Lipid storage droplet-2</u> : Lipid droplet membrane associated protein (PAT family). Control lipolysis and lipid storage in lipid droplets.
<i>mag</i> FBgn0036996	<u>Magro</u> : Encodes a lipase induced by the product of Hr96 in the intestine, where it acts in the lumen to degrade dietary triglycerides for fatty acid absorption.
<i>Mef2</i> FBgn0011656	<u>Myocyte enhancer factor 2</u> : Transcriptional factor that act as an immune response - lipid metabolic switch in the fat body.
<i>mdy</i> FBgn0004797	<u>Midway</u> (diacylglycerol acyltransferase; DGAT1): triglycerides synthesis. Involved on the formation of small lipid droplets.
<i>Phospholipase A1</i> FBgn0039471	<u>Phospholipase A1</u> : Lipase activity.
<i>SREBP</i> FBgn0261283	<u>Sterol regulatory element binding protein</u> : Transcriptional factor master-regulator of lipogenesis.

1.7 Different insecticide classes produce oxidative stress

Oxidative stress might be an important and neglected component of insecticide toxicity in insects, that could have major impacts on lipid and energy metabolism. Different insecticide classes, such as organochlorines, organophosphates, carbamates and permethrins were shown to produce some level of oxidative stress, causing deleterious effects *in vitro* and *in vivo* in different model organisms (Karami-Mohajeri and Abdollahi 2011; Lukaszewicz-Hussain 2010; Vontas, Small, and Hemingway 2001; Wang et al. 2016). In *Drosophila*, pyrethroid exposure can increase the production of peroxidized lipids (Terhzaz et al. 2015). Such oxidized products are further degraded by phospholipases (Afsar et al. 2016; Grossmann and Wendel 1983) which leads to the release of free fatty acids, lysolipids and glycerophospholipids in water fleas exposed to pyrethroids (Taylor et al. 2010). Duzguner and Erdogan (2010) showed that imidacloprid causes oxidative stress and inflammation in the central nervous system and liver of mammals. Rats exposed to imidacloprid have an increase in nitric oxide concentration in brain, liver and plasma samples and depletion of intracellular glutathione in liver and brain. These findings show insecticides ability to perturb the oxidative status. However, there is a limited amount of data available demonstrating that insecticides generate oxidative stress in insects and no mechanism has been shown to explain the mechanism by which they could do so.

Oxidative stress is especially harmful to the lipid environment and lipid peroxidation represents major damage to cell membranes. During lipid peroxidation, a methylene group is oxidized, creating a carbon radical ($-\dot{\text{C}}\text{H}-$) which initiates a chain reaction altering the fluidity and shape of biological membranes. Such damage can disrupt cell membranes and calcium homeostasis which is essential for intracellular signaling. Peroxidized lipids can also damage DNA and proteins (Magder 2006). Peroxidation of lipids like polyunsaturated fatty acids can lead to the production of bioactive aldehydes like 4-hydroxynonenal (4-HNE) and malondialdehyde. Such products have been related to decrease neuronal viability in humans (Hernández et al. 2016). 4-HNE is an unsaturated aldehyde that forms covalent adducts with proteins (Uchida 2003). These stable reactive products can propagate damage to cells and tissues (Milatovic et al. 2006). Oxidative stress is associated with numerous brain disorders including Parkinson's disease,

schizophrenia and multiple sclerosis (Gotti and Clementi 2004; Quik et al. 2007; Zhao et al. 2016).

Since organochlorines are highly lipophilic and tend to accumulate in body adipose tissues and brain, they elevate levels of TAG, fatty acids, and fasting glucose. This process increases the ratios of β -oxidation of fatty acids within peroxisomes and endothelial reticulum, leading to exaggerated production of ROS and oxidative damage. Oxidative stress can affect the electron transport chain in mitochondria, leading to more ROS production and affecting energy metabolism. These phenomena elevate energy demand activating gluconeogenesis and PKC, which regulates the secretion of glucagon and insulin (Karami-Mohajeri and Abdollahi 2011). The increased energetic demand depletes carbohydrate stocks in early stages, whilst lipids and proteins stocks are used in the later phases. In mammalian models, oxidative stress caused by the organochlorines injures organs including the pancreas, brain, and liver, leading to more impairment in the metabolism of lipids, carbohydrates, and proteins. Additionally, organochlorines were shown to specifically depress metabolic activity in adipose tissue. Together these features are linked to obesity and increased risk of Type 2 Diabetes Mellitus in humans (Karami-Mohajeri and Abdollahi 2011).

Based on the evidence that insecticides can generate oxidative stress, and with considerable foresight, Farooqui (2013) predicted that chronic exposures to neonicotinoids and amine-based pesticides could impair cholinergic and octopaminergic receptors in honeybees, increasing Ca^{2+} flux in neurons. Farooqui hypothesized that this would lead to mitochondrial impairment and generation of ROS, thereby activating oxidative stress pathways, damaging neurons, and resulting in olfactory dysfunction, thus contributing for the Colony Collapse Disorder. Farooqui's hypothesis has gained limited attention and the mechanisms by which these insecticides exert their low dose effects has largely been ignored. The effect of insecticide exposure on Ca^{2+} flux into neurons, the generation of oxidative stress and its downstream effects are systematically investigated in this study.

1.8 Oxidative stress, lipids, and neurodegeneration

Neurodegeneration, defined by a progressive death of neuronal and glial cells, is often associated with mitochondrial defects, increased ROS production, the accumulation of protein aggregates, proteasome or autophagosome dysfunction, and endolysosomal defects. This process is conserved among vertebrates and invertebrates (Jaiswal et al. 2012; Liu et al. 2017; Nixon 2013). Liu et al. (2015) found that, in the *Drosophila* nervous system, mitochondrial dysfunction leading to oxidative stress promotes the accumulation of LDs in glial cells, which are further peroxidized triggering neurodegeneration. The oxidative stress activated the stress pathway c-Jun-N-terminal Kinase (JNK) and Sterol Regulatory Element Binding Protein (SREBP) in neurons, promoting the lipid droplets accumulation. These results were replicated in mice, revealing an evolutionary conserved mechanism (Liu et al. 2015).

Liu et al. (2017) showed that the mechanism responsible for the accumulation of LDs in neurons in the face of oxidative stress is dependent on several elements involved in lipid metabolism. The glial and neuronal monocarboxylate transporters (MCTs), fatty acid transport proteins (FATPs), and apolipoproteins are involved. As shown in **Figure 1.4**, MCTs shuttle lactate from glia to neurons. This is converted to pyruvate and acetyl-CoA. Under circumstances of oxidative stress and mitochondrial dysfunction acetyl-CoA is preferably used in the lipid synthesis pathway. The excess fatty acids produced are then transferred back to glia by FATP and apolipoproteins. In the glia they accumulate as LDs. Even in the presence of oxidative stress, the neuronal knockdown of the pyruvate dehydrogenase PDHA which converts pyruvate to acetyl-CoA, reduces the accumulation of LDs in glia and delays the neurodegeneration (Liu et al. 2017).

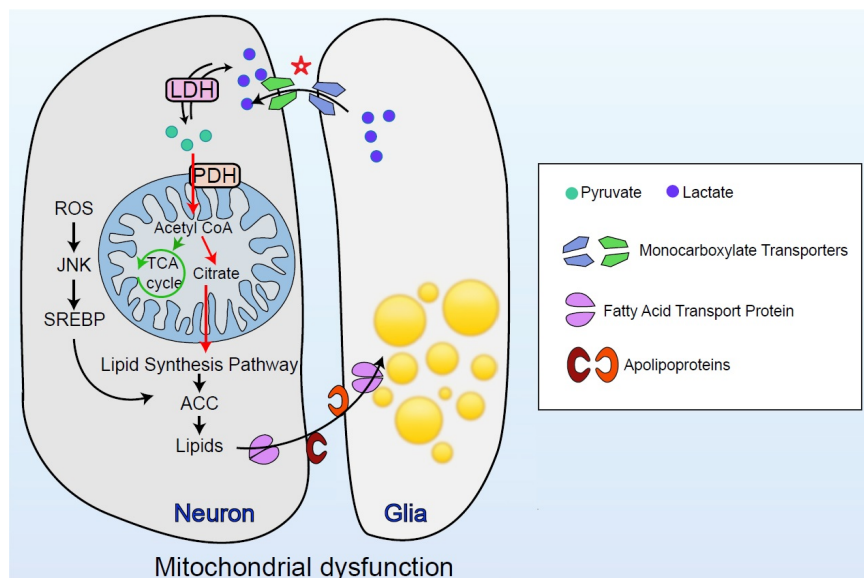


Figure 1.4. Model for the mechanism of lactate and lipid transfer and accumulation in glia and neurons (Liu et al., 2017). Lactate is transported from glial to neuronal cells by monocarboxylate transporters (MCTs). Lactate is then converted to pyruvate and acetyl-CoA. Under oxidative stress lipid synthesis is stimulated using acetyl-CoA as a substrate. Lipids are transferred back to glia by the fatty acid transporter protein (FATP) and apolipoproteins. Lipids accumulate as lipid droplets (Liu et al. 2017).

The molecular targets of insecticides such as the nAChRs are often in neuronal cells. Insecticides often lead to the impairment of lipid metabolism and the generation of oxidative stress (sections 1.5 and 1.7). So, insects exposed to chronic sublethal doses of insecticides could also face challenges such as the onset of neurodegeneration, a possibility that merits thorough investigation.

1.9 Study aims and hypothesis

Insect populations are plummeting around the world, threatening agriculture and ecosystems, given their dependence on insects for pollination and bio-geo-chemical services. Available evidence indicates that this is a multi-factorial problem. Insecticide exposure is suggested to be a major contributor, along with climate change, pathogens, and habitat loss. Insecticide research has focused on pest control, lethal doses and evolved resistances that overcome them. Around 600,000 tonnes of insecticide are used annually around the world (Müller 2018). They contaminate water and soil and thus, exposing beneficial insects to small doses. The study of collateral biological impacts of low dose insecticide exposures upon non-pest species has been limited with most interest being

devoted to behavioural studies, particularly in *Apis mellifera* (Kiljanek et al. 2016). There have been very few studies on impacts on metabolism and those that have been conducted indicate significant perturbations of metabolism that warrant detailed and systematic investigation (Brinzer et al. 2015). Most of the research on neurotoxic insecticides focuses on the brain (Perry et al. 2011), neglecting impacts outside of the nervous system in organs such as the fat body. There is real need to more fully understand the mode of action of insecticides at low dose, to define the mechanism by which low doses of insecticides may exert toxic effects at the levels of tissues and biochemical/physiological pathways.

This study systematically investigated the impacts of low dose exposures to the neurotoxic neonicotinoid insecticide imidacloprid and the spinosyn spinosad on insect biology, using *Drosophila melanogaster* as an experimental organism. Neonicotinoids account for 27% of the global insecticide market and the organic alternative, the spinosyns account for 3% (Sparks and Nauen 2015). Here, low dose exposures to imidacloprid and spinosad were shown to have pleiotropic impacts, damaging multiple organs, impairing the nervous system, the lipid environment and reducing energy metabolism. Given that the targets of these insecticides and the metabolic pathways involved are conserved among insects, exposures to low doses of these insecticides may be a threat to populations of other non-pest insect species.

Experiments involving imidacloprid and spinosad were all performed simultaneously and compared against the same controls, but these experiments are described in separate chapters. **Chapter Two** investigates the acute and chronic impacts of low doses exposures to imidacloprid, as well as its mechanism of action. **Chapter Three** investigates the acute and chronic impacts of low doses exposures to spinosad, as well as its mechanism of action. In **Chapter Four** the data for imidacloprid and spinosad are brought together for comparison. The differences and similarities between their mechanisms of action are considered. The discussion is then widened to consider the implications of this study in assessing the potential for these insecticides to inflict harm on natural populations of non-pest insects and in shaping research on the mechanisms of action of other insecticides.

Chapter Two

The impacts of low dose exposures to imidacloprid on insect biology

2.1 Introduction

Neurotoxic insecticides can create extensive perturbations in insect physiology, binding to their targets, triggering a variety of downstream responses (Perry et al. 2011). Neurotoxic insecticides kill insects by acting on primary targets in the nervous system (Casida and Durkin 2013). Imidacloprid (**Figure 2.1**) is a neonicotinoid insecticide. The term ‘neonicotinoid’ was initially proposed by Izuru Yamamoto to differentiate a new class of synthetic compounds that have activity on nAChRs from older nicotinoid insecticides, such as nicotine. Other neonicotinoids include nitenpyram, thiacloprid and acetamiprid (Thany 2010).

Neonicotinoids exhibit a high degree of selectivity towards insect nAChRs in the nervous system. Such selectivity is a result of electronegative interactions of the compounds with specific sub-sites unique to insect nAChRs (Anderson et al. 2015; Thany 2010). Studies on cockroach neurons demonstrated that imidacloprid is an nAChR agonist and it displaces α -bungarotoxin binding from central nervous system membranes (Thany 2010). In *Drosophila melanogaster*, imidacloprid targets the subunits $\alpha 1$ and $\beta 2$ of nAChRs, but loss of function mutants for these genes have only moderate resistance (Perry et al. 2008), suggesting that other nAChR subunits are targeted by imidacloprid. More recent unpublished data implicate the $\alpha 2$ and $\beta 1$ subunits (Perry et al. unpubl.). Acute exposure to high doses of imidacloprid cause uncontrolled neuronal discharges, leading to failure in propagating nervous impulses, excitotoxicity and rapid death (Anderson et al. 2015; Jeschke and Nauen 2008; Thany 2010).

Neonicotinoids are among the most widely used insecticides, accounting for 27% of the world’s insecticide market (Sparks and Nauen 2015). Worldwide declines in honeybees colonies (i.e. Colony Collapse Disorder) have prompted the scientific community to study the involvement of neonicotinoids (Brandt et al. 2016; Bryden et al. 2013; Goulson 2013; Kerr 2017; Wu-Smart and Spivak 2016).

Imidacloprid is used to control a wide range of insect pests of agriculture and horticulture and is variously sprayed (concentrations 50-100 parts per million [ppm]), applied to soil for uptake into plant tissues (350-2800 ppm) or used as seed-coating that allow the insecticide to be translocated into the tissues of plants as they develop (Bayer

2019). Imidacloprid has a greater persistence in the soil (half-life = 174 days) compared with many other insecticides (e.g. organophosphorus, carbamate, and pyrethroid insecticides) (DiBartolomeis et al. 2019). In addition, imidacloprid exhibits high water solubility. This creates a greater potential for the exposure of insects consuming pollen, nectar, plant exudates and tissues or for aquatic insects dwelling in runoff water (DiBartolomeis et al. 2019). Girolami et al. (2009) showed that leaf guttation drops on corn germinated from imidacloprid-coated seeds contained up to 200 ppm of this insecticide. Doses of imidacloprid found in pollen and nectar samples vary greatly, depending on the origin of the samples and analysis methods used, ranging from 0.7 to 39 parts per billion (ppb) (Calvo-Agudo et al. 2019).

Low doses of imidacloprid have been shown to produce oxidative stress (Balieira et al. 2018; Nareshkumar et al. 2018) and to impact behaviour and fitness in some non-pest species (Crall et al. 2018; Goñalons and Farina 2015). But there is no mechanistic understanding of how, or even if, this connects to the interaction of imidacloprid with its nAChR targets. In this Chapter the mode of action and the impacts of imidacloprid in insect biology are investigated in detail for both acute and chronic low dose exposures.

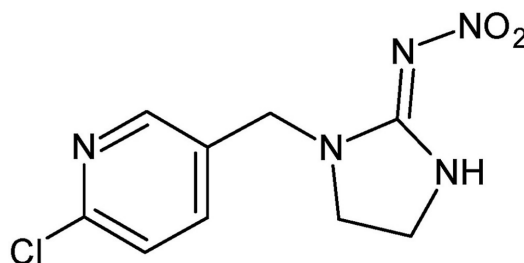


Figure 2.1. Imidacloprid chemical structure. 1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine, molar mass 255.661 g/mol.

2.2 Material & Methods

2.2.1 Fly strains and rearing

Armenia¹⁴ (Line 14), an isofemale line derived from Armenia⁶⁰ (Drosophila Genomics Resource Center #103394) (Perry et al. 2008), was used as the susceptible wild type line for all assays except the following. Expression of nAChR-D α 6 gene in larval brains: D α 6 T2A Gal4 (BDSC #76137) was crossed with UAS-GFP.nls (BDSC #4775). Insecticide impact on mitochondrial turnover: the MitoTimer line (Gottlieb and Stotland 2015) was used. Knock down of mitochondrial genes in the central nervous system: UAS ND42 RNAi (BDSC #28894) and UAS Marf RNAi (BDSC #55189) were crossed with Elav-Gal4 (BDSC #8760). As a control UAS Luciferase RNAi (BDSC #31603) was crossed with Elav-Gal4 (BDSC #8760). GCaMP experiment: UAS-tdTomato-P2A-GCaMP5G (III) (Daniels et al. 2014; Wong et al. 2014) was crossed with D α 6 T2A Gal4 (BDSC #76137). Changes in *GstD1* expression and JNK signalling in response to insecticide exposure: a transgenic reporter expressing GFP under the control of a 2.7kb *GstD1* promoter sequence (Sykietis and Bohmann 2008) were crossed to another reporter line expressing RFP (DsRed) under the control of four tandemly arranged copies of the TRE (tetradecanoylphorbol acetate response element) that provide binding sites for the *Jun/Fos* transcription factor complex (Chatterjee and Bohmann 2012) to create a homozygous double reporter line *GstD1*-GFP; TRE-DsRed. A CRISPR knockout mutant for the *GstD1* gene and its genetic background control line (Wxac - Actin-Cas9 (BDSC #54590) with w1118 X chromosome) were used for a larval motility assay (Wiggle Index assay) to test responses to acute insecticide exposure. A strain containing a deletion of the nAChR *D α 1* subunit gene (created with CRISPR in the Armenia¹⁴ background) (Perry et al. 2008) was used as a resistant line to test the effect of imidacloprid on the larval lipid environment.

For experiments involving larvae, flies were reared on standard food media (described in 2.2.3) sprinkled with dried yeast and maintained at 25°C. For experiments involving adults, flies were reared in molasses food (described in 2.2.3) and maintained at 25°C. In all experiments involving adult flies only females were used to maintain consistency.

2.2.2 Collecting third instar larvae

3 to 5 days old male (6) and female (30) adult flies were placed into vials on standard food media. The flies were left undisturbed for 24 hours for oviposition and then cleared from vials, which were maintained at 25°C for 5 additional days at which point in time third instar larvae were collected. Larvae were recovered from the food using sucrose extraction (Nichols et al. 2012). Briefly, this involved pouring 20 mL of 20% w/v sucrose solution (non-Analytical Reagent) into each vial. The top layer of food was then gently disrupted with a metal rod in order to release the larvae, which float in the sucrose solution. The solution was then carefully poured onto a fine cloth mesh to isolate the larvae and transferred onto grape juice agar plates (described in 2.2.3).

2.2.3 Food media recipes

Fly medias used were prepared as described:

<i>Standard Food (1L)</i>		<i>Grape Juice Plates (1L)</i>		<i>Molasses Food (1L)</i>	
<i>H₂O</i>	987 mL	<i>H₂O</i>	720 mL	<i>H₂O</i>	800 mL
<i>Potassium Tartrate</i>	8.0 g	<i>Agar</i>	20 g	<i>Molasses</i>	160 mL
<i>Calcium Chloride</i>	0.5 g	<i>Apple Juice</i>	200 mL	<i>Maize meal</i>	60 g
<i>Agar</i>	5.0 g	<i>Brewer's Yeast</i>	7.0 g	<i>Dried active yeast</i>	15 g
<i>yeast</i>	12 g	<i>Glucose</i>	52 g	<i>Agar</i>	6.0 g
<i>Glucose</i>	53 g	<i>Sucrose</i>	26 g	<i>Acid mix</i>	7.5 mL
<i>Sucrose</i>	27 g	<i>Tegosept</i>	6.0 mL	<i>Tegosept</i>	5.0 mL
<i>Semolina</i>	67 g				
<i>Acid Mix</i>	12 mL				
<i>Tegosept</i>	15 mL				

2.2.4 Insecticide and hydrogen peroxide dilution and exposure

Pure imidacloprid (Sigma Aldrich®) was used in all assays. The chemical was diluted with dimethyl sulfoxide (DMSO) to create a 1000 ppm stock solution which was kept on freezer (-20°C). Before exposures, 5x stocks were generated for the dose being used by diluting the 1000 ppm stock in 5% Analytical Reagent Sucrose (Chem Supply) solution. An equivalent volume of DMSO replaced the insecticide for controls. For larval acute assays, third instar larvae were exposed in 5% w/v sucrose (Chem Supply) in NUNC cell

plates (Thermo-Scientific) dosed with the desired insecticide concentration for 2 hr or 4 hr. For adult exposures, the insecticide was diluted in lukewarm food. An equivalent volume of DMSO replaced the insecticide for controls. A new batch of treated food was cooked every week and vials were replaced every second day. Adult flies chronically exposed were kept in dark at 25°C. For hydrogen peroxide (H₂O₂) exposures a 5x stock solution was diluted in 5% sucrose solution from an original 30% H₂O₂ stock solution (Chem Supply). For H₂O₂ exposures, controls were performed with larvae exposed to 5% sucrose solutions only.

2.2.5 Larvae movement assay

Larvae movement in response to insecticide exposure was quantified by Wiggle Index Assay, as described by Denecke et al. (2015). Briefly, 25 third instar larvae were used for a single biological replicate and four replicates were tested for each exposure condition. Larvae in NUNC cell plates (Thermo-Scientific) in 5% Analytical Reagent Sucrose (Chem Supply) solution were filmed for 30 seconds before insecticides exposure. After adding insecticide solution larvae were filmed again at the desired time-points. Videos are analysed in R software (v.3.4.3) using the Wiggle Index script. The motility in response to each insecticide is expressed in terms of Relative Movement Ratio (RMR), normalized to motility prior to addition of the insecticide.

2.2.6 Larvae viability and adult survival tests

For all tests 5 replicates of 20 individuals (100 individuals) per condition were used. In assessing third instar larval viability and metamorphosis following insecticide exposure, individuals were rinsed three times with 0.5% w/v sucrose (Chem Supply) and placed in vials on insecticide-free food medium. For the following 10 days the vials were scored for dead larvae, pupae, or adults. Cumulative adult emergence from larvae exposed to 2.5 ppm imidacloprid for 2 hr was analysed using Kolmogorov–Smirnov test. The corrected percentage survival of larvae exposed to 24 ppm for 4 hr or 48 ppm for 2hr was analysed using Abbots' correction. To examine the survival of adult flies chronically exposed to 4 ppm imidacloprid, 5 replicates of 20 females (3-5 days old) were exposed for 25 days. The same number of flies was used for the control group. Statistical analysis was based on the Kaplan-Meier method and data were compared by the Log-rank Mantel-Cox test.

2.2.7 Tissue dissection

Third instar larvae recovered from food media using sucrose extraction (Nichols et al. 2012) were placed in PBS buffer (Ambion) on slides. While under a stereo microscope (Zeiss, Stemi 2000-C) larvae were held down just posterior to the brain with forceps, and the head was removed with a second pair of forceps. The remaining part of the larva was held by the posterior spiracles with one pair of forceps and pushed inward slowly while the other pair slid the cuticle from the point of incision towards the spiracles until about half of the larval fat body was exposed. The posterior end was firmly held, and the other pair of forceps was used to loosely hold the cuticle. The posterior end was pulled away slowly so that the cuticle and the attached intestine slid through the gap. Using the forceps, intestine and Malpighian tubules were disconnected from fat body, which was then completely separated from other structures. For brain dissection, the head was held sideways with two pairs of forceps, which were gently pulled apart from each other to tear the cuticle. The surrounding trachea and tissues were removed from the brain using the forceps.

2.2.8 GCaMP assay

Third instar larvae were rinsed in 70% ethanol and then distilled water before dissection. Brains were quickly (< 2 min/brain) and carefully dissected in sterile Schneider's insect media (Sigma) supplemented with 10% heat inactivated FBS, Antibiotic-Antimycotic (Gibco), and 0.5 mM Ascorbic acid (Sigma). 4 brains were dissected to generate the ideal number of cells for 3 plates. Dissected brains were submitted to 15 min digestion with papain (5 U/ml) and L-cysteine (0.067 mg/ml) dissolved in HL-3 (HL-3: 70 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 20 mM MgCl₂, 10 mM NaHCO₃, 115 mM sucrose, 5 mM trehalose, and 5 mM HEPES; pH 7.2). Brains were then transferred to microtubes containing Schneider's media and submitted to three cycles of centrifugation (1 min, 100 x g), refreshing the media at every step. Brain cells were dissociated by pipetting up and down 100 times and the resulting cell suspension was divided among three culture plates (35 mm glass-bottom dishes with 10 mm bottom well (Cellvis), coated with concanavalin A (Sigma)). Cells were allowed to redevelop their neuronal morphology at room temperature for 4 days with the media refreshed daily. For GCaMP imaging, cells were kept in bath solution: HL-3: 70 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 20 mM MgCl₂, 10

mM NaHCO₃, 115 mM sucrose, 5 mM trehalose, and 5 mM HEPES; pH 7.2. Carbachol (100 μ M) was used as cholinergic agonist and thapsigargin (5 μ M) as SERCA inhibitor to release Ca²⁺ stores from the endoplasmic reticulum. Recording setup: Nikon A1 confocal microscope, 40x air objective, sequential 488nm and 561nm excitation, measurements at 3 second intervals. At least 50 neuronal cells were evaluated per treatment. Cytosolic Ca²⁺ levels were reported as GCaMP5G signal intensity divided by tdTomato signal intensity. Signal was recorded for 60 sec before the addition of 2.5 ppm imidacloprid to the bath solution. 5 min after that both imidacloprid and control groups were stimulated by carbachol (100 μ M) added to the bath solution, and finally thapsigargin (5 μ M) was added after a further 1 min. The data were analysed using a t-test.

2.2.9 Expression of $\alpha 6$ nAChRs in larval brains

The expression pattern of nAChR-D $\alpha 6$ gene in larval brains was assessed in the crossing between D $\alpha 6$ T2A Gal4 (BDSC #76137) and UAS-GFP.nls (BDSC #4775). Larval brains were dissected as described in 3.2.7 fixed in 4% paraformaldehyde (PFA-Electron Microscopy Science) in PBS for 20 minutes at room temperature. PFA was removed and tissues were washed 3 times in PBS. Samples were mounted in Vectashield (Vector Laboratories). Images were obtained with a Leica TCS SP8 (DM600 CS), software LAS X, 400x magnification, using GFP channel. Images were analysed using the software ImageJ.

2.2.10 Evaluation of mitochondrial turnover

Larvae of the MitoTimer line were exposed to 2.5 ppm imidacloprid for 2 hr. Control larvae were exposed to 2.5ppm DMSO. Midguts and brains were dissected in PBS and fixed in 4% PFA (Electron Microscopy Science) and mounted in Vectashield (Vector Laboratories). 20 proventriculus and 20 pairs of optical lobes were analysed for each condition. Confocal microscopy images were obtained in Leica SP5 Laser Scanning Confocal Microscope at 200x magnification for both green (excitation/emission 488/518 nm) and red (excitation/emission 543/572 nm) signals. Three independent measurements along the z stack were analysed for each sample. Fluorescence intensity was quantified on ImageJ software and data were analysed using a t-test.

2.2.11 Systemic mitochondrial aconitase activity

Relative mitochondrial aconitase activity was quantified using the colorimetric Aconitase Activity Assay Kit from Sigma (#MAK051), following manufacturer's instructions. A total of 6 samples (25 whole larvae per sample) were exposed to 2.5 ppm imidacloprid for 2 hr, whilst 6 control samples (25 whole larvae per sample) were exposed to DMSO for 2 hr. The final reaction absorbance was measured at 450 nm in a FLUOstar OPTIMA (BMG Labtech) microplate reader using the software OPTIMA. Sample absorbance was initially normalized to the respective sample weight and then absorbance levels of exposed animal samples were normalized to the absorbance levels for controls. The data were analysed using a t-test.

2.2.12 Systemic ATP levels

Relative ATP levels were quantified fluorometrically using an ATP assay kit (Abcam, #83355), following manufacturer instructions. A total of 6 samples (20 larvae per sample) were exposed to 2.5 ppm imidacloprid for 2 hr, whilst 6 control samples (20 larvae per sample) were exposed to DMSO for 2 hr. The final reaction fluorescence was measured at excitation/emission = 535/587 nm in FLUOstar OPTIMA (BMG Labtech) microplate reader using the software OPTIMA. Samples fluorescence was initially normalized to the respective sample weight and then fluorescence levels of exposed animal samples were normalized to absorbance levels in controls. The data were analysed using a t-test.

2.2.13 Measurement of superoxide (O_2^-) levels

To evaluate the production of reactive oxygen species (ROS) we stained tissues with dihydroethidium (DHE – Sigma-Aldrich), as described in (Owusu-Ansah et al. 2008). Briefly, larvae were dissected in Schneider's *Drosophila* Medium 1x (GIBCO) and incubated in the same medium containing 30 μ M DHE at room temperature on an orbital shaker for 7 minutes in the dark. Three washing steps of five minutes each were performed with Schneider's *Drosophila* Medium 1x. Tissues were fixed in 8% PFA (Electron Microscopy Science) for 5 minutes at room temperature on an orbital shaker in dark. Tissues were then rinsed once with PBS (Ambion) and mounted in Vectashield (Vector Laboratories). Confocal microscopy images were obtained in a Leica SP5 Laser

Scanning Confocal Microscope at 200x magnification (excitation/emission 518/605 nm). Third instar larvae were exposed to 2.5 ppm imidacloprid for 1 or 2 hr. Controls were exposed to equivalent doses of DMSO. A total of 15 brains and 15 midguts were assessed for each condition. Three independent measurements along the z stack were analysed for each sample. Fluorescence intensity was quantified on ImageJ software and data were analysed using a t-test.

2.2.14 Generating oxidative stress in brains

To test whether the generation of oxidative stress in the brain could trigger disturbances in other tissues, such as fat body and Malpighian tubules, two mitochondrial genes were independently knocked down in brains. *ND42* (NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10) encodes a subunit of complex I of the mitochondrial electron transport chain and *Marf* (mitochondrial assembly regulatory factor) encodes a GTPase that mediates outer mitochondrial membrane tethering and fusion. Knockdown of these genes in brains has been previously shown to cause the accumulation of LDs in glial cells in the retinas of adult flies (Liu et al. 2015). To knockdown these genes UAS ND42 RNAi (BDSC #28894) and UAS Marf RNAi (BDSC #55189) were independently crossed with the neuronal driver Elav-Gal4 (BDSC #8760). UAS Luciferase RNAi (BDSC #31603) was crossed with Elav-Gal4 (BDSC #8760) to provide a control. To measure impacts outside central nervous system the fat bodies and Malpighian tubules were dissected from 10 larvae per group, stained with Nile red before using confocal microscopy to visualize and count the number of lipid droplets (described in 2.2.16). Images were obtained with a Leica TCS SP8 (DM600 CS), software LAS X and analysed using ImageJ. The data were analysed using t-test.

2.2.15 Nile red staining of adult retinas

For whole mount staining of fly adult retinas, heads were dissected in cold PBS (Ambion) and fixed in 37% formaldehyde overnight. Subsequently, the retinas were dissected and rinsed several times with 1× PBS and incubated for 15 minutes at 1:1000 dilution of PBS with 1 mg/ml Nile Red (Sigma). Tissues were then rinsed with PBS and immediately mounted with Vectashield (Vector Labs) for same-day imaging. In total three sections of 625 μm^2 per retina per fly were accounted, the number of lipid droplets per section was

divided by the number of ommatidium contained in that same section. For checking the effects of mitochondrial genes knockdown in retinas (described in 2.2.14) 5 retinas from 5 adult female flies were analysed per group. For checking the effects of chronic exposures 8 retinas from 8 adult female flies were analysed per condition (imidacloprid 4 ppm and control) per day (after 1, 5, 10, 15 and 20 days of exposure). Images were obtained with a Leica TCS SP8 (DM600 CS), software LAS X, 600x magnification, and analysed using ImageJ. The data were analysed using t-test.

2.2.16 Evaluation of lipid environment of metabolic tissues in larvae

Fat bodies, midguts and Malpighian tubules were dissected in PBS (Ambion) and subjected to lipid staining with Nile Red N3013 Technical grade (Sigma-Aldrich). For insecticide exposures three biological replicates were performed for each exposure condition. Each replicate consisted of a single tissue from a single larva. A Nile Red stock solution (100 µg/mL in acetone) was prepared. A 10 µg/mL intermediate PBS dilution was used to prepare 1 µg/mL PBS use solution. Tissues were fixed in 4% paraformaldehyde (PFA-Electron Microscopy Science) in PBS for 20 minutes at room temperature. PFA was removed and tissues were washed three times in PBS. Samples were stained with 0.5 µg/mL Nile Red/PBS for 20 minutes in dark and agitation and washed five times. Slides were mounted in Vectashield (Vector Laboratories) and analysed using a Leica SP5 Laser Scanning Confocal Microscope at 400x magnification. Red emission was observed with 540 ± 12.5 nm excitation and 590 LP nm emission filters. Images were analysed using ImageJ software. For fat bodies, the number, size and percentage of area occupied by lipid droplets was measure in 5 different random sections of 2500 µm² per sample (three samples per group). For Malpighian tubules number of lipid droplets was measure in five different random sections of 900 µm² per sample (three samples per group). For midgut samples, lipid droplets were not quantified, rather zones containing lipid droplets were identified by microscopy. The data were analysed using t-test.

2.2.17 Lipid quantification in larvae hemolymph

Extracted hemolymph lipids were measured using the sulfo-phospho-vanillin method (Cheng et al. 2011). 30 third instar larvae were used for a single biological replicate and

7 replicate samples were prepared for each exposure condition. Larvae were wounded with a sterile needle and placed into a microtube with a hole at the bottom, which was placed inside another microtube and centrifuged for 5 minutes at 9.500 rpm. In total 4 μ L of hemolymph were collected for each replicate. Hemolymph was dissolved in 100 μ L of chloroform/methanol solvent (2:1 ratio). Solvent was evaporated at 95°C for 10 minutes. 100 μ L of concentrated sulfuric acid was added and then the solution was incubated for 20 minutes at 95°C. After cooling in ice for two minutes, 500 μ L of vanillin reagent (0.2% vanillin in 67% ortho-phosphoric acid) were added. The final reaction absorbance was measured at 540 nm in a CLARIOstar® (BMG LABTECH) microplate reader using MARS Data Analysis Software (version 3.10 R3). Cholesterol (Sigma-Aldrich) was used for the preparation of standard curves. The data were analysed using a t-test.

2.2.18 Antioxidant treatment

The antioxidant, N-acetylcysteine amide (NACA – AD4) was freshly prepared from powder before use by dissolving in distilled water. For acute exposure assays (Wiggle index and lipid droplet numbers in fat body) larvae were treated with 75 μ g/mL of AD4 in 5% Analytical Reagent Sucrose (Chem Supply) solution for 5 hr prior to insecticide exposure in the same NUNC cell plates (Thermo-Scientific). For chronic exposure assays (adult survival and climbing) 75 μ g/mL of AD4 was dissolved in the molasses food, cooked once a week and replaced every second day.

2.2.19 Evaluation of JNK and GstD1 activity

Larvae of the double reporter line, *GstD1*-GFP; TRE-DsRed, were exposed to 2.5 ppm imidacloprid for two hours. Controls were performed exposing larvae to 2.5ppm of DMSO. Midguts were dissected as described above and samples were fixed in 4% PFA as described above and mounted in Vectashield (Vector Laboratories). 40 proventriculus + gastric caeca + anterior midgut regions were analysed for each condition. Images were acquired in Leica M205 FA microscope with Leica DFC7000T camera, using the software Leica Application Suite X (LAS X) at 30x magnification for both green (excitation/emission 488/518 nm) and red (excitation/emission 543/572 nm) signals. GstD1 and JNK pathways activation was then assessed analysing the fluorescence intensity on ImageJ software. The data were analysed using t-test.

2.2.20 Lipid extraction for LC-MS

Third instar larvae exposed for 2 hr to 2.5 ppm imidacloprid (2.5 ppm DMSO for controls) were transferred to cryomill tubes and frozen in liquid nitrogen. Three biological replicates were prepared for each condition; each replicate composed of 10 larvae and kept at -80°C until lipid extraction. Larval weight was recorded to normalise lipid quantification. For sample extraction 200 µL of methanol containing 0.001% BHT (butylated hydroxytoluene, an organic lipophilic antioxidant) and 0.01 g/mL 13C5 Valine (IS) were added to each cryomill tube. Samples were subsequently homogenized (three × 45 seconds at 6100 rpm, with 30 seconds rest between) using a Cryomill (Bertin Technologies) at -10°C. Then 400 µL of chloroform was added to each tube and samples were incubated for 15 min at room temperature in a shaker at 1200 rpm. Samples were then centrifuged for 15 minutes, at 13000 rpm at room temperature; the supernatants were removed and transferred to new 1.5 mL microtubes. For a second wash, 100 µL of methanol (0.001% BHT and 0.01 g/mL 13C5 Valine) and 200 µL of chloroform were added to cryomill tubes, followed by vortexing and centrifuging as before. Supernatants were transferred to the previous 1.5 mL microtubes. 300 µL of 0.1 M HCl was added to pooled supernatants, microtubes were then vortexed and centrifuged (15 minutes, room temperature, 13000 rpm). Upper phases (lipid phases) were collected and transferred to clean 1.5 mL microtubes, as well as the lower phases (polar phases). Microtubes with lower phase were washed with 200 µL of chloroform, centrifuged as before and the new upper phases were transferred to clean 1.5 mL microtubes, whereas the lower phases were combined with the lower phases from previous wash. Once the final upper and lower phases were extracted and sorted in different microtubes, 50 µL aliquot from each upper phase sample were pooled together to make a PBQC (pooled biological quality control sample). The same was done to create a PBQC from lower phase samples. For each sample, 300 µL of the lower phase was transferred into glass inserts and dried down then re-suspended in 100 µL of 50% methanol:water (v/v) for LC-MS analysis. The rest of the lower fractions were transferred to HPLC vials for the LC-MS analysis. Samples from the upper phase were dried down in glass inserts for lipid analysis (speed vac at minimum vacuum of 200 bar to avoid chloroform bubbling). Upper phase samples were re-constituted in 100 µL 1:1 buthanol:methanol with 100 mM ammonium formate. All samples were kept at -20°C until analysis. For LC-MS analysis at Metabolomics Australia, microtubes were shaken for 30 minutes at 30°C, then centrifuged at 100rpm

for 10 minutes at room temperature after which the supernatants were transferred to LC vials. Extracts were used for lipid analysis as well as for peroxidized lipid analysis.

2.2.2/ Lipid analysis using LC-MS

Lipids were separated by injecting 5 μ L aliquots of lipid extract onto a 50 mm $\times$ 2.1 mm $\times$ 2.7 μ m Ascentis Express RP Amide column (Supelco, Sigma, St Louis, USA) at 35 $^{\circ}$ C using an Agilent LC 1200 (Mulgrave, Australia), and eluted at 0.2 mLmin⁻¹ over a 5 min gradient of water/ methanol/ tetrahydrofuran (50:20:30, v/v/v) to water/ methanol/ tetrahydrofuran (5:20:75, v/v/v), with the final buffer held for 3 min. Lipids were analysed by electrospray ionisation-mass spectrometry (ESI-MS) using an Agilent Triple Quad 6410 (Mulgrave, Australia). Lipid species from each class were identified using precursor ion scanning from 100 -1000 m/z, in positive ion mode, phosphatidylcholines (PC, precursors of m/z 184.1), ceramides (CER, m/z 264.6), cholesterol esters (CE, m/z 369.4), phosphatidylglycerols (PG, m/z 189) and in negative ion mode phosphatidylinositols (PI, m/z 241). Neutral loss scanning was used to identify phosphatidylethanolamines (PE, in positive ion mode, neutral loss of m/z 141) and phosphatidylserines (PS, negative ion mode, m/z 87). Diacylglycerol (DG) and Triacylglycerol (TG) species were identified according to the neutral loss of fatty acyl moiety. Identified lipid species were quantified using multiple reaction monitoring (MRM) with a 20 ms dwell time for the simultaneous measurements of ~20 to 50 compounds and the chromatographic peak width of 30 sec to 45 sec, the minimum data points collected across the peak was 12 to 16. Optimised parameters for capillary, fragmentor, and collision voltages were 4000 V, 140 - 380, and 15–60 V, respectively. In all cases, the collision gas was nitrogen at 7 Lmin⁻¹. Lipid standards (Avanti Polar Lipids, Alabaster, USA) were prepared by combining equal volumes of individual lipid stock solutions. The standard solution was then diluted to provide a set of calibration solutions ranging in concentration from 0.1 to 10 μ M. Calibration curves were constructed by least squares linear regression, fitting reverse phase peak area of the analyte against the concentration of the lipid in the reference standards. The concentration of each lipid species in the larvae extract samples was estimated by using the regression model to convert normalized peak area to lipid concentration. Detected lipid species were annotated as follows; lipid class (sum of carbon atoms in the two fatty acid chains:sum of double bonds in the fatty acid chains). The LC/MS ESI-MRM data was processed using

Agilent MassHunter quantitative software (version 6) (Mulgrave, Australia). For statistical analysis the concentration of lipid compounds was initially normalized to sample weight. Principal Components Analysis (PCA) was calculated to verify the contribution of each lipid compound in the variance of each treatment. PCA was calculated using the first two principal component axes. To discriminate the impacts of imidacloprid on the accumulation of specific lipid compounds we performed a One-way ANOVA test with post-hoc Tukey's HSD ($p < 0.05$).

2.2.22 RNA isolation and transcriptomics analysis

Third instar larvae exposed to 2.5 ppm imidacloprid for 2 hr were submitted to a tissue specific transcriptomic analysis. Brains and fat bodies were dissected and independently used for RNA isolation. In total 3 biological samples were prepared per tissue per treatment. 40 larvae were dissected for each biological sample. Samples were individually stored at -80°C in 800 μL of TRIsure (Bioline). RNA was extracted using 0.2 volumes of chloroform and then precipitated using 1 volume of isopropanol, following manufacturer's instructions. The RNA pellet was washed with 75% ethanol before being resuspended in RNase-free water (Sigma). To remove DNA traces, RNA was treated with RQ1 RNase-free DNase (Promega) and the reaction was stopped using RQ1 Stop solution (Promega). Sample quality was tested by running a 1% agarose gel electrophoresis. RNA purity and concentration were evaluated by spectrophotometry (NanoDrop® ND-1000, NanoDrop Technologies). All samples presented at least 2 μg of total RNA, showed no signs of degradation and OD 260/280 ratio ≥ 1.9 . Samples were then diluted in 20 μL of nuclease-free water and transferred to GenTegra® tubes, where RNA was further dried using SpeedVac. Library preparation (250-300bp cDNA insert) and Illumina sequencing (150bp paired end) were carried out by Novogene Bioinformatics Technology in Singapore. For analysis, reads were aligned to the *Drosophila* genome and exons using Tophat2. Reads representing gene transcripts were counted using HTSeq, and transcript per kilobase per million (TPM) was calculated for gene expression levels. Differential expression analysis was performed using DESeq2 and for significant DEGs, adjusted p -value was set to $p < 0.05$.

2.2.23 Gene Ontology and KEGG pathway analysis

Novogene provided preliminary Gene Ontology (GO) and KEGG pathway analysis considering all genes significantly affected by insecticide treatment. But alternatively, the list of differentially expressed genes (DEGs) provided by Novogene was also used to perform a more personalized GO and KEGG analysis using the software DAVID (Bioinformatics Resources 6.8). In this case, only DEGs presenting foldchange $>|2|$ and $P_{adj} < 0.05$ were used for that purpose. The GO and KEGG terms generated were selected based on the criteria of presenting the Modified Fisher Exact P -value < 0.01 .

2.2.24 Electrophysiology of the retina

In preparation for the electroretinogram (ERG) adult flies were anesthetized and glued to a glass slide. A reference electrode was inserted in the back of the fly head and the recording electrode was placed on the corneal surface of the eye, both electrodes were filled with 100 mM NaCl. Flies were maintained in the darkness for at least 5 min prior to a series of 1 s flashes of white light delivered using a halogen lamp. During screening 8 to 10 flies per treatment group were tested. For a given fly, amplitude and ON transient measurements were averaged based on the response to the 3 light flashes. Responses were recorded and analysed using AxoScope 8.1. The data were analysed using t-test.

2.2.25 Adult brain histology (Hematoxylin & Eosin staining)

Adult fly heads were fixed in 8% glutaraldehyde (EM grade) and embedded in paraffin. Sections (10 μ m) were prepared by a microtome (Leica) and stained with Hematoxylin and Eosin as described (Chouhan et al. 2016). At least three animals were examined for each group (20 days exposure to 4 ppm imidacloprid plus control group). The data were analysed using t-test.

2.2.26 Transmission Electron Microscopy (TEM)

Both retinas and laminae of adult flies chronically exposed for 20 days were processed for TEM imaging as described (Luo et al. 2017). Samples were processed using a Ted Pella Bio Wave microwave oven with vacuum attachment. Adult fly heads were dissected at 25 °C in 4 % paraformaldehyde, 2 % glutaraldehyde, and 0.1 M sodium cacodylate

(pH 7.2). Samples were subsequently fixed at 4 °C for 48 hr. 1 % osmium tetroxide was used for secondary fixation with subsequent dehydration in ethanol and propylene oxide. Samples were then embedded in Embed-812 resin (Electron Microscopy Science, Hatfield, PA). 50 nm ultra-thin sections were obtained with a *Leica UC7* microtome and collected on Formvar-coated copper grids (Electron Microscopy Science, Hatfield, PA). Specimens were stained with 1 % uranyl acetate and 2.5 % lead citrate and imaged using a JEOL JEM 1010 transmission electron microscope with an AMT XR-16 mid-mount 16 mega-pixel CCD camera. For quantification of ultrastructural features, electron micrographs were examined from 3 different animals per treatment. The data were analysed using t-test.

2.2.27 Bang Sensitivity

Bang sensitivity phenotype was tested after 1, 10 and 20 days of chronic exposure to 4 ppm imidacloprid. 5 adult female flies were placed into a clean vial and allowed to rest for 30 min (in total, n = 100 flies/treatment). They were then vortexed on a VWR vortex at maximum strength for 10 s. The time required for flies to flip over and regain normal standing posture was then recorded. The data were analysed using the non-parametric Wilcoxon test.

2.2.28 Climbing assay

Climbing phenotype was tested after 1, 10 and 20 days of exposure to 4 ppm imidacloprid. 5 adult female flies were placed into a clean vial and allowed to rest for 30 min (in total, n = 100 flies/treatment). Vials were tapped against a pad and the time required for the flies to climb up to a pre-determined height (7 cm) was recorded. Flies that did not climb the pre-determined height within 30 seconds failed the test. The data were analysed using the non-parametric Wilcoxon test.

2.2.29 Graphs and Statistical analysis

All graphs were created, and all statistical analysis were performed in the software R (v.3.4.3). Figures and schematics were designed using the free image software Inkscape (0.92.4).

2.3 Results & Discussion

2.3.1 Low imidacloprid doses reduce larvae motility without affecting survival

As a starting point to study the systemic effects of sublethal insecticide exposure a dose of imidacloprid that could reduce the movement of third instar larvae by 50% upon a 2 hr exposure was determined. This parameter was chosen based on the expectation that such a dose would be affecting the nervous system (evidenced by the reduction of movement), but would be unlikely to cause mortality during, or immediately after, this exposure period. Larvae exposed in this way would provide excellent material for a search for both impacts on the brain and downstream on metabolism. Unless otherwise stated, the fly strain Line 14 (*Drosophila* Genomics Resource Center #103394) was used for all experiments. The larval movement assay, known as Wiggle Index (Denecke et al. 2015), which consists of filming and quantifying larvae movement over time, was used to identify the desired acute low dose of imidacloprid. Doses of 6 ppm and 3 ppm were initially tested (**Figure 2.2a**) but caused more than a 50% reduction in movement. The dose of 2.5 ppm matching the selection criteria (**Figure 2.2b**) was used in the subsequent experiments with 3rd instar larvae. Even though this dose causes close to 50% reduction in movement after only 30 min of exposure, it causes no significant impact on total adult emergence (**Figure 2.2c**) when larvae are rinsed in 5% sucrose solution, placed back onto insecticide-free media and allowed to develop. It causes however, a developmental delay.

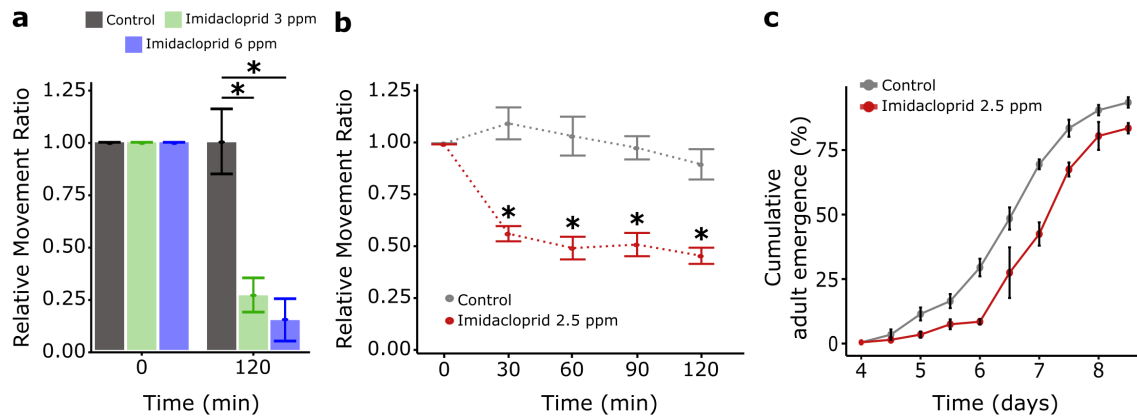


Figure 2.2. Toxicology and viability assays with larvae exposed to different doses of imidacloprid for 2 hr. **a**, Dose response to 3 ppm and 6 ppm of imidacloprid by an assay of larval movement, the Wiggle Index. Values are expressed in terms of Relative Movement Ratio (RMR) in response to a 120 min exposure ($n = 25$ flies/ replicate; 4 replicates/ treatment). **b**, Dose response (Wiggle Index) to 2.5 ppm imidacloprid ($n = 25$ flies/ replicate; 4 replicates/ treatment). **c**, Cumulative adult emergence (%) of larvae exposed to 2.5 ppm imidacloprid for 2 hr ($n = 100$ larvae/ treatment). Error bars in **a**, **b** and **c** represent standard error (t-test; * $P < 0.01$). No significant difference was found in **c** (Kolmogorov–Smirnov test; $P > 0.05$).

2.3.2 Low dose of imidacloprid elicits a low but enduring Ca^{2+} flux into nAChR neurons

Imidacloprid is a nAChR agonist, binding with high affinity to nAChRs causing channel opening and cationic flux (Anderson et al. 2015; Thany 2010). The investigation of Ca^{2+} influx is of particularly interest because excess intracellular Ca^{2+} leads to oxidative stress by a variety of mechanisms (Brookes et al. 2004), which could precipitate a cascade of damage. In order to quantify changes in Ca^{2+} influx caused by 2.5 ppm imidacloprid dose a GCaMP assay was performed. GCaMP is an engineered protein which fluoresces green when Ca^{2+} binds to it (Daniels et al. 2014; Wong et al. 2014). It therefore allows the opening of nAChR channels and Ca^{2+} influx into neurons to be measured. The UAS GCaMP fly strain used constitutively expresses the tdTomato protein, which was used to normalize GCaMP fluorescence. This strain was crossed with the D α 6 T2A Gal4 driver strain. Even though it is not the target of imidacloprid, the D α 6 subunit is expressed in the majority of cells in the adult brain (66%) (Croset et al. 2018) and in the larval brain (**Figure 2.3**). A high percentage of cells expressing D α 6 subunits also co-express at least one of the target subunits of imidacloprid, since D α 1 (58%), D α 2

(26%), D β 1 (83%) and D β 2 (35%) are also highly expressed in brain cells (Croset et al. 2018).

GCaMP fluorescence was measured in cultured cells obtained from larval brains. The data generated indicated a small, but sustained, Ca²⁺ influx which lasted for 5 minutes. This may have continued, but was interrupted by the addition of an acetylcholine analogue, carbachol (**Figure 2.4a, b**). The enduring influx is consistent with the continuous binding of imidacloprid, which unlike the natural ligand acetylcholine, is not hydrolysed (Anderson et al. 2015; Buckingham et al. 1997). A 2.2-fold decrease in cholinergic response, after carbachol exposure, in cells treated with insecticide is thus, explained by reduction in the number of sites on nAChRs free to bind this drug. Finally, cells were also exposed to thapsigargin, a drug that causes Ca²⁺ release from the endoplasmic reticulum. The purpose of this was to test whether the neuronal cells remained alive throughout the assay. The GCaMP results observed in response to thapsigargin indicated a normal physiological response from both control and imidacloprid-treated cells (**Figure 2.4a, b**).

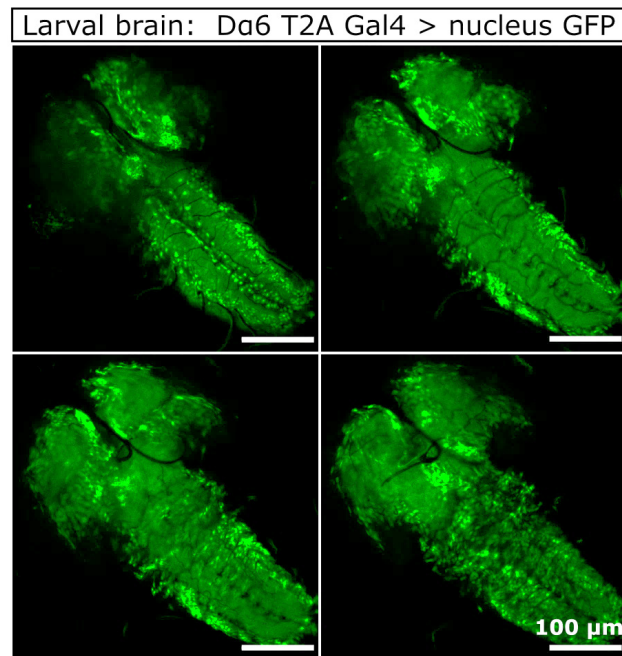


Figure 2.3. nAChR subunit D α 6 expression in *Drosophila* larval brain. D α 6 T2A Gal4 crossed with UAS nuclear GFP, showing expression pattern of nAChR subunit D α 6 in the 3rd instar larval brain. Different cross sections through anterior-posterior axis are displayed (400x magnification). Leica SP8 Laser Scanning Confocal Microscope.

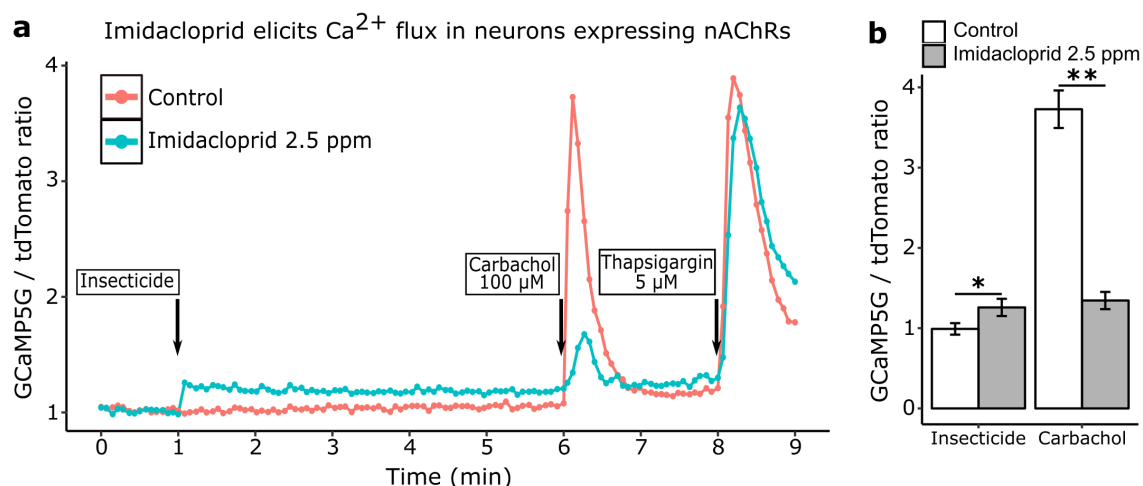


Figure 2.4. Imidacloprid elicits Ca^{2+} flux into neurons and impairs cholinergic response. **a**, Ca^{2+} flux measured by GCaMP in neuronal cells expressing nAChRs. Measurement is expressed in terms of the GCaMP5G signal divided by the tdTomato signal (constitutive expression). After 1 min the imidacloprid group was exposed to 2.5 ppm of this insecticide. At 6 min and 8 min imidacloprid and control groups were exposed to 100 μM carbachol and 5 μM thapsigargin, respectively. Each point represents the average of at least 50 cells. **b**, Ca^{2+} influx peak responses to imidacloprid and carbachol. Error bars represent mean $\pm$ s.e.m. (t-test; * $P < 0.05$; ** $P < 0.01$).

2.3.3 Low exposures impact mitochondria and give rise to oxidative stress

Following the observation of increased Ca^{2+} flux in nAChR expressing cells exposed to imidacloprid, experiments were conducted to detect oxidative stress. To assess the levels of ROS in acutely exposed larvae, the accumulation of superoxide anion (O_2^-) in brains and midguts after 1 hr and 2 hr exposure was measured. Dihydroethidium (DHE) staining showed an average signal increase in the brain of 45% and 47% after 1 hr and 2 hr exposures, respectively (**Figure 2.5a, b**). No increase in DHE signal was measured in anterior midgut after 1 hr, but a 59% increase was measured after 2 hr (**Figure 2.5a, b**). These data showed that oxidative stress is initially generated in the brain. This could be explained by the persistent Ca^{2+} flux into neurons expressing nAChRs induced by imidacloprid. However, damage is not confined to the brain, spreading to other organs within 2 hr.

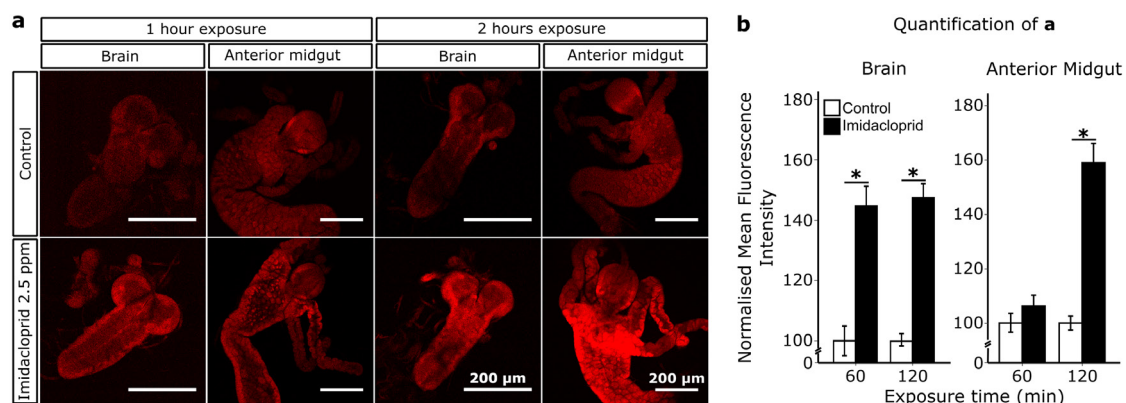


Figure 2.5. Imidacloprid induces the accumulation of ROS in brains and midguts. a, Superoxide levels in brain and anterior midgut exposed to 2.5 ppm imidacloprid for either 1 hr or 2 hr. Images stained with DHE and obtained in Leica SP5 Laser Scanning Confocal Microscope, 200x magnification. **b,** Quantification of **a**, normalized mean fluorescence intensity (n = 15 larvae/ treatment; 3 image sections/ larva). Error bars represent mean $\pm$ s.e.m. (t-test; *P < 0.01).

Mitochondrial defects arise as a result of oxidative stress, as perturbations in the electron transport chain leading to electron leakage are the main source for ROS production in eukaryotic cells (Magder 2006). Superoxide is the primary ROS produced by mitochondria (Magder 2006), hence its accumulation in the brain and midgut points to possible mitochondrial impairment. Therefore, mitochondria turnover was assessed using the reporter line MitoTimer. MitoTimer is an engineered protein, which shifts fluorescence signal from red to green as mitochondria age (Gottlieb and Stotland 2015). While MitoTimer green signal is determined principally by MitoTimer synthesis, mitochondrial biogenesis, or protein import, the red signal is mainly affected by degradative pathways such as autophagy, mitochondrial proteases, or the ubiquitin–proteasome system (Ferree et al. 2013).

A 2hr exposure to 2.5 ppm imidacloprid led to an increase of 22% and 20% for the green and red signals in the optical lobes of larval brains (**Figure 2.6a, b**). An 11% increase was observed for both red and green signals in a localized region of the alimentary canal, the proventriculus (**Figure 2.6a, b**). That both the red and green signal increase is suggesting of increases in levels of both mitochondrial biogenesis and impairment. Under situations of oxidative stress in highly energy demanding cells, such as cardiac and striated muscle, ROS promote mitochondrial biogenesis (Chang et al. 2010), a mechanism that can compensate for the damaged mitochondria and thus,

maintain ATP production. Here both the observed mitochondrial biogenesis and impairment may be induced by the oxidative stress caused by imidacloprid. This hypothesis can account for the MitoTimer observations in the brain, where imidacloprid binds to nAChRs, increasing Ca^{2+} flux into neurons which is known to elevate levels of ROS, oxidative stress, and mitochondrial damage. But what of the increases in the proventriculus? One possible explanation is that ROS generated in the brain escape from this organ, reaching neighbouring tissues.

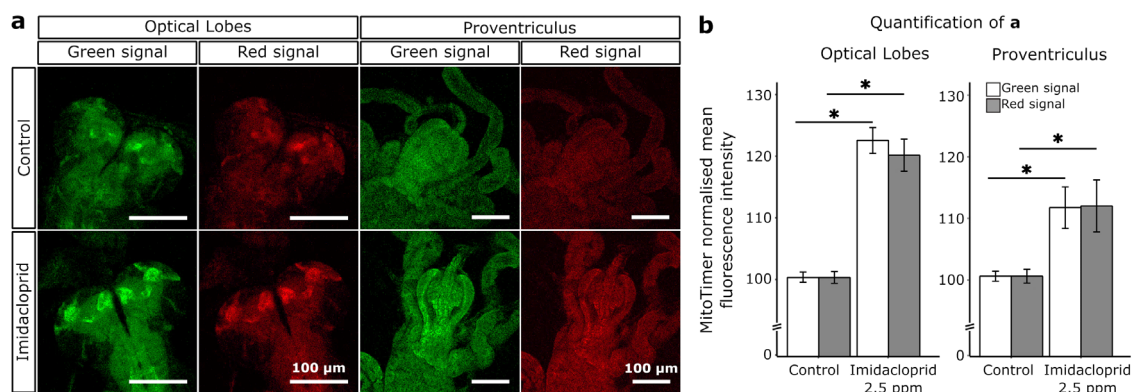


Figure 2.6. Mitochondrial turnover is increased in larvae exposed to 2.5 ppm imidacloprid for 2 hr. MitoTimer reporter fly strain. **a**, Optical lobes in the larval brain and proventriculus in the larval alimentary canal. Insecticide exposure increased the signal of healthy (green) and unhealthy (red) mitochondria (n = 20 larvae/ treatment; 3 image sections/ larva). Images obtained in Leica SP5 Laser Scanning Confocal Microscope, 200x magnification. **b**, Quantification of **a** in terms of normalized mean fluorescence intensity. Error bars indicate standard error (t-test; *P < 0.01).

In assessing the level of oxidative stress, two mitochondrial phenotypes were also further investigated in larvae treated with the same exposure conditions: mitochondrial aconitase activity and ATP levels. Mitochondrial aconitase is a ROS sensitive enzyme, which catalyses the stereo-specific isomerization of citrate to isocitrate; the activity of this enzyme is inversely proportional to oxidative stress levels (Yan et al. 1997). Exposed larvae had a 32% mean reduction in systemic levels of mitochondrial aconitase activity (**Figure 2.7a**). A 21% mean reduction in systemic ATP levels was also observed in exposed larvae (**Figure 2.7b**). These data confirm that low doses of imidacloprid can quickly increase oxidative stress levels, impairing mitochondrial activity and leading to a significant reduction of energy levels.

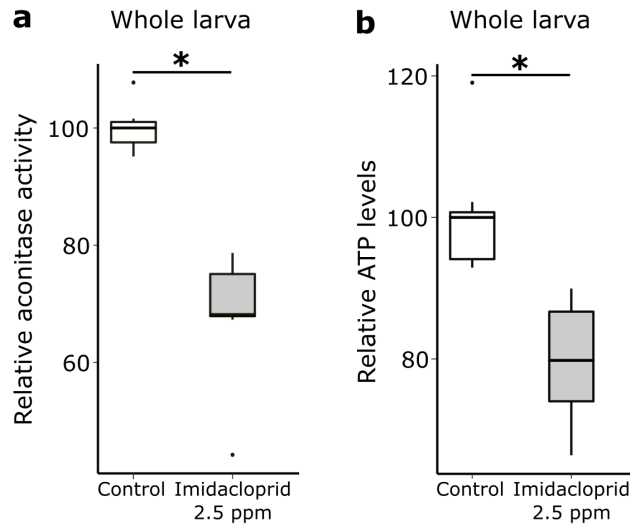


Figure 2.7. Imidacloprid reduces systemic levels of mitochondrial aconitase activity and ATP. Larvae were exposed to 2.5 ppm imidacloprid for 2 hr. **a**, Relative mitochondrial aconitase activity (n = 25 larvae/ replicate; 6 replicates/ treatment). **b**, Relative ATP levels (n = 20 larvae/ replicate; 6 replicates/ treatment). (t-test; *P < 0.01).

2.3.4 Oxidative stress generated in the brain precipitates changes in the lipid environment of metabolic tissues

Oxidative stress has been shown to induce the accumulation of lipid droplets (LDs) in glial cells in *Drosophila* (Liu et al. 2015, 2017), as well in other tissues and models (Bailey et al. 2015). Here ROS was detected in the brain prior to other tissues, raising the question of whether ROS generated in the brain could impact LD dynamics outside the central nervous system. The *elav* neuronal driver was used to knockdown the expression of two genes encoding proteins required for mitochondrial electron transport chain, *ND42* (NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10) and *Marf* (mitochondrial assembly regulatory factor) using RNAi. *Marf* encodes a GTPase that mediates outer mitochondrial membrane tethering and fusion. Its loss causes mitochondrial fragmentation and endoplasmic reticular stress. *ND42* encodes a subunit of complex I of the mitochondrial electron transport chain. Knockdown of these genes has been previously shown to cause the accumulation of LDs in glial cells in the retina of adult flies (Liu et al. 2015) and, in the case of *Marf*, also reduce LD numbers in ring glands (Sandoval et al. 2014). Thus, as an internal control, accumulation of LDs in retinas was the first phenotype checked. As expected, *ND42* knockdown in neurons caused a

significant accumulation of LDs in glial cells of one day old adult flies. Knockdown of *Marf*, under the conditions tested, was pupal lethal (**Fig 2.8a, b**).

While the knockdown of these genes was limited to neurons in the larval brain, significant changes in LD numbers were observed in the Malpighian tubules and fat body. Malpighian tubules have a functional overlap with vertebrate kidneys and play an extensive role in metabolizing and excreting insecticides (Harrop et al. 2014). The portion of Malpighian tubules analysed was the main segment where LDs are localized. This segment is responsible for fluid secretion (O'Donnell et al. 1996). As expected, Malpighian tubules LDs showed a more homogeneous and smaller size and were much reduced in number when compared to LDs in fat bodies. Therefore LDs numbers were counted in Malpighian tubules, whereas in fat bodies three different measures were used - the total area covered by LDs as well as the number of LDs in two different size ranges: small LDs (ranging from 1.5 μm to 10 μm) and large LDs (ranging from 10 μm to 20 μm). The number of LDs in Malpighian tubules was significantly reduced by *Marf* or *ND42* knockdown. The opposite effect was observed in fat bodies, which showed an increase in the area occupied by LDs (**Figure 2.8a, c and d**). The two independent knockdowns also caused increased number of small LDs, and reduced number of large LDs in fat bodies (**Figure 2.8e, f**). This result suggests that oxidative stress causes an accumulation of LDs of altered size in fat bodies and could be splitting large LDs into smaller ones. Such response could act to improve the protection against ROS, since smaller surfaces are physically less exposed than larger ones. Most importantly, these data demonstrate the ability of oxidative stress generated in the brain to cross the boundaries of this organ, affecting the lipid environment of metabolic tissues. ROS are typically unstable, but ROS can react with lipids to produce peroxidised lipids that are more stable and able to inflict damage to several cell components (Magder 2006; Valko et al. 2007). Peroxidized lipid species could be transported through hemolymph and trigger oxidative stress in other tissues. The small body size as well as the proximity of organs in larvae also must be considered here. The larval brain is closely associated with the proventriculus and the fat body.

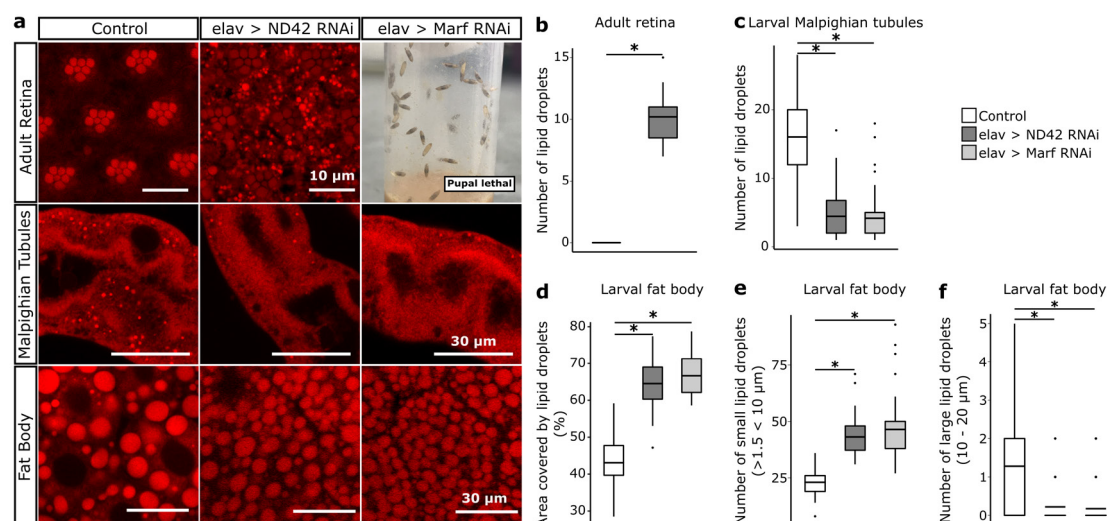


Figure 2.8. Inducing ROS in the brain impacts the lipid environment of metabolic tissues. **a**, Knockdown of mitochondrial components ND42 and Marf in neurons caused the accumulation of LDs in retinas of one day old female flies (Marf knockdown is pupal lethal), accumulation of LDs in larval fat bodies and reduction of LDs in larval Malpighian tubules. Tissues stained with Nile red and images obtained in Leica SP8 Laser Scanning Confocal Microscope, 400 x magnification for larval tissues and 600 x magnification for adult retinas. **b**, Number of LDs per ommatidium in adult retinas (n = 5 adults/ group; 5 image sections/ adult). **c**, Number of LDs in larval Malpighian tubules (sections of 30 μ m x 30 μ m). **d**, Percentage of area covered by LDs in larval fat bodies (sections of 50 μ m x 50 μ m). **e**, Number of small LDs (> 1.5 μ m < 10 μ m) in larval fat bodies (sections of 50 μ m x 50 μ m). **f**, Number of large LDs (10 μ m - 20 μ m) in larval fat bodies (sections of 50 μ m x 50 μ m). For **c**, **d**, **e**, and **f** (n = 10 larvae/ group; 5 image sections/ larva). (t-test; *P < 0.01).

2.3.5 Oxidative stress generate by imidacloprid impacts the metabolic tissues and larvae motility

Having used genetic tools to show that the generation of elevated levels of ROS in neurons precipitated changes in metabolic tissues, imidacloprid was tested to determine whether it could create a similar phenotype. Larvae exposed to 2.5 ppm imidacloprid for 2 hr showed a significant 19% mean increase in the area occupied by LDs in fat body (**Figure 2.9a, b**). Recapitulating the results with RNAi in the brain, imidacloprid caused a significant increase in the number of small LDs (27% on average) and reduction in the number of large LDs (30% on average) in fat bodies (**Figure 2.9c, d**). Taken together these experiments suggest that oxidative stress generated in the larval brain by imidacloprid exposure may cause oxidative stress in other tissues. To test if the LD phenotype was indeed a response to the oxidative stress generated by insecticide exposure a potent antioxidant, N-acetylcysteine Amide (AD4), was used (Schimel et al. 2011).

AD4 is a non-toxic glutathione precursor and therefore acts to boost levels of glutathione (Massey and Racz 1981). Larvae pre-treated with AD4 for a period of five hours prior to insecticide exposure showed a control-like phenotype (**Figure 2.9a-d**), not displaying impacts on LD numbers. The pre-treatment with AD4 also improved the movement of larvae exposed to imidacloprid. Pre-treated larvae showed a mean 27% reduction in motility, compared to the 50% reduction of non-pre-treated animals (**Figure 2.10**). These data support the hypothesis that oxidative stress induced by imidacloprid is the cause of the observed impacts on the larval central nervous system and the lipid environment.

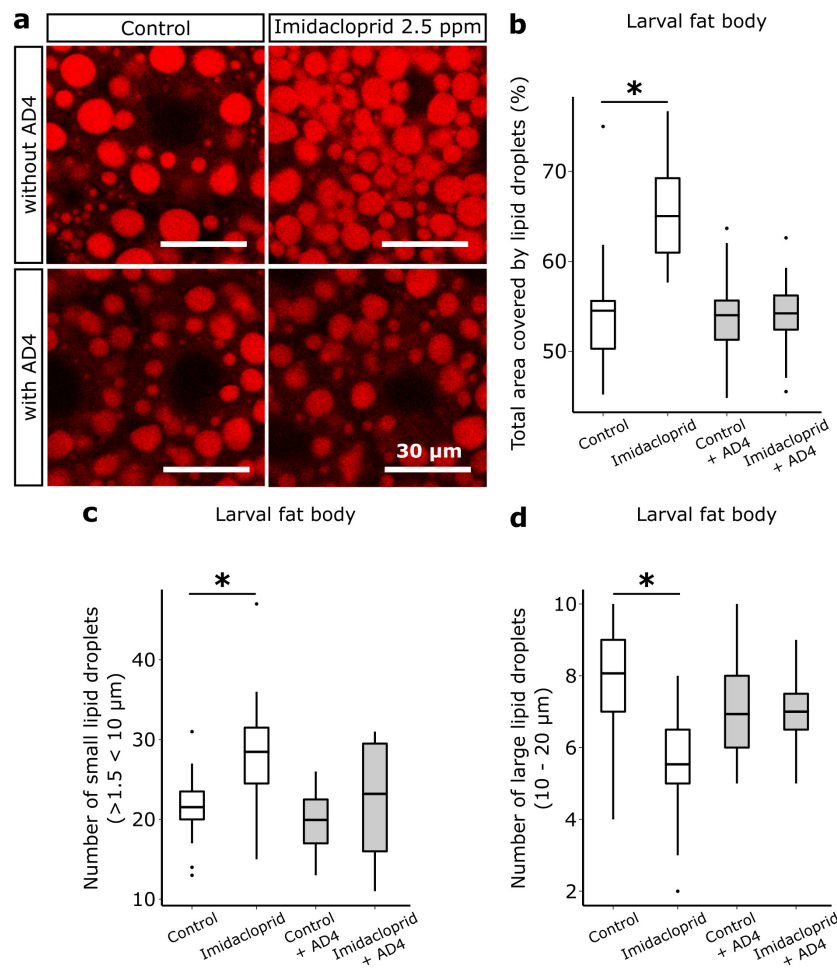


Figure 2.9. Imidacloprid increases lipid storages in fat body and antioxidant treatment reduces this accumulation. **a**, Larvae treated with 75 μ g/mL of antioxidant N-acetylcysteine amide (AD4) for 5 hrs prior to a 2 hrs exposure to 2.5 ppm of imidacloprid show no significant changes in the area occupied by LD in fat body. Nile red staining. Images obtained in Leica SP5 Laser Scanning Confocal Microscope, 400x magnification (n = 3 larvae/ treatment; 5 image sections/ larva). **b**, Percentage of area occupied by LD in fat body (sections of 50 μ m x 50 μ m). **c**, Number of small LD (> 1.5 μ m < 10 μ m) in larval fat bodies (sections of 50 μ m x 50 μ m). **d**, Number of large LD (10 μ m - 20 μ m) in larval fat bodies (sections of 50 μ m x 50 μ m). (t-test; *P < 0.05).

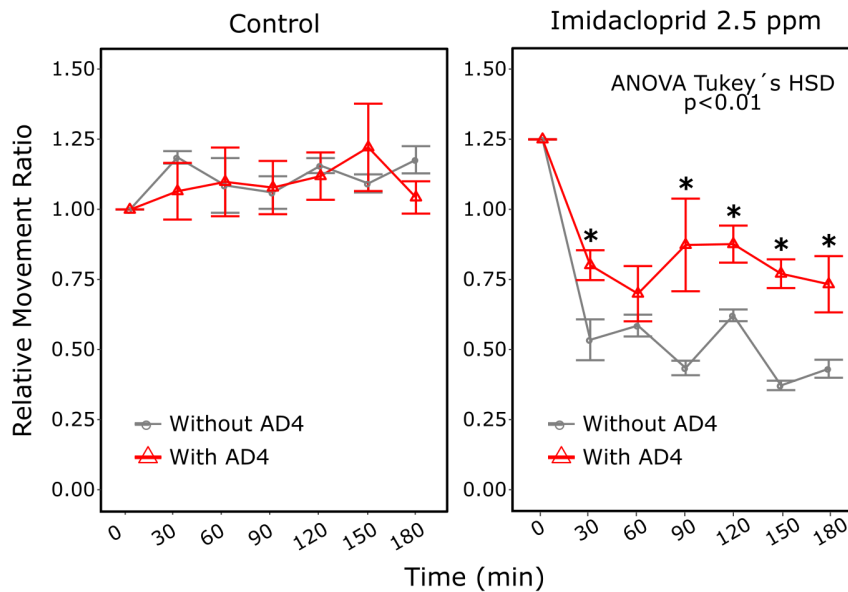


Figure 2.3. Antioxidant treatment improves the movement of imidacloprid exposed larvae. Larvae treated with 75 $\mu\text{g/mL}$ of antioxidant N-acetylcysteine amide (AD4) for 5 hrs prior to the exposure to 2.5 ppm of imidacloprid. Dose response to insecticide by Wiggle Index analysis. Results are expressed in terms of Relative Movement Ratio (RMR) values in response to the exposure time in minutes ($n = 25$ flies/ replicate; 4 replicates/ treatment). All points correspond to the mean RMR values with standard error (t-test; * $P < 0.01$).

Higher doses of imidacloprid were tested to see whether this would lead to a greater accumulation of LDs in fat bodies of exposed larvae. To select the doses to test, survival assays were performed to find the highest acute doses of imidacloprid that do not significantly impact adult emergence. Third instar larvae were exposed to different doses for a 2 hr or 4 hr period, rinsed and placed back onto insecticide-free media and allowed to develop to adulthood. Among the doses tested 24 ppm for 4 hr and 48 ppm for 2 hr were selected for matching the criteria established (**Figure 2.11a, b**). These doses caused an average 23% and 22% increase in the area occupied by LDs, respectively (**Figure 2.12a, b**). Besides that, the characteristic increase in the number of small LDs and reduction in the number of large LDs was once again observed (**Figure 2.12c, d**).

The decrease in LD numbers in Malpighian Tubules observed with the mitochondrial gene knockdown in brains was also observed after exposure to the higher doses of imidacloprid. Both doses of 24 ppm for 4 hr and 48 ppm for 2 hr caused an approximately 90 % average reduction in the total number of LDs (**Figure 2.13a, b**). A

similar impact in the midgut was also observed with these doses. The midgut portion analysed was the alkaline zone of posterior midgut, where most of insect digestion takes place (Terra and Ferreira 1994). Unlike fat bodies and Malpighian tubules, LDs were not homogeneously distributed along the length of the midguts. However, in all control replicates performed it was possible to identify zones of LDs accumulation. Such areas were not present or were smaller in the midgut of larvae exposed to imidacloprid (**Figure 2.13c**). Similarities between these phenotypes and the ones caused by the knockdown of mitochondrial genes in neurons, reinforce the view that the impact of imidacloprid in metabolic tissues is likely to be driven by ROS generated in the brain.

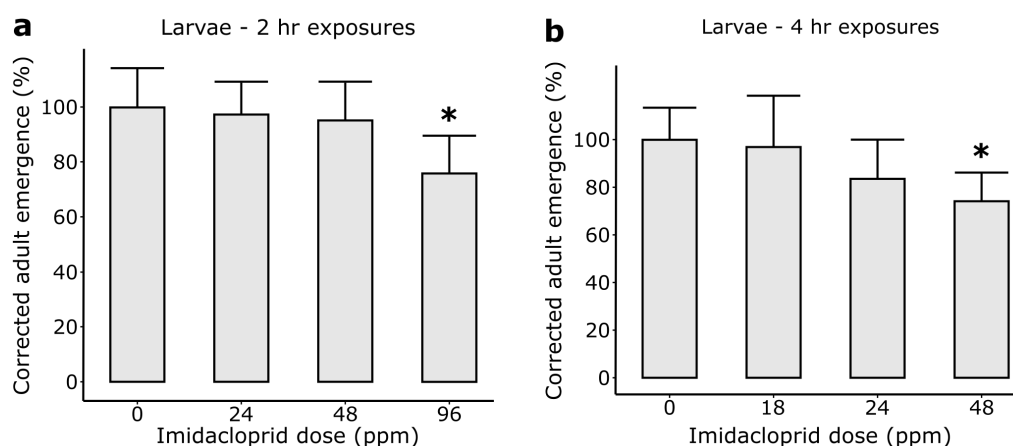


Figure 2.4. Impacts on adult emergence after larval exposure to imidacloprid. Exposed larvae were rinsed in 5% sucrose and placed back in insecticide-free media for quantification of adult emergence. **a**, Larvae exposed to 24, 48 or 96 ppm of imidacloprid for 2 hrs. Exposure to 48 ppm of imidacloprid for 2 hrs was selected as the highest dose that does not interfere with adult emergence. **b**, Larvae exposed to 18, 24 or 48 ppm of imidacloprid for 4 hrs. Exposure to 24 ppm of imidacloprid for 4 hrs was selected as the highest dose that does not interfere with adult emergence. Grey bars indicate corrected percentage survival (Abbott's correction) (n = 60 animals/ treatment). Error bars indicate 95% confidence interval (One-way ANOVA, Turkey's HSD; *P < 0.05).

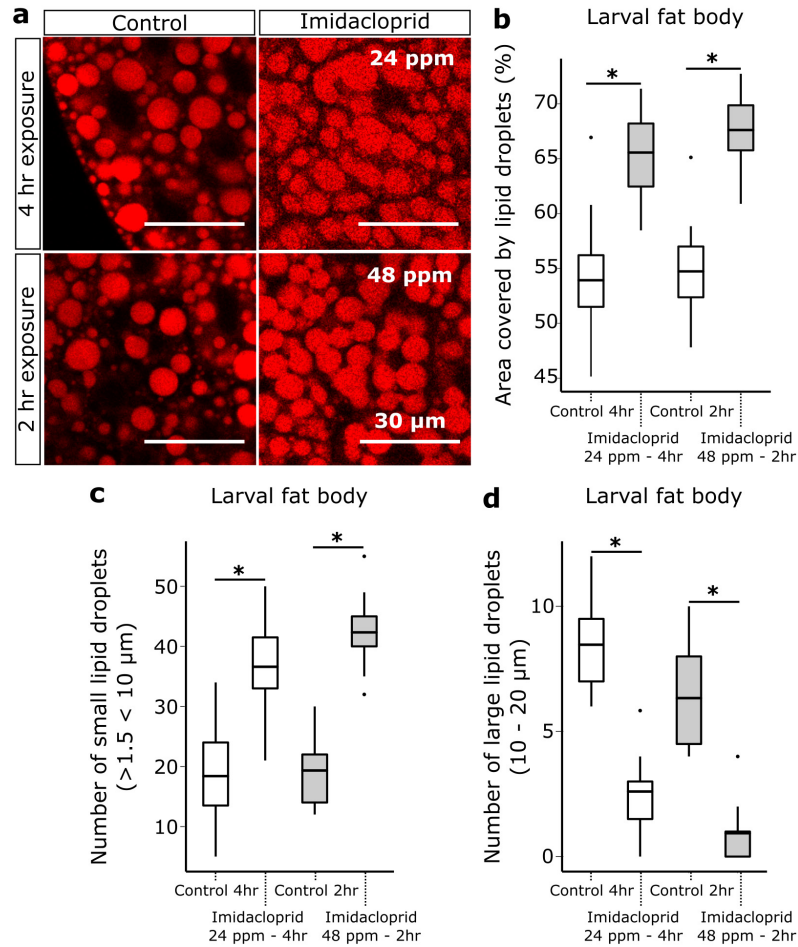


Figure 2.5. Higher doses of imidacloprid cause greater accumulation of lipid storage in fat body. **a**, Larvae exposed to either 24 ppm for 4 hr or 48 ppm for 2 hr. Nile red staining. Images obtained in Leica SP5 Laser Scanning Confocal Microscope, 400x magnification (n = 3 larvae/ treatment; 5 image sections/ larva). **b**, Percentage of area occupied by LD in fat body (sections of 50 μm x 50 μm). **c**, Number of small LD (> 1.5 μm < 10 μm) in larval fat bodies (sections of 50 μm x 50 μm). **d**, Number of large LD (10 μm - 20 μm) in larval fat bodies (sections of 50 μm x 50 μm). (t-test; *P < 0.01).

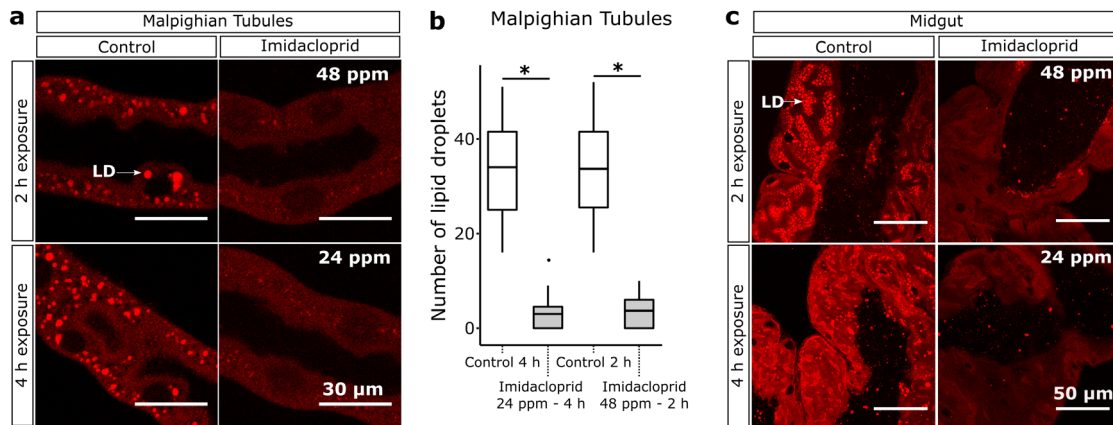


Figure 2.6. Imidacloprid reduces lipid stocks in Malpighian tubules and midguts. Larvae exposed to either 48 ppm of imidacloprid for 2 hr, or 24 ppm for 4 hr. Nile red staining. Images obtained in Leica SP5 Laser Scanning Confocal Microscope, 400x magnification (n = 3 larvae/ treatment; 5 image sections/ larva). **a**, Malpighian Tubules main segment microscopy. White arrow indicates a LD. **b**, Number of lipid droplets per Malpighian Tubule (sections of 30 μ m x 30 μ m) (n = 3 larvae/ treatment; 5 sections/ larva). **c**, Posterior Midgut. White arrow indicates LDs. Zones with LD accumulation were only found in non-exposed animals (n = 3 larvae/ treatment). (t-test; *P < 0.01).

The accumulation of lipid stores in fat body and reduction in Malpighian tubules and midguts raised the hypothesis of lipid mobilization between organs induced by imidacloprid exposure. To test this hypothesis lipid levels in hemolymph were measured after exposure to 2.5 ppm imidacloprid. This concentration was chosen to test whether the lowest dose used in experiments so far would cause perturbations. Using the Vanillin assay, which measure total lipid levels (Cheng et al. 2011), an average 4.4 μ g/ μ L of lipid in hemolymph of non-exposed larvae was detected (**Figure 2.14**). As early as 15 minutes exposure to imidacloprid induced an average 10% decrease on lipid levels; while an average 20% decrease was observed after 2 hr exposure (**Figure 2.14**). These changes suggest that lipid mobilization between tissues is occurring. Ioannou et al. (2019) showed that the overstimulation of rat hippocampal neurons with N-methyl-D-aspartate leads to ApoE lipid particles transferring fatty acids to astrocytic LDs (Ioannou et al. 2019). In *Drosophila* ApoE/D is ROS sensitive and regulates lipid transport between neurons and glia (Liu et al. 2017). This same mechanism may explain the changes in lipid levels in the hemolymph and metabolic tissues in larvae observed here; insecticide-driven oxidative stress could be affecting lipid transport mediated by ApoE/D.

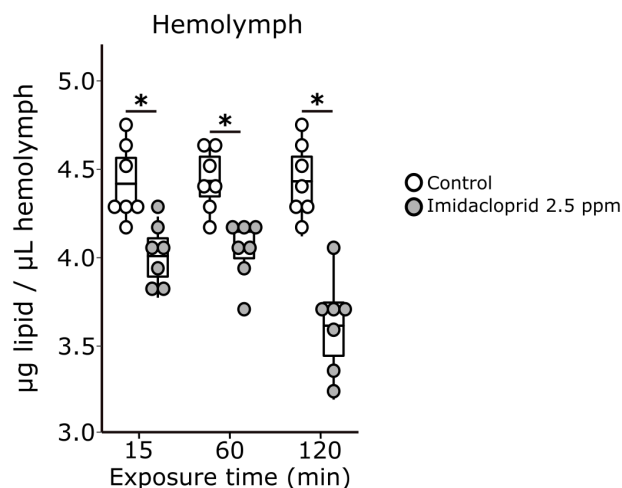


Figure 2.7. Imidacloprid reduces lipid levels in hemolymph. Amount of lipids in the hemolymph ($\mu\text{g}/\mu\text{L}$) of larvae exposed to 2.5 ppm of imidacloprid for 15 min, 1 hr, or 2 hr. Measured using the colorimetric vanillin assay ($n = 7$ replicates/ treatment/ time point; 30 larvae/ replicate). (t-test; * $P < 0.01$).

2.3.6 Hydrogen peroxide exposure induces similar disturbances on the lipid environment

In parallel to the RNAi experiments on the brain and insecticide exposures, larvae were also exposed to sublethal doses of hydrogen peroxide (H_2O_2), 1000 ppm or 5000 ppm for 2 hr. The aim of this experiment was to test whether an oxidizing agent by itself is able to impact the lipid environment of larvae in the same way imidacloprid did. As expected, results obtained with H_2O_2 were very similar to the ones obtained with RNAi and the imidacloprid exposures (**Figure 2.15a, b**). An average 29% and 48% increase in the area covered by LDs was observed in fat bodies after exposures to 1000 ppm and 5000 ppm H_2O_2 , respectively. Once again, a significant increase in the number of small LDs and reduction in the number of large LDs was registered in fat bodies (**Figure 2.15c, d**).

The ability of this oxidizing agent to disturb lipid levels in hemolymph was also tested. The levels of lipids in hemolymph were increased by 13% and 28% after exposures to 1000 ppm and 5000 ppm H_2O_2 respectively (**Figure 2.15e**). Even though the response obtained with H_2O_2 exposure is opposite to the response obtained with imidacloprid, the result still corroborates that oxidative stress by itself can affect lipid levels in the insect blood.

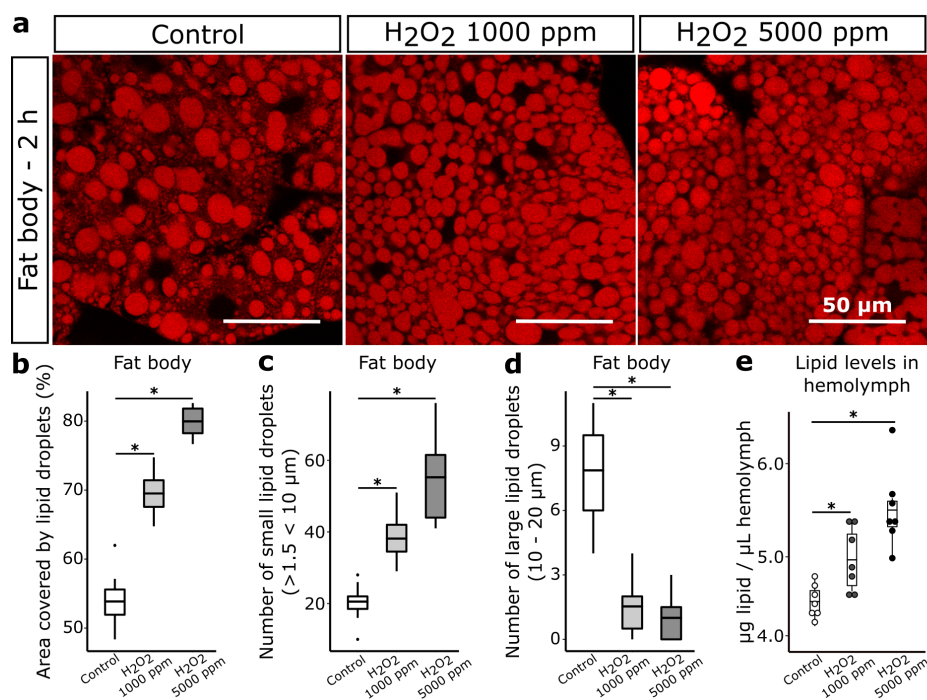


Figure 2.8. Hydrogen peroxide (H₂O₂) induces lipid accumulation in larval fat body and in hemolymph after 2 hr exposure. **a**, Exposure to either 1000 ppm or 5000 ppm of H₂O₂ for 2 hr induces accumulation of LD in fat body. Tissues stained with Nile red and images obtained in Leica SP5 Laser Scanning Confocal Microscope, 400 x magnification. **b**, Percentage of area covered by LD in larval fat bodies (sections of 50 μm x 50 μm). **c**, Number of small LD (> 1.5 μm < 10 μm) in larval fat bodies (sections of 50 μm x 50 μm). **d**, Number of large LD (10 μm - 20 μm) in larval fat bodies (sections of 50 μm x 50 μm). **e**, Levels of lipid in hemolymph (μg/ μL). (t-test; *P < 0.01).

2.3.7 Knockout of GstD1 increases imidacloprid susceptibility

The effect of a loss of Glutathione-s-transferase D1 (GSTD1) activity on the motility of imidacloprid-exposed larvae was tested. GSTs are a large family of multifunctional enzymes conserved in aerobic organism with a vital role in the detoxification of a wide range of endogenous and exogenous substrates (Enayati et al. 2005). Increased GST activity has been correlated with resistance to all the major insecticide classes (Enayati et al. 2005; Vontas et al. 2001). GSTs are also able to metabolize insecticides by facilitating their reductive dehydrochlorination or by conjugating reactions with reduced glutathione (GSH), to produce water-soluble metabolites and thus facilitating their excretion (Enayati et al. 2005; Oakley 2011). *GstD1* is one of the most highly expressed genes in *Drosophila*. It is expressed in most tissues and all stages of the life cycle and has the broadest substrate specificity of all of the GST's

in this species (Saisawang et al. 2012). GSTD1 glutathione has peroxidase activity, meaning that it can catalyse the reduction of hydrogen peroxide to water and oxygen, reducing levels of ROS (Tang and Tu 1994). GSTD1 has been shown to bind imidacloprid using Nuclear Magnetic Resonance (Chen & Gooley unpubl.). The *GstD1* knockout mutant larvae are significantly more sensitive to imidacloprid exposure, showing a greater reduction in movement when compared to the respective genetic background control line (**Figure 2.16**). The greater insecticide resistance in larvae expressing *GstD1* corroborates the role of this enzyme in protecting flies against the damage inflicted by imidacloprid exposure either by directly binding to imidacloprid or by removing ROS and oxidized compounds produced by imidacloprid exposure. Considered together, the set of results presented here and on sections 2.3.5 and 2.3.6 create strong evidence that the impacts of low dose imidacloprid exposures are caused by the generation of oxidative stress initiated in the brain.

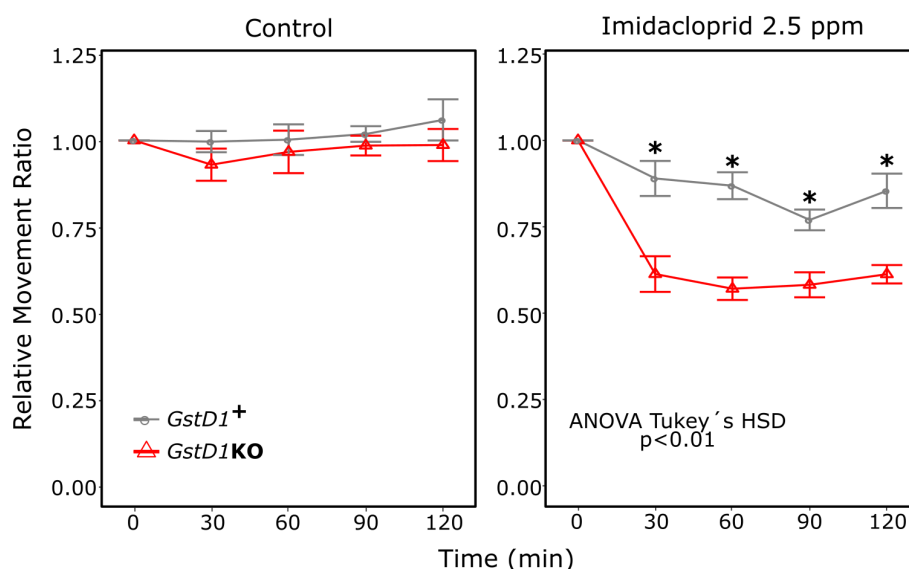


Figure 2.9. Knockout of an antioxidant enzyme increases sensitivity to imidacloprid. *GstD1* background control (*GstD1*⁺) larvae and *GstD1* knockout (*GstD1*^{KO}) larvae exposed to 2.5 ppm of imidacloprid for 2 hr. Dose response to insecticide by Wiggle Index analysis. Results are expressed in terms of Relative Movement Ratio (RMR) values in response to the exposure time in minutes (n = 25 flies/ replicate; 4 replicates/ treatment). All points correspond to the mean RMR values with standard error (t-test; *P < 0.01).

2.3.8 The existence of multiple nAChR targets to imidacloprid hinders the rescue of the LD exposure phenotype

Perry et al. (2008) showed that knockout of nAChR subunits D α 1 and D β 2 confer a moderate level of resistance to imidacloprid in *Drosophila*, meaning that other subunits could also be targeted. Subsequent unpublished research by Perry et al. has shown that at least two other subunits (D α 2 and D β 1) are involved. Imidacloprid, therefore almost certainly targets a number of different nAChR subtypes. Given that it is proposed here that the downstream impacts on metabolic tissues are triggered by an interaction between imidacloprid and its targets, the ideal experiment would be to examine whether a loss of the targets leads to an amelioration of these impacts. Numerous attempts at creating genotypes with multiple nAChR knockout mutations show that a genotype with knockout mutations for the four genes encoding subunits targeted by imidacloprid would not be viable (Perry et al. unpubl). Nonetheless, experiments with *D α 1* knockout mutants were still performed, to check whether the absence of one of the imidacloprid targeted subunits could prevent the development of phenotypes outside the CNS in exposed animals. *D α 1* knockout larvae generated by CRISPR in the same background line used in most experiments so far (line 14) were exposed to 2.5 ppm imidacloprid for 2 hr and; and as a positive control, to 2.5 ppm spinosad (which targets D α 6 subunits) for 2 hr. A significant accumulation of LDs was still observed in fat bodies of third instar larvae exposed to imidacloprid, as well as spinosad (**Figure 2.17a-d**). Even though presenting some level of resistance to imidacloprid, results suggest that the presence of other target subunits could still lead to the cascade of damage initiated with Ca²⁺ flux into neurons and resulting in the observed phenotype. Assays with mutants are resumed in the next Chapter with spinosad exposures, where the presence of a single nAChR target allows for more experimentation.

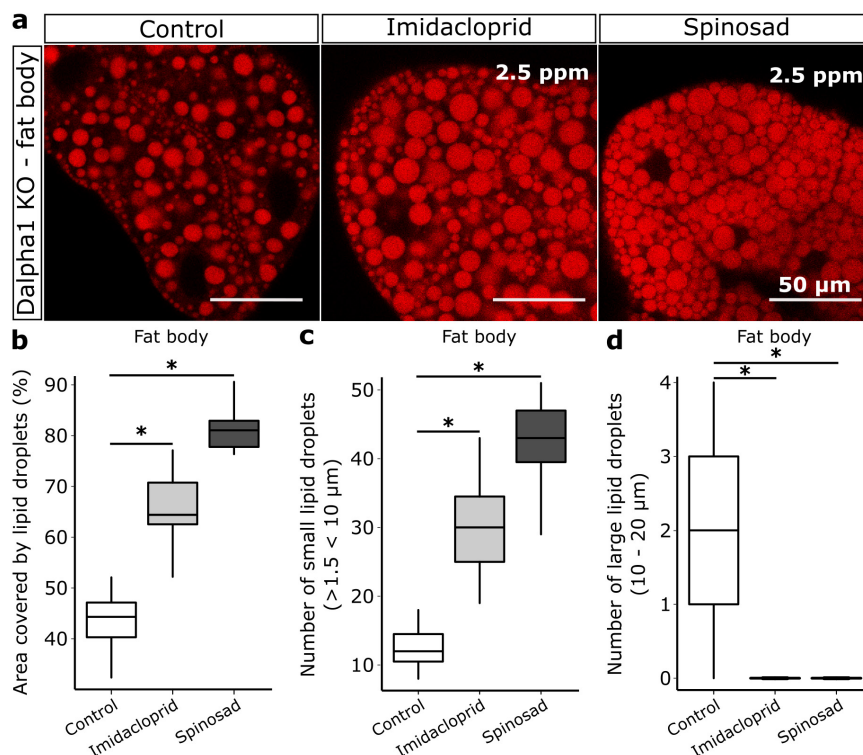


Figure 2.10 Larvae resistant to imidacloprid (Dα1 knockout) show lipid accumulation in fat body when exposed to this insecticide. **a**, Fat bodies of Dα1 KO larvae exposed to 2.5 ppm of either imidacloprid or spinosad for 2 hr. Nile red staining. Images obtained in Leica SP8 Laser Scanning Confocal Microscope, 400x magnification (n = 3 larvae/ treatment; 5 image sections/ larva). **b**, Percentage of area occupied by LD in fat body (sections of 50 μm x 50 μm). **c**, Number of small LD (> 1.5 μm < 10 μm) in larval fat bodies (sections of 50 μm x 50 μm). **d**, Number of large LD (10 μm - 20 μm) in larval fat bodies (sections of 50 μm x 50 μm). (t-test; *P < 0.01).

2.3.9 Low dose exposure to imidacloprid induces the activation of major stress response pathways

2.5 ppm imidacloprid also increased the expression of *GstD1* and the JNK signalling pathway in midgut after a 2 hr exposure. JNK (c-Jun NH2-terminal Kinase), encoded by the basket (*bsk*) gene in *D. melanogaster*, is a key enzyme in a MAP kinase signalling pathway (Wang et al. 2003). It is known to be involved in embryonic development, wound healing, apoptosis, and various stress responses. Once activated JNK phosphorylates the transcription factor c-Jun which forms heterodimers with other transcription factors, including Fos. The c-Jun:c-Fos heterodimer binds to TRE elements in the promoter regions of many genes, activating them (Wang et al. 2003). *GstD1* is induced via the Cnc (Nrf2)/ Keap1 pathway which provides a defence against oxidative stress (Sykietis and Bohmann 2008). Imidacloprid induced a mean increase in *GstD1*

expression of 52% and a similar increase (54%) for the JNK pathway (**Figure 2.18a, b**). The two pathways have distinct spatial patterns of activation. The proventriculus shows predominant JNK activation, the gastric caeca show predominant activation of *GstD1*, whilst both JNK pathway and *GstD1* were activated in the anterior midgut (**Figure 2.18c**). Both pathways are activated by a range of chemical inducers known to cause or enhance oxidative stress (Chatterjee and Bohmann 2012; Pitoniak and Bohmann 2015). The anterior midgut region is involved in the regulation of lipid metabolism (Sieber and Thummel 2012). The *DHR96* nuclear receptor, which is involved in TAG homeostasis in *Drosophila* (Sieber and Thummel 2009) and *Magro*, a target of DHR96 which encodes a lipase, are expressed in anterior midgut and the proventriculus (Sieber and Thummel 2012). The activation of JNK and *GstD1* suggest that imidacloprid can affect the genes involved in the regulation of lipid metabolism and antioxidant response in anterior midguts of *D. melanogaster*.

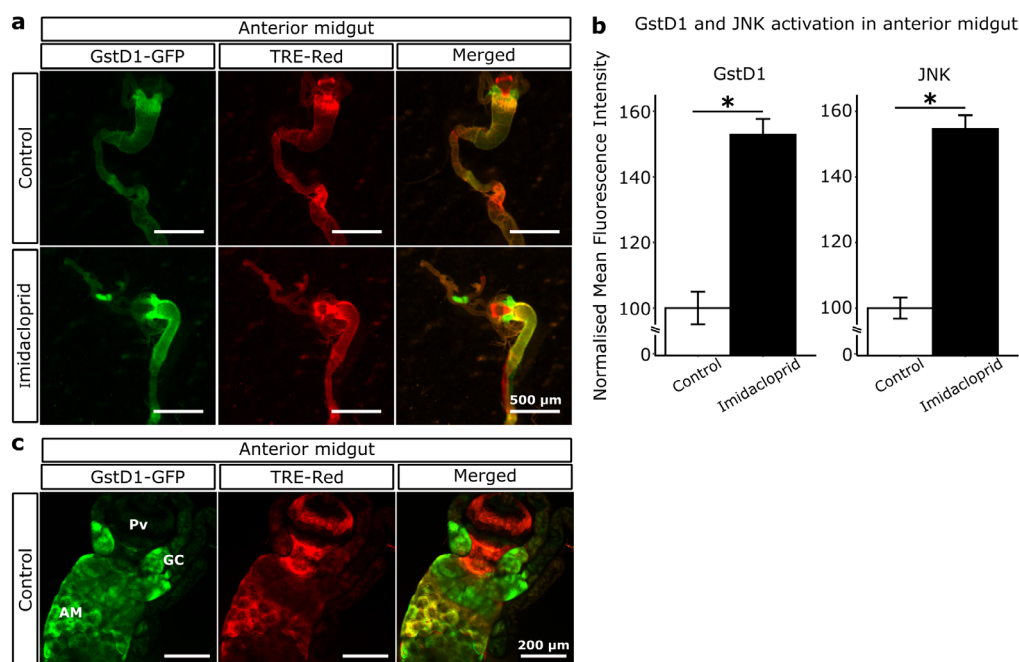


Figure 2.11. Imidacloprid increases activation of GstD1 and JNK in anterior midgut. Double reporter (GstD1-GFP; TRE-DsRed) larvae exposed to 2.5 ppm of imidacloprid for 2 hr (n = 40 larvae/ treatment). **a**, Activation of GstD1 and JNK in anterior midgut. Images were acquired in Leica M205 FA microscope with Leica DFC7000T camera, using the software Leica Application Suite X (LAS X) at 30x magnification. **b**, Mean Fluorescence Intensity of GstD1 and JNK in anterior midgut, gastric caeca and proventriculus. **c**, Activation pattern of GstD1 and JNK in proventriculus (Pv), gastric caeca (GC) and anterior midgut (AM) in control GstD1-GFP; TRE-Red larvae. Images obtained in Leica SP5 Laser Scanning Confocal Microscope, 400x magnification. (t-test; *P < 0.01).

2.3.10 Imidacloprid triggers major alterations in the lipidome

The disruption of the lipid environment of metabolic tissues in response to low acute imidacloprid exposure led to the investigation of lipidomic profiles. Larvae exposed to 2.5 ppm imidacloprid for 2 hr were used for MS-LC lipidomic analysis, performed in biological triplicate. While the lipidomic analysis of whole larvae lacked the capacity to detect differences in specific tissues, or even diluted the differences emerging from tissues with opposite phenotypes (accumulation of lipids in fat body and reduction in tubules and guts), it was possible to detect a more systemic response to imidacloprid exposure. 18 major lipid classes were detected: triacylglycerols (TAG), phosphatidylcholines (PC), diacylglycerols (DAG), phosphatidylethanolamines (PE), odd chain phosphatidylcholines (oddPC), phosphatidylinositols (PI), lysophosphatidylcholines (LPC), lysophosphatidylethanolamines (LPE), phosphoethanolamines alkyl ether (PE(O)), phosphoethanolamines 1Z-alkenyl ether (PE(P)), phosphatidylserines (PS), phosphatidylcholines alkyl ether (PC(O)), phosphatidylglycerols (PG), cholesterol esters (CE), lysophosphatidylcholines alkyl ether (LPC(O)), cardiolipins (CL), dihydroceramides (dhCer) and modified phosphatidylcholines (modPC). Three major classes of oxidized compounds were also identified and grouped together as: oxidized phosphatidylcholines, oxidized phosphatidylethanolamines and oxidized phosphatidylglycerols. 80% of all content of lipids quantified were from the TAG, PC, DAG, and PE classes.

Significant changes were observed in the levels of 34 lipids out of 378 detected by mass spectrometry (**Figure 2.19a**; **Supplementary Table I**). Multivariate analysis (**Figure 2.19b**) indicates that even though only 34 out of 378 detected lipids were significantly impacted by imidacloprid, the overall lipidomics profile of exposed larvae cluster together and away from control samples. A significant portion of the observed impacts corresponds to a reduction in some PC and PE species in exposed larvae. In *Drosophila* larvae, approximately 90% of membrane lipid is composed of glycerophospholipids; PE represents 65% of it, followed by PC (Guan et al. 2013). The reduction in overall PE levels is consistent with impaired membrane function. PE is an important determinant of membrane fluidity, regulating the lipid bilayer rigidity (Dawaliby et al. 2016). PCs are also key regulators of LD dynamics (Krahmer et al. 2011) which, along with observed alterations in some TAG species (Liu and Huang 2013), may

underpin the changes in the LDs in the different metabolic tissues. A significant increase in three oxidized phospholipid species was also observed (HPOT keto 34:2-PG-/16:0 neg, HPOD keto 34:3-PG-/16:0 neg and M34:2-PE-/18:2 neg) as well as a significant decrease in another (HOT 36:6-PC-/18:3). A large decrease (48.5%) in the total levels of the identified cardiolipins (**Figure 2.19c**) was also observed. Cardiolipins are a class of lipids located in the inner mitochondrial membrane that anchor electron transport chain proteins. Reduction in cardiolipins levels is indicative of defects in electron transport Complex I (Kagan et al. 2014; Ren et al. 2014). Loss of Complex I function is associated with increased ROS (Quintana et al. 2010), which is in accordance with the observed reduction in mitochondrial aconitase activity and ATP levels. nAChR activity and trafficking to membranes is strongly affected by the surrounding lipid environment of membranes (Baenziger et al. 2015; Barrantes 2004; Barrantes et al. 2010). nAChRs have also been shown to actively change the lipid composition of membrane domains where they are present (daCosta et al. 2009). Such effect might result from the activity of the M4 transmembrane α -helix component of nAChRs subunits, which is thought to perform a lipid sensor function (daCosta and Baenziger 2009). It means that, at least part of the observed impacts on phospholipids might be due to changes on nAChRs activity caused by the insecticides. The converse may also be true.

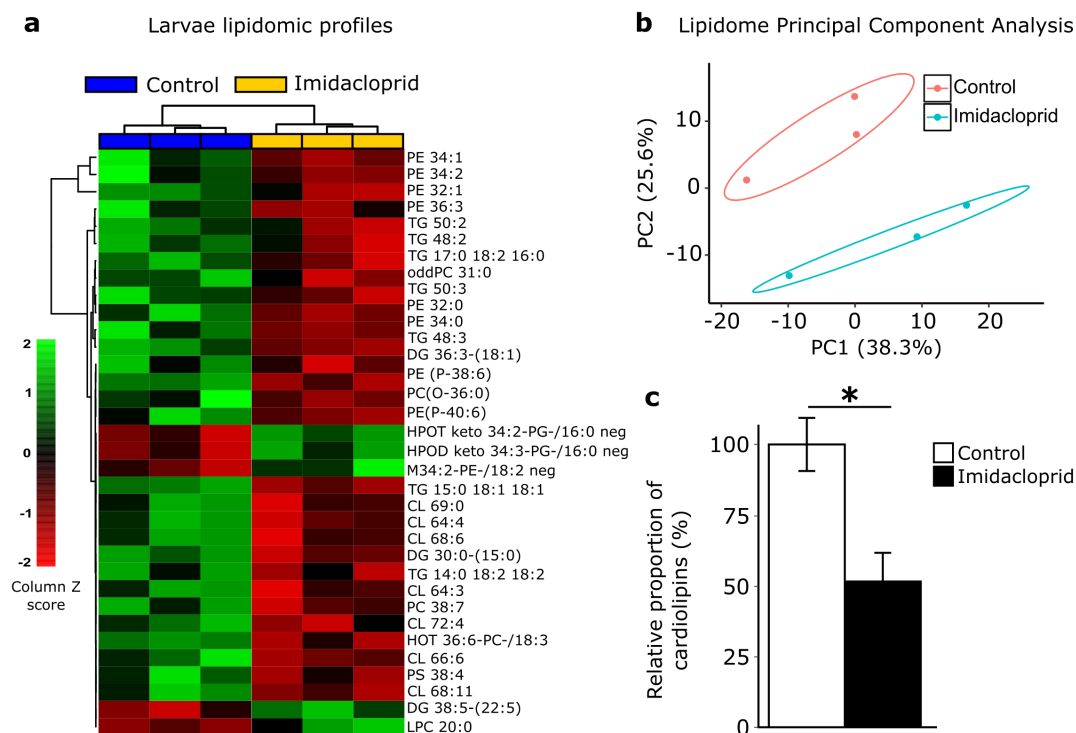


Figure 2.19. Imidacloprid disturbs lipid profiles of exposed larvae. Lipidomic profile of larvae exposed to 2.5 ppm imidacloprid for 2 hr ($n = 10$ larvae/ replicate; 3 replicates/ treatment). **a**, 34 lipid species out of 378 were significantly affected by insecticide treatment (One-way ANOVA, Turkey's HSD, $P < 0.05$). The column Z score is calculated subtracting from each value within a row the mean of the row and then dividing the resulting values by the standard deviation of the row. The features are colour coded by row with red indicating low intensity and green indicating high intensity. **b**, Principal Component Analysis of 378 lipid species. **c**, Relative proportion of cardiolipins in exposed animals versus control. Each dot represents the lipidome data sum of each sample. First component explains 38.3% of variance and second component explains 25.6% of variance. Error bars in **c** represent mean $\pm$ s.e.m. (t-test; * $P < 0.01$).

2.3.11 Low acute exposures to imidacloprid perturbs gene expression in the brain and fat body

RNAseq was performed in biological triplicate on RNA extracted from brains and fat body dissected from acutely exposed larvae (2.5 ppm – 2 hr) (**Supplementary Table II**). For the brain samples (**Figure 2.20a**) a total of 9,640 transcripts were identified, from which 371 were down regulated (foldchange $>|2|$, adj p-value < 0.05), whilst 425 were up regulated in imidacloprid samples (foldchange $>|2|$, adj p-value < 0.05). These lists of differentially expressed genes (DEGs) were used to generate Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses, using the

software DAVID (6.8). GO and KEGG terms were considered based on p-value ($P < 0.01$). Terms enriched in brains after imidacloprid exposure included metabolism of xenobiotics by cytochrome P450s, oxidation-reduction process, metabolism of amino acids and several terms related to immune response. Terms down regulated in brains of exposed larvae included metabolism of carbohydrates and neuroactive ligand-receptor interaction (**Figure 2.20a**). For the fat body samples, a total of 9,564 transcripts were identified, of which 149 were down regulated in exposed animals (foldchange $>|2|$, adj p-value < 0.05), whilst 272 were up regulated (foldchange $>|2|$, adj p-value < 0.05) (**Figure 2.20b**). GO and KEGG analyses pointed to the up regulation of oxidation-reduction process, and a downregulation of xenobiotics metabolism by cytochrome P450s and immune response in exposed animals (**Figure 2.20b**).

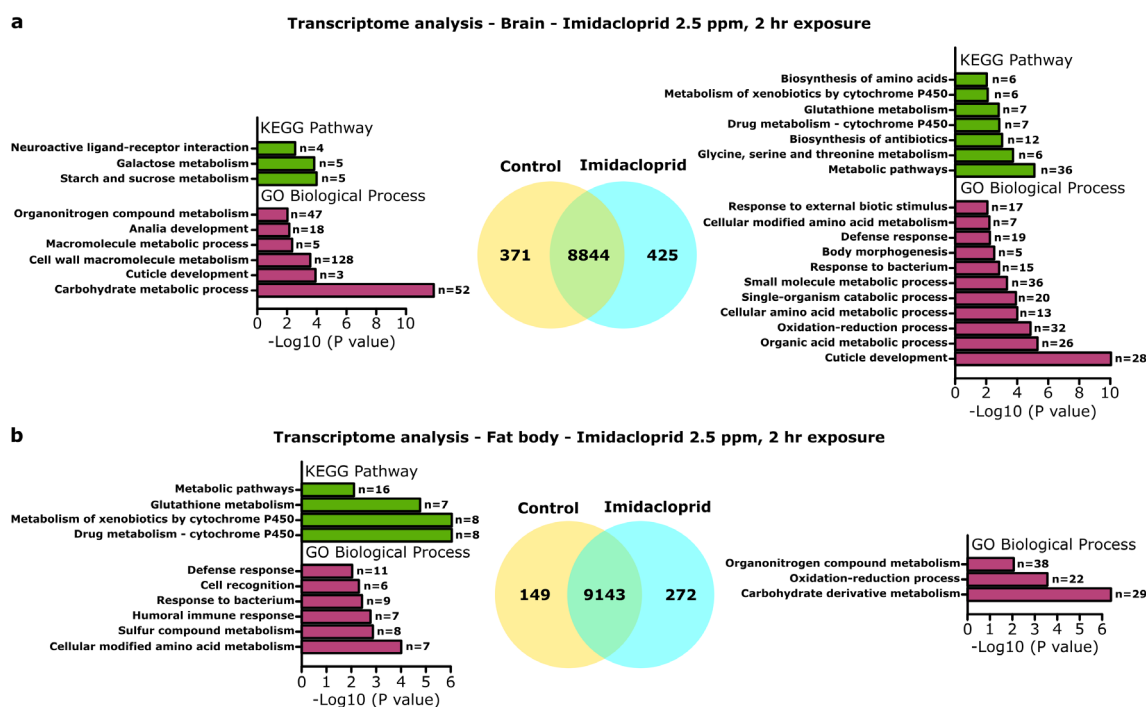


Figure 2.12. Transcriptome analysis of brains and fat bodies of larvae acutely exposed to imidacloprid. Third instar larvae exposed to 2.5 ppm imidacloprid for 2 hr ($n = 40$ larvae/ sample; 3 samples/ treatment). **a**, Transcriptomic analysis of brains, 371 genes downregulated and 425 genes upregulated in imidacloprid samples (foldchange $>|2|$, P adj < 0.05). **b**, Transcriptomic analysis of fat bodies, 149 genes downregulated and 272 genes upregulated in imidacloprid samples (foldchange $>|2|$, P adj < 0.05). KEGG and Gene Ontology analysis were performed in DAVID (Bioinformatics Resources 6.8), the Modified Fisher Exact P-value was transformed using $-\log(10)$ scale. The “n” values in front of the bars indicate the number of genes associated to each KEGG Pathway or GO Biological Process in the dataset.

The transcriptomics results strongly corroborate the data obtained so far. The previous experiments involving low dose exposures that did not impact survival showed extensive perturbations in metabolism and physiology. The transcriptomic analysis revealed perturbation on the expression profiles of genes involved in oxidative stress and metabolism. Among the GO terms with contrasting responses in the two tissues was the one related to the metabolism of xenobiotics by cytochrome P450s. P450s comprise a large multigene family that encodes a diverse class of heme-thiolate enzymes found in all insects (Feyereisen 1999). P450s fulfill many important roles in development and xenobiotic detoxification by incorporating an atom of oxygen into a wide range of substrates. They are involved in the synthesis and degradation of hormones such as ecdysteroids and juvenile hormone. In the case of ecdysteroids, synthesis is dependent on lipid metabolism. The process starts with the conversion of cholesterol to 7-dehydrocholesterol (Feyereisen 1999). Only a subset of the *Drosophila* P450 genes are well-characterized. The ones that have roles in development are best known, but several have been implicated in xenobiotic detoxification (Chung et al. 2009). Some P450s capable of metabolizing imidacloprid have been identified in various insect species (Bass et al. 2011; Karunker et al. 2008; Puinean et al. 2010), including *Drosophila* (Fusetto 2017; Denecke et al. 2017). Only a limited number of P450s are known to be induced by insecticide exposures (Giraud et al. 2010; Willoughby et al. 2006). However, it should be noted that much of the work on induction has been done on whole animals, meaning that changes in expression in individual tissues may have been masked.

Since a perturbation in P450s expression was suggested by the GO analysis, these genes were further investigated in the transcriptomics. Of the 86 P450 genes present in *Drosophila* (Chung et al. 2009), 15 had their expression perturbed by imidacloprid. In most cases this was modest, though significant increase in expression (**Table 2.1**). Most of these P450 genes had their expression induced in the brain. The overexpression of one of these, Cyp6g2, has already been associated with imidacloprid metabolism and resistance (Denecke et al. 2017). The midgut, Malpighian tubules and fat body are considered to be major centres of detoxification and, therefore, one would expect that these tissues have a significant role in protecting the brain from neurotoxic insecticides. However, research presented earlier suggests that it is likely that at least some imidacloprid reaches the brain prior to reaching these tissues (**Figure 2.5**). Clearly a capacity to metabolise imidacloprid in the brain would be advantageous under such

circumstances. This has been shown to be the case for the Cyp6g1 (Denecke et al. 2017), which did not show altered expression in this study. It should be noted that of the 11 P450s that were overexpressed in the brain, only CYP6G2 has a demonstrated capacity to metabolize imidacloprid. The other 10 are yet to be tested. P450 expression changes in the fat body were very minor. While this tissue is an important metabolic centre, not all metabolic functions reside there. The insect gut and Malpighian tubules also have vital functions that are not captured by the fat body transcriptome. P450s are more likely to be expressed in these tissues than in the fat body (Harrop et al. 2014). Further, while the transcriptomics analysis here was performed in larvae exposed for 2 hr in order to allow comparisons with other experiments, the optimal time for induction of genes encoding detoxification enzymes seems to be 4 hr (Willoughby et al. 2006).

GST genes were also investigated in further detail since they are major components of xenobiotics detoxification and antioxidant response (section 2.3.7 and 2.3.9). Among the 35 genes encoding GST enzymes in *Drosophila*, 20 showed altered expression in brain, fat body or both (**Table 2.1**). 6 GSTs had increased expression in the brain and 5 showed a decrease, with these changes being small. For the fat body there was a more consistent trend with 9 of the 11 genes having a reduced level of expression. It is noteworthy that *GstD1* had a reduced level of expression in both the brain and the fat body, while at this same time its expression is significantly increased in the anterior midgut (**Figure 2.18**). Clearly there are large gaps in our knowledge of how metabolic responses to xenobiotics are regulated within tissue and the extent of any coordination between tissues. It is known that a biological circuit between brain and fat body is responsible for coordinating several aspects of insect life (Li et al. 2019).

Table 2.1. Imidacloprid perturbs the expression of P450s and GSTs. List of genes of interest affected by 2.5 ppm imidacloprid exposure for 2 hr. Brain and fat body transcriptomic analysis (↓ gene downregulated; ↑ gene upregulated).

Flybase ID	Gene	Brain - Imidacloprid			Fat body - Imidacloprid		
		response	fold change	adj p-value	response	fold change	adj p-value
FBgn0035154	Cyp9b2	↓	-0.64	0.018314	--	--	--
FBgn0005655	Cyp6v1	↓	-0.76	0.000000	--	--	--
FBgn0038006	Cyp313a2	↑	6.67	0.001044	--	--	--
FBgn0039519	Cyp6a18	↑	6.40	0.000000	↑	2.39	0.000024
FBgn0033065	Cyp6w1	↑	3.17	0.000000	--	--	--
FBgn0000473	Cyp6a2	↑	2.62	0.000000	--	--	--
FBgn0013771	Cyp6a9	↑	2.07	0.000000	--	--	--
FBgn0013772	Cyp6a8	↑	1.96	0.006559	↑	2.25	0.005318
FBgn0000473	Cyp6g2	↑	1.91	0.000000	--	--	--
FBgn0013773	Cyp6a22	↑	1.80	0.011175	--	--	--
FBgn0038037	Cyp9f2	↑	1.47	0.000000	--	--	--
FBgn0033775	Cyp9h1	↑	1.46	0.000000	--	--	--
FBgn0033302	Cyp6a14	↑	1.41	0.028973	--	--	--
FBgn0015038	Cyp9b1	--	--	--	↓	-0.56	0.008736
FBgn0034053	Cyp4aa1	--	--	--	↑	4.92	0.035861
FBgn0001149	GstD1	↓	-1.20	0.000000	↓	-1.95	0.002778
FBgn0010039	GstD3	--	--	--	↓	-3.71	0.000762
FBgn0010040	GstD4	--	--	--	↓	-3.80	0.000000
FBgn0010041	GstD5	--	--	--	↓	-2.42	0.000006
FBgn0010043	GstD7	↑	4.38	0.000000	--	--	--
FBgn0038020	GstD9	--	--	--	↓	-2.75	0.002660
FBgn0063498	GstE2	↑	1.19	0.019430	--	--	--
FBgn0063497	GstE3	↑	1.43	0.003285	--	--	--
FBgn0063496	GstE4	↑	2.80	0.005891	--	--	--
FBgn0063494	GstE6	--	--	--	↓	-3.06	0.000000
FBgn0063493	GstE7	--	--	--	↓	-3.63	0.012443
FBgn0063491	GstE9	↑	2.58	0.000000	--	--	--
FBgn0034354	GstE11	↓	-1.65	0.000000	↓	-1.80	0.013776
FBgn0033381	GstE13	↓	-1.17	0.004813	--	--	--
FBgn0035904	GstO3	↓	-1.41	0.000000	--	--	--
FBgn0010226	GstS1	↓	-1.71	0.000478	--	--	--
FBgn0050000	GstT1	--	--	--	↓	-1.60	0.049502
FBgn0050005	GstT2	--	--	--	↑	1.70	0.041407
FBgn0031117	GstT3	↑	1.22	0.018348	--	--	--
FBgn0030484	GstT4	--	--	--	↑	2.10	0.000056

Intriguingly, the GO and KEGG analysis performed with genes showing foldchange $>|2|$ and adj p-value < 0.05 did not reveal perturbations in lipid metabolism. Such perturbation was expected after observing the effects of imidacloprid on the lipid environment of metabolic tissues. Hence, the expression profiles of genes involved in lipid metabolism in *Drosophila* (Chapter One - **Table 1.1**) were investigated in more detail in the transcriptomics data (**Table 2.2**). Genes involved with lipid droplet accumulation in the brain and neurodegeneration, as well as genes related to oxidative stress response were also investigated (**Table 2.2**). There were no significant changes in expression level of lipid synthesis genes in the fat body. The expression of the most

important genes for lipid biosynthesis, such as *mdy* and *Dgat2* (TAG synthesis), *ACC* (catalyses malonyl-CoA, the rate-limiting substrate for fatty acid synthesis), and *FASN1*, *FASN2* and *FASN3* (fatty acid synthases) were not altered. The expression, however, of *LRPI* (encodes a cell receptor for low-density lipoprotein transported in the hemolymph), *apolpp* (encodes a protein involved in hemolymph lipid transport) and *Apoltp* (encodes a lipid transport particle which transfer lipids from gut to circulating lipophorins, and from them to peripheral tissues) were upregulated (**Table 2.2**). These data, in combination with measures of lipid levels in hemolymph, suggest that even though lipid synthesis could still be occurring in the fat body, the main process responsible for the accumulation of lipids in this organ is lipid mobilization. Lipid may be mobilized from the midgut and Malpighian tubules, which showed a reduction in lipid droplet storage, and then transported through the hemolymph to the fat body.

The data also suggest that expression of several genes involved in lipid homeostasis is affected in the brains of exposed organisms (**Table 2.2**). These include the transcription factors SREBP and Mef2 that were upregulated in the brain. SREBP coordinates fatty acid synthesis and is inhibited by PE production (Liu and Huang 2013), the levels of which were reduced according to the lipidomic analysis – **Fig 2.19**. Mef2 promotes the expression of lipogenic and glycogenic enzymes and upon infection, also promotes the expression of antimicrobial peptides (Azeez et al. 2014). Several of these genes involved in lipid metabolism are also involved in the onset of neurodegeneration (Liu et al. 2017). *Glial lazarrillo* encodes a lipid binding protein that protects against stress; *ldh* encodes a lactate dehydrogenase (converts lactate into pyruvate); *pdha*, *pdhb*, and *pdp* encode pyruvate dehydrogenases (convert pyruvate into acetyl-CoA); and *kdn* encodes a citrate synthase (converts acetyl-CoA into citrate - used for lipid synthesis). *Silnoon* and *outsiders* encode monocarboxylate transporters, and *Basigin* encodes an accessory protein to them, facilitating their role in the transport of citrate to glial cells. *Shakin B* encodes a *Drosophila* innexin which forms heteromeric and heterotypic gap junctions. Liu et al. (2017) showed that knockdown of these genes in *Drosophila* reduces the accumulation of LDs in glial cells linked with oxidative stress and neurodegeneration (Liu et al. 2015). All these genes were up regulated in brains of larvae exposed to imidacloprid (**Table 2.2**), strongly suggesting that lipid metabolism is impaired in brain and that genetic mechanisms that lead to neurodegeneration are induced. Evidence of

neurodegeneration will be presented later in this Chapter for adults chronically exposed to low doses of imidacloprid.

Genes involved in the regulation of stress pathways were also further investigated using the transcriptomic data (**Table 2.2**). The transcriptomic analysis also revealed elevated expression of *bsk* (JNK), *Hep* and *kay*. *Hep* encodes a kinase that activates JNK, which in turn activates the transcription factor c-jun encoded by the gene *Jra*. c-jun forms heterodimers with other transcription factors, including fos (encoded by *kay*), binding to TRE elements in the promoter regions of many genes, activating them (Wang et al. 2003). The genes encoding the transcription factor Maf-s and the protein Keap1 also showed elevated expression in brains of exposed organisms and in the case of Keap1, also in fat bodies. These genes are involved in the activation of the cnc (cap-n-collar) pathway, which coordinates the major xenobiotic response pathway in insects, antioxidant response and mitochondria homeostasis (Bryan et al. 2013; Misra et al. 2013). Keap1 is associated to cnc in the cytoplasm and is phosphorylated in the presence of stressors such as ROS, releasing cnc to translocate to the nucleus forming a heterodimer with Maf-s, binding to AREs (Anti-oxidant Response Elements) in target gene activating antioxidant response genes (Bryan et al. 2013; Misra et al. 2013).

The brain and fat body expression of other genes encoding antioxidant enzymes was also perturbed by imidacloprid exposure. The gene encoding superoxide dismutase *Sod2*, responsible for the detoxifying superoxide radicals produced in mitochondria had elevated expression in brain of exposed larvae, whereas catalase (*Cat*), which clears hydrogen peroxide (Magder 2006) was downregulated.

Table 2.2. Imidacloprid affects the expression of genes involved in lipid metabolism, neurodegeneration, and oxidative stress. List of genes of interest affected by 2.5 ppm imidacloprid exposure for 2 hr. Brain and fat body transcriptomic analysis (↓ gene downregulated; ↑ gene upregulated).

Flybase ID	Gene	Brain - Imidacloprid			Fat body - Imidacloprid		
		response	fold change	adj p-value	response	fold change	adj p-value
FBgn0053087	LRP1	--	--	--	↑	1.58	0.048743
FBgn0087002	apolpp	↑	1.81	0.000000	--	--	--
FBgn0032136	Apoltp	↑	2.90	0.000000	--	--	--
FBgn0029994	Ldsdh1	↑	1.69	0.000000	--	--	--
FBgn0051092	LpR2	↑	1.26	0.000000	--	--	--
FBgn0066101	LpR1	↑	1.41	0.000000	--	--	--
FBgn0026718	Agpat2	↑	1.21	0.003232	--	--	--
FBgn0039114	Lsd-1	↑	1.64	0.000000	--	--	--
FBgn0030608	Lsd-2	↑	1.05	0.036574	--	--	--
FBgn0037913	fabp	↓	-1.59	0.000000	--	--	--
FBgn0040212	Dhap-at	↑	1.16	0.013088	--	--	--
FBgn0263593	Lipin	↑	1.12	0.000041	--	--	--
FBgn0039471	CG6295	↓	-2.91	0.000000	--	--	--
FBgn0036996	mag	↑	2.75	0.000000	--	--	--
FBgn0036449	bmm	↑	1.65	0.000000	--	--	--
FBgn0000568	Eip75B	↑	1.16	0.000000	--	--	--
FBgn0261283	SREBP	↑	1.10	0.000354	--	--	--
FBgn0011656	Mef2	↑	1.48	0.000000	--	--	--
FBgn0033799	Glial lazarrillo	↑	1.56	0.000000	--	--	--
FBgn0028325	Pdha	↑	1.36	0.000000	--	--	--
FBgn0039635	Pdha	↑	1.20	0.000000	--	--	--
FBgn0029958	Pdp	↑	1.15	0.000898	--	--	--
FBgn0001258	Ldh	↑	1.41	0.000000	--	--	--
FBgn0261955	kdn	↑	1.44	0.000000	--	--	--
FBgn0033657	Silnoo	↑	1.45	0.000000	--	--	--
FBgn0261822	Basigin	↑	1.21	0.000000	--	--	--
FBgn0085387	Shaking B	↑	1.73	0.000000	--	--	--
FBgn0265187	fatp2	↓	-1.65	0.019004	--	--	--
FBgn0000229	bsk (JNK)	↑	1.10	0.000255	--	--	--
FBgn0010303	Hep	↑	1.10	0.003549	--	--	--
FBgn0001297	kay (Fos)	↑	1.17	0.000000	--	--	--
FBgn0038475	Keap1	↑	1.21	0.023706	↑	2.20	0.000210
FBgn0034534	maf-S	↑	1.10	0.030073	--	--	--
FBgn0010213	Sod2	↑	1.11	0.000120	--	--	--
FBgn0000261	Cat	↓	-1.08	0.002721	--	--	--

Finally, the transcriptomic analysis revealed the impact of imidacloprid exposures on several genes involved in immune response (**Table 2.3**). Other studies have noted the adverse effects of insecticide exposure on the immune system of non-target insects (Brandt et al. 2016, 2017; Di Prisco et al. 2013), a process for which there is currently no clear mechanism. The immune response in *Drosophila* is mediated by the production of antimicrobial peptides (AMPs). Seven distinct AMPs, along with their isoforms, have been identified in *Drosophila*: Drosomycin, Defensin, Diptericin, Metchnikowin, Attacin-A, Cecropin A2 and Drosocin (Buchon et al. 2014; Lemaitre and Hoffmann

2007). In insects, NF- κ B transcription factors Relish, Dorsal and DIF regulate the expression of AMP genes via two distinct pathways: Toll and immune deficiency (IMD) pathways. Relish acts in the IMD pathway, whereas Dorsal and DIF act downstream of the Toll pathway (Buchon et al. 2014; Lemaitre and Hoffmann 2007). The genes encoding the AMPs are among those most affected by imidacloprid exposure. The Diptericin A gene showed more than a 40-fold reduction in expression in the brain and more than a 200-fold reduction in fat body. Drosomycin-like 2 showed more than a 500-fold reduction in the brain. Several other components of innate immune response in *Drosophila* were also affected including the factors *Relish* and *Dorsal* which showed increased expression in the brain (**Table 2.3**). While the increased expression of *Relish* and *Dorsal* contrasts with the downregulation of AMPs is worth noticing that these transcription factors showed only a very mild induction. It raises the possibility that other factors were involved in the repression of AMPs in exposed insects. Regardless of the factors involved, the large reductions in the expression of AMPs suggests that imidacloprid exposure could render insects more susceptible to pathogens and parasites.

Altogether, imidacloprid has a significant impact on the larval transcriptome. An acute 2 hr exposure that does not impact survival, was sufficient to affect metabolism, xenobiotic and immune response, besides indicating the onset of neurodegeneration.

Table 2.3. Imidacloprid affects the expression of genes involved in immune response. List of genes of interest affected by 2.5 ppm imidacloprid exposure for 2 hr. Brain and fat body transcriptomic analysis (↓ gene downregulated; ↑ gene upregulated).

Flybase ID	Gene	Brain - Imidacloprid			Fat body - Imidacloprid		
		response	fold change	adj p-value	response	fold change	adj p-value
FBgn0010388	<i>Drosocin</i>	↓	-12.75	0.000035	↓	-24.68	0.002660
FBgn0004240	<i>Diptericin A</i>	↓	-46.83	0.000000	↓	-249.92	0.000203
FBgn0034407	<i>Diptericin B</i>	↓	-10.98	0.008850	↓	-40.03	0.001063
FBgn0052279	<i>Drosomycin-like 2</i>	↓	-566.41	0.000000	--	--	--
FBgn0035434	<i>Drosomycin-like 5</i>	--	--	--	↓	-3.99	0.000005
FBgn0014865	<i>Metchnikowin</i>	↓	-9.69	0.007991	↓	-9.59	0.005256
FBgn0012042	<i>Attacin-A</i>	↑	4.02	0.035974	--	--	--
FBgn0041579	<i>Attacin-C</i>	↓	-1.98	0.039617	--	--	--
FBgn0014018	<i>Relish</i>	↑	1.16	0.000038	--	--	--
FBgn0260632	<i>dorsal</i>	↑	1.14	0.003056	--	--	--
FBgn0033367	<i>Prophenoloxidase 2</i>	↑	1.23	0.000000	--	--	--
FBgn0261363	<i>Prophenoloxidase 3</i>	--	--	--	↑	2.09	0.035861
FBgn0004425	<i>Lysozyme B</i>	↓	-8.93	0.000000	--	--	--
FBgn0004426	<i>Lysozyme C</i>	↓	-6.47	0.000000	--	--	--
FBgn0004427	<i>Lysozyme D</i>	↓	-6.70	0.000000	--	--	--
FBgn0004430	<i>Lysozyme S</i>	↑	1.12	0.037193	--	--	--
FBgn0004431	<i>Lysozyme X</i>	↑	8.25	0.035271	--	--	--
FBgn0043576	<i>PGRP-SC1a</i>	↓	-13.57	0.000012	↓	-22.20	0.000014
FBgn0033327	<i>PGRP-SC1b</i>	↓	-18.33	0.000000	-	-	-
FBgn0043578	<i>PGRP-SB1</i>	--	--	--	↓	-4.10	0.000000
FBgn0035806	<i>PGRP-SD</i>	--	--	--	↓	-3.47	0.000642
FBgn0043841	<i>vir-1</i>	↑	1.74	0.000000	--	--	--
FBgn0030051	<i>spirit</i>	↑	3.36	0.000000	--	--	--
FBgn0053329	<i>Sp212</i>	↑	2.27	0.026942	↑	6.20	0.000000
FBgn0034647	<i>pirk</i>	↓	-2.16	0.001338	--	--	--
FBgn0033593	<i>Listericin</i>	↓	-9.23	0.000015	--	--	--
FBgn0261560	<i>Thor</i>	↑	1.66	0.000000	--	--	--
FBgn0002930	<i>necrotic</i>	↑	2.12	0.000000	--	--	--
FBgn0040653	<i>IM4</i>	--	--	--	↑	5.23	0.000194
FBgn0031561	<i>IM33</i>	↑	2.86	0.000000	--	--	--
FBgn0005660	<i>Ets21C</i>	↑	1.57	0.000014	--	--	--
FBgn0026760	<i>Tehao</i>	↓	-3.63	0.000863	--	--	--
FBgn0016675	<i>Lectin-galC1</i>	↑	1.89	0.000000	--	--	--
FBgn0038134	<i>wntD</i>	↑	9.76	0.004648	--	--	--
FBgn0031055	<i>eye transformer</i>	↑	1.84	0.000000	--	--	--
FBgn0037515	<i>Serine protease 7</i>	↑	1.27	0.000847	--	--	--
FBgn0039494	<i>grass</i>	↑	1.56	0.000015	--	--	--
FBgn0041182	<i>Tep2</i>	↑	1.43	0.000000	--	--	--
FBgn0032638	<i>SPH93</i>	↑	2.31	0.000000	--	--	--
FBgn0243514	<i>eater</i>	↑	1.49	0.000016	--	--	--
FBgn0052185	<i>edin</i>	--	--	--	↓	-100.61	0.000363
FBgn0267339	<i>p38c</i>	--	--	--	↓	-5.05	0.000000
FBgn0014000	<i>Helical Factor</i>	↑	2.31	0.000000	--	--	--
FBgn0038973	<i>Pebp1</i>	↑	2.06	0.000000	--	--	--
FBgn0039102	<i>SPE</i>	↑	2.52	0.021559	--	--	--

2.3.12 Adult flies chronically exposed to imidacloprid show signs of neurodegeneration

Moving on from acute exposure assays in larvae, the effects of chronic exposures in adults were assessed. The investigation of these impacts is vital in understanding the threats that many species, including honeybees, may be subjected to in the field. Chronic exposures may create cumulative damage with enduring effects.

In order to examine the effects of chronic imidacloprid exposure a dose that could kill 50% of adult female flies within 20 to 25 days was determined (**Figure 2.21**). At such a dose the insecticide is killing the insect very slowly. Lifespan is truncated, but it is longer than the average lifespan of this species in the field. Among the doses tested, the dose of 4 ppm imidacloprid was the one that matched the selected criteria (**Figure 2.21**). This dose has no significant impact on survival for the first 10 days. This allows the identification of functional and morphological defects that precede death, identifying impacts that may occur when non-pest insects are exposed to low concentrations of this insecticide in the field.

The effects of chronic exposure were initially tested using two different behavioural paradigms: the bang sensitivity assay and the climbing assay. Bang sensitivity measures the time it takes for flies to recover to a standing position following a mechanical shock and is typically associated with defects in mitochondrial function (Pavlidis and Tanouye 1995). The bang sensitivity phenotype (i.e. taking a long time to recover) often leads to seizures that can be reversed with human anti-seizure medication (Reynolds et al. 2004). Significant differences between exposed flies and unexposed controls were first observed after 10 days, but these increased substantially after 20 days, time when around 15% of flies did not recover to standing positions 30 seconds after the mechanical shock (**Figure 2.22**).

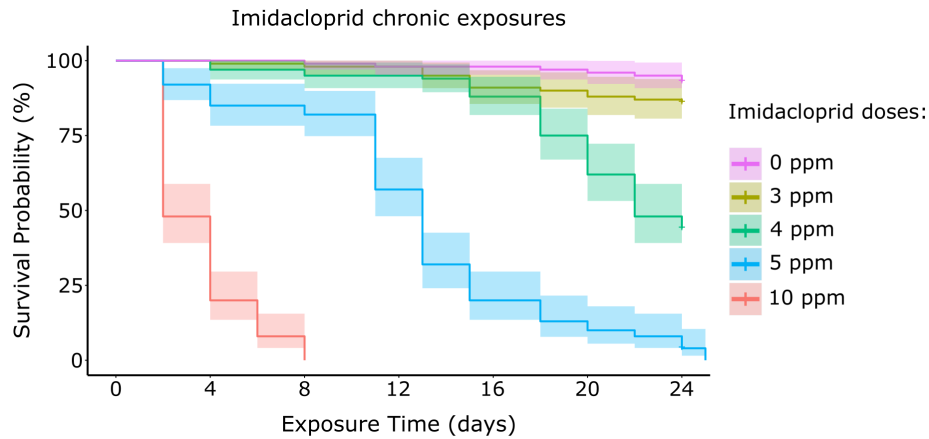


Figure 2.13. Adult chronic exposures to imidacloprid. Females adults (2-5 days old) were exposed to different concentrations of imidacloprid for 25 days (n = 25 flies/replicate; 4 replicates/ treatment). The dose of 4 ppm was selected as the dose that kills 50% of flies within 20 to 25 days. Shaded areas represent 95% confidence interval (Kaplan-Meier method and the Log-rank Mantel-Cox test).

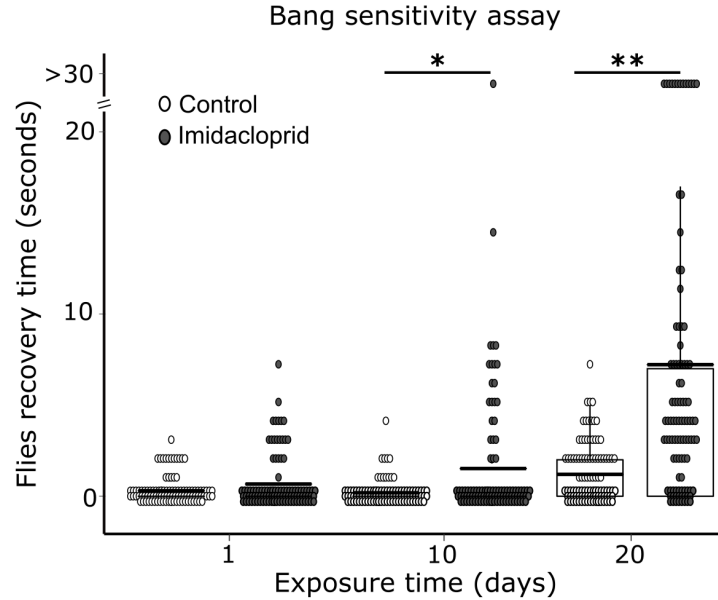


Figure 2.14. Chronic exposure to imidacloprid increases bang sensitivity. Flies exposed to 4 ppm of imidacloprid for 20 days. Bang sensitivity assay of adults at days 1, 10 and 20. Groups of 5 flies were vortexed in a clear vial for 10 seconds at maximum speed and recovery time (time for flipping back) for each fly was recorded (n = 100 flies/time point/ treatment). (Wilcoxon test; *P < 0.05, **P < 0.05).

Exposed flies also performed poorly in climbing assays. This assay measured the ability of flies to climb past a pre-determined mark of 7 cm height on the vial during the allotted time period of 30 seconds after the vial is tapped strongly on a hard surface, causing the flies to fall to the bottom of the vial. Not climbing the pre-determined height within the 30 seconds was considered failure. Climbing requires coordination and failure in this test is often linked to neurodegenerative disorders in the fly model, such as Parkinson's disease (McGurk et al. 2015). 6%, 44% and 68% of flies failed to climb after 1, 10 and 20 days of exposure, respectively (**Figure 2.23**). The ability of antioxidant treatment to ameliorate this phenotype was also tested. Climbing ability was significantly less impacted in flies exposed to the same dose of imidacloprid, but concomitantly treated with the antioxidant AD4. The failure ratios were 7%, 13% and 31% for the respective time points of 1, 10 and 20 days (**Figure 2.23**).

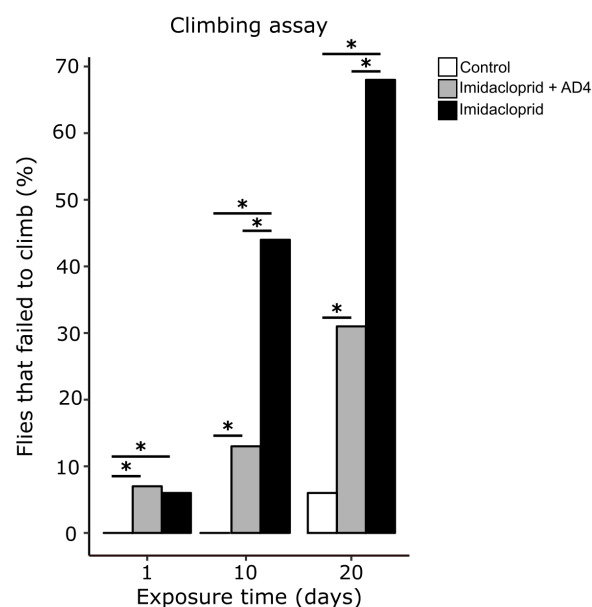


Figure 2.15. Chronic exposure to imidacloprid reduces climbing and antioxidant treatment rescues the phenotype. Flies exposed to 4 ppm imidacloprid with and without concomitant treatment with 75 μ g/mL of antioxidant N-acetylcysteine amide (AD4). The flies treated with AD4 are less impacted after 10 and 20 days of exposure than flies exposed only to imidacloprid. Percentage of adult flies that failed climbing assay at days 1, 10 and 20 of exposure (n = 100 flies/ time point/ treatment). (Wilcoxon test; *P < 0.01).

Similarly, the longevity of flies treated with imidacloprid was rescued in the presence of AD4, going from 47% to 83% survival at day 25 (**Figure 2.24**). Similar observations have recently been reported for silk worms (Gao et al. 2018). Together, these data suggest the presence of mitochondrial defects and neurodegeneration, both associated with the generation of oxidative stress.

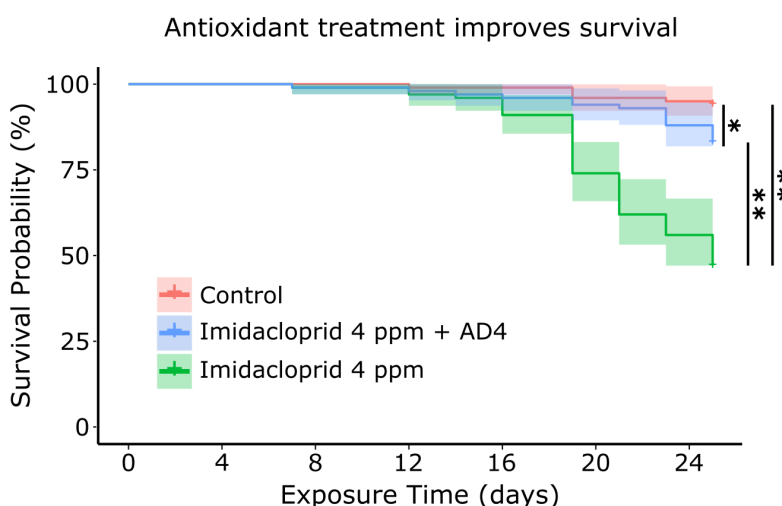


Figure 2.16. Antioxidant treatment rescues survival. Flies exposed to 4 ppm imidacloprid but concomitantly treated with 75 $\mu\text{g/mL}$ of antioxidant N-acetylcysteine amide (AD4) show a better survival than flies exposed to insecticide only. Shaded areas represent 95% confidence interval ($n = 25$ larvae/ replicate; 4 replicates/ treatment) (Kaplan-Meier method and the Log-rank Mantel-Cox test; * $P < 0.05$, ** $P < 0.01$).

2.3.13 The visual system shows severe lesions and neurodegeneration in response to chronic exposures

To address the impact of imidacloprid on nervous system morphology and function, a suite of nervous system phenotypes was assessed. The fly visual system was used for this purpose as it is a well-established model (Liu et al. 2015, 2017). It has the advantage that the function of the eye can be easily tested in live flies.

The *Drosophila* visual system can be didactically divided into three components: retina, lamina, and medulla. The retina is composed of approximately 800 individual eyes - ommatidia (Cagan 2009; Kumar 2012; Neriec and Desplan 2016). Each ommatidium contains eight photoreceptor cells (**Figure 2.25**). Each photoreceptor cell contains an

array of densely stacked membranes called rhabdomeres, the rhodopsin light-gathering structures, responsible for visual signalling. The R1–R6 photoreceptors initiated in the retina terminate in the lamina neuropil and relay motion information to lamina neurons (L1–5) which gather in synaptic units called lamina cartridges. This information is in turn transmitted to the medulla by the axonal projections of lamina neurons, each with its unique arborizations in specific medulla layers. R7 and R8 photoreceptors send colour information directly to medulla layers M6 and M3, respectively. Motion and colour information converges in medulla columns where it is further processed (Cagan 2009; Kumar 2012; Neriec and Desplan 2016) (**Figure 2.26**).

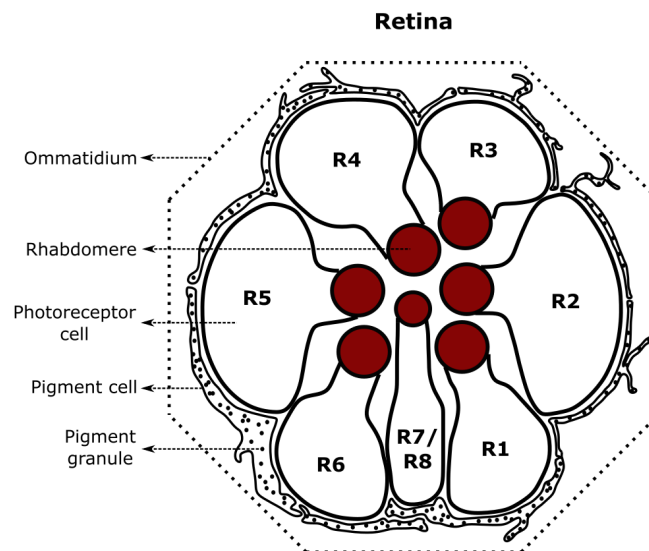


Figure 2.17. Representation of a transverse section of the adult fly ommatidium. A total of 8 photoreceptor cells (R1-R8) are present per ommatidium. Each photoreceptor cell contains an array of densely stacked membranes called rhabdomeres, with exceptions of R7 and R8 which sit on the top of each other and contain a single rhabdomere. Photoreceptors are surrounded by pigment cells (glial cells) which contain the pigment granules that optically insulate each photoreceptor cell. Around 800 ommatidia are found per retina.

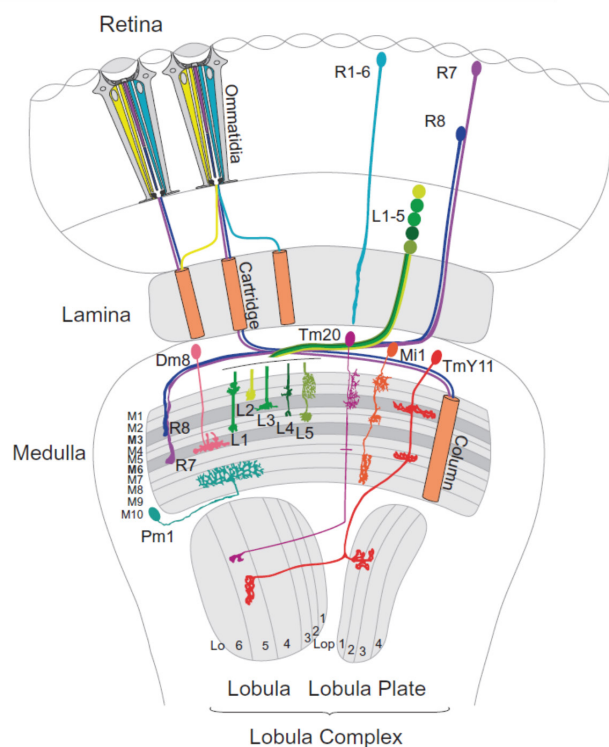


Figure 2.18. Representation of a longitudinal section through the *Drosophila* adult visual system. Showing from top to bottom: retina, lamina and medulla (Plazaola-Sasieta et al. 2017). The major types of neurons in the optic lobe and their synaptic organization and relationship are shown.

The retinas of chronically exposed adult female flies were checked for signs of degeneration. The generation of oxidative stress in the central nervous system can lead to the accumulation of LDs in glial cells in the retinas of adult flies (Liu et al. 2015). This is a transient phenotype and an early marker of neurodegeneration. LDs that accumulate in retinas are believed to burst and propagate oxidative stress, triggering more neurological lesions through a cascade of oxidative damage initiated by peroxidised lipids (Liu et al. 2015, 2017). Inspection of chronically exposed flies showed the presence of LDs in retinas after 1 day of exposure, aligning with the climbing assay phenotype, demonstrates the early presence of oxidative stress at a time when there is no impact on survival (**Figure 2.27a**). Over the following 20 days of exposure when retinas were assessed, the LDs phenotype was not observed again. Instead, a diffuse lipid accumulation was observed, which became denser over time (**Figure 2.27b**). It can be speculated that in the absence of LDs in glial cells which burst after the first days of exposure, and due to the ongoing generation of oxidative stress by imidacloprid, lipids would accumulate diffusely through the retina, rather than being localized in specific organelles.

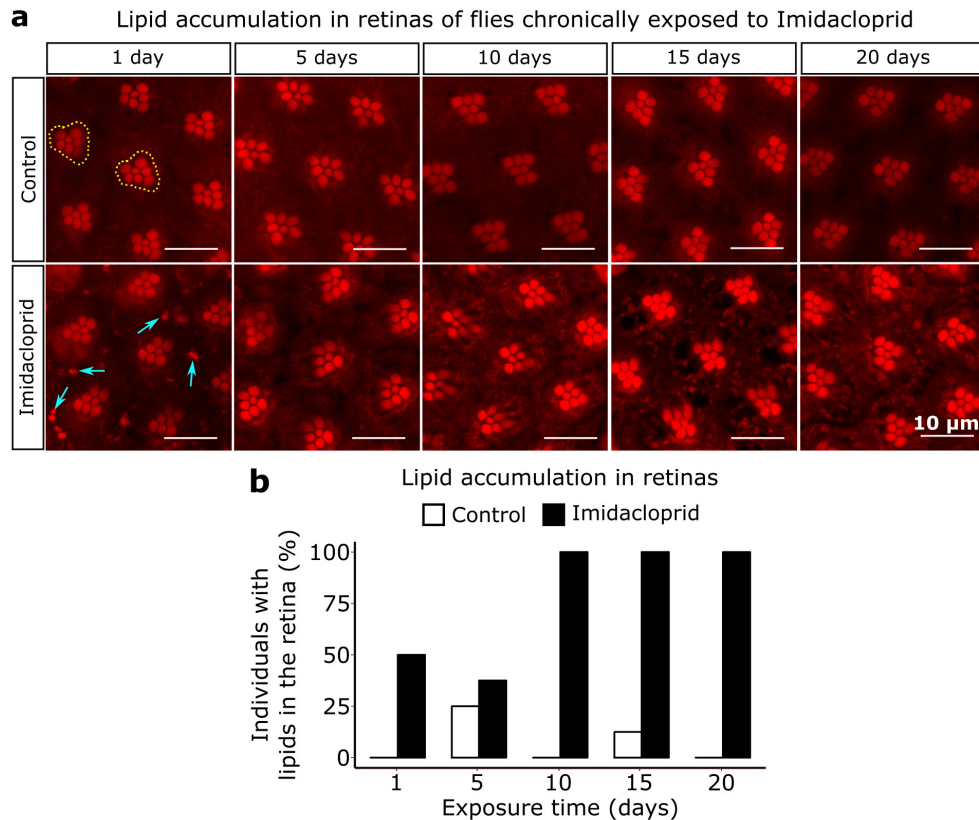


Figure 2.19. Chronic exposure to 4 ppm of imidacloprid causes accumulation of lipids in retinas of flies. **a**, Fly retinas showing the clusters of rhabdomeres (light trapping structures). Day 1 – control, two clusters of rhabdomeres are delimited with yellow dotted-lines. Day 1 – imidacloprid exposure, blue arrows point to LD accumulating in glial cells. LD disappear after day 1 and a diffuse lipid accumulation is observed from day 5 onwards. Nile red staining. Images obtained in Leica SP8 Laser Scanning Confocal Microscope, 600 x magnification. **b**, Percentage of animals that presented accumulation of lipids in retinas (n = 8 flies/ treatment/ time point).

Transmission Electron Microscopy (TEM) was used to investigate the retina in more detail. Transverse sections of fly retinas were analysed after 20 days of chronic exposure. TEM indicated the accumulation of pigment granules and an increased number of mitochondria (**Figure 2.28a-i**). Damaged mitochondria, enlarged and with abnormal cristae, were also identified. These results corroborate the findings with MitoTimer reporter line in larvae, supporting the hypothesis that mitochondrial biogenesis is induced, probably to compensate for the cellular stress induced by Ca^{2+} and ROS (Wenz 2013). Most importantly, a severe vacuolisation of glial cells was also observed with TEM. Glial cells perform a vital role by providing support and protection for neurons and cleansing metabolites and toxic compounds generated by neuronal activity (Freeman 2015). The

vacuolation of glial cells in retina is detrimental for vision and a defining marker of neurodegeneration (Liu et al. 2015, 2017).

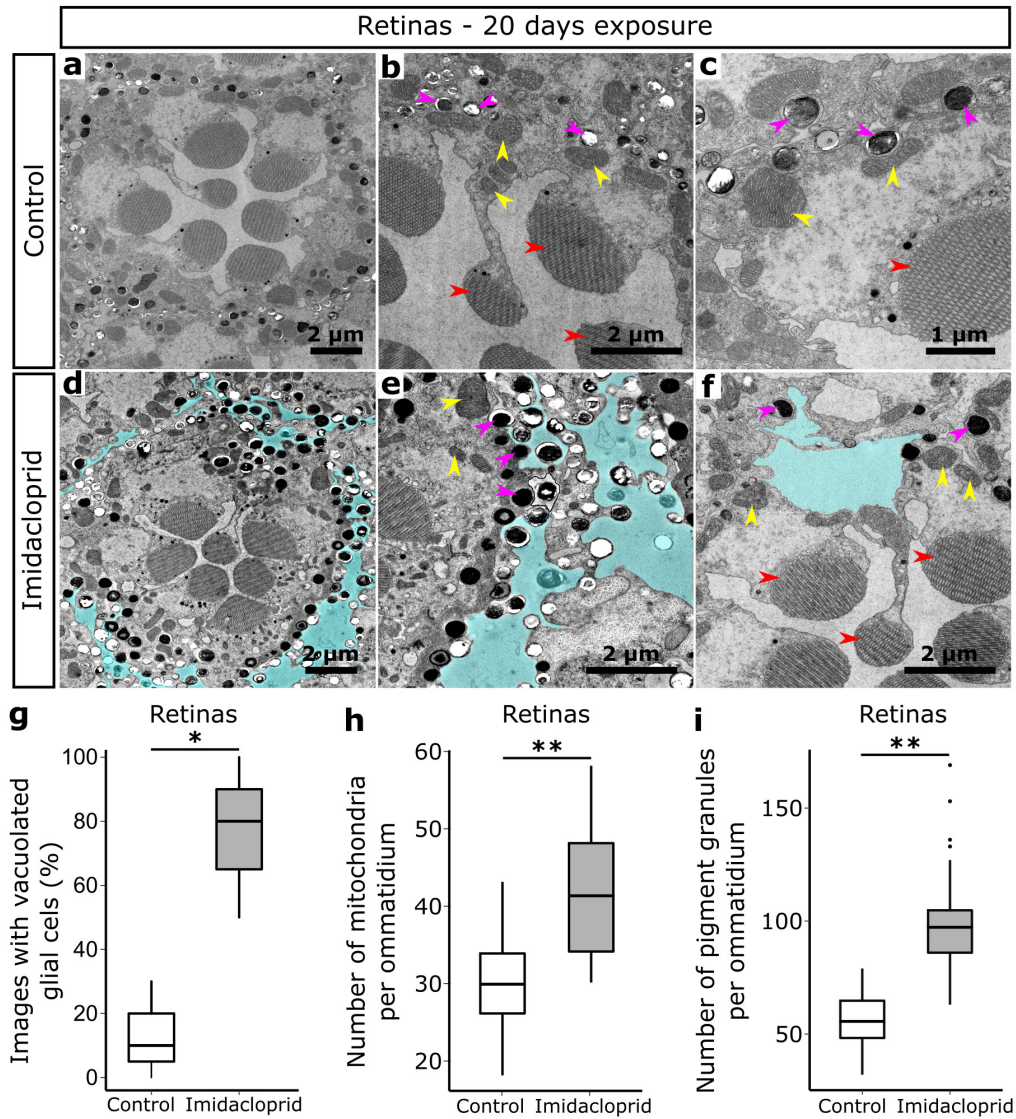


Figure 2.20. Chronic exposure to imidacloprid creates defects in retina. Electron microscopy of retinas of flies exposed to 4 ppm of imidacloprid for 20 days. **a**, Retina of a control fly showing a typical ommatidium. **b-c**, Ommatidia, red arrowheads indicate rhabdomeres, yellow arrowheads indicate mitochondria, pink arrowheads indicate pigment granules. **d**, Retina of an imidacloprid exposed fly showing an ommatidium, areas highlighted in blue indicate vacuolated glial cells. **e-f**, Ommatidia, red arrowheads indicate rhabdomeres, yellow arrowheads indicate mitochondria, pink arrowheads indicate pigment granules, areas highlighted in blue indicate vacuolated glial cells. **g**, Percentage of images that show vacuolated glial cells in retinas (10 images/ fly; 3 flies/ treatment). **h**, Number of mitochondria per ommatidium (16 ommatidia/ fly; 3 flies/ treatment). **i**, Number of pigment granules per ommatidium (16 ommatidia/ fly; 3 flies/ treatment). (t-test; *P < 0.05, **P < 0.01).

Transverse sections of lamina were also assessed by TEM after 20 days of chronic exposure. A representation of the laminar cartridge is shown in **Figure 2.29**, indicating the disposition of photoreceptor terminals and laminar neurons and post-synaptic processes. Defects observed in the lamina were milder than those detected in the retina (**Figure 2.30a-f**), but they were still significant. Again, defective mitochondria were observed, but there were no significant differences in mitochondrial numbers in exposed flies compared to controls. The lamina of exposed flies also showed an increased number of capitate projections and active zones. Capitate projections are endocytic glial invaginations involved in synaptic vesicle regeneration (Fabian-Fine et al. 2003). The lower number of capitate projections in exposed animals suggests decreased vesicle trafficking and recycling, which might be a consequence of the defective glial cells. Active zones, identified by the presence of a tetrad of synapses, are the zones where synapses occur (Van Vactor and Sigrist 2017). It can be speculated that a higher number of active zones in exposed flies is a compensatory mechanism for malfunctioning nAChRs. Some vacuoles were also observed in lamina but were less common than in the retina. Lamina vacuoles were found surrounding the cartridges, and could have emerged from dead glial cells, as in retinas.

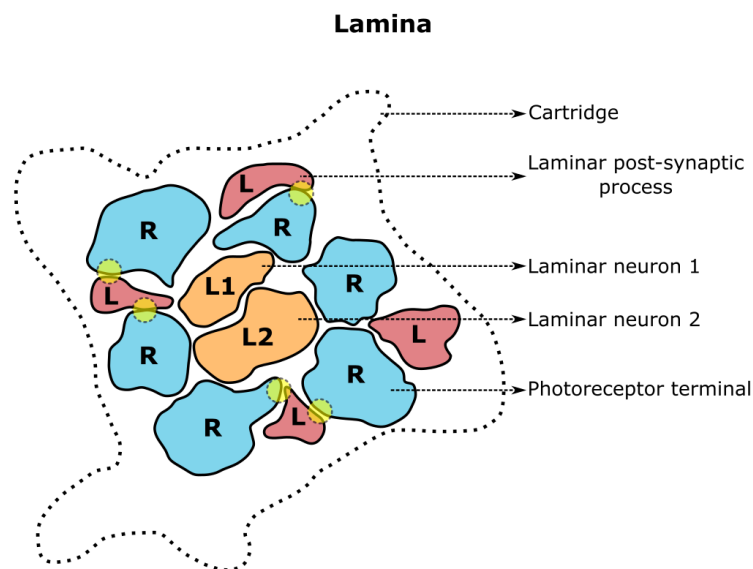


Figure 2.21. Representation of a transverse section of the adult fly laminar cartridge. A total of 6 individual photoreceptor terminals (R1-R6) are packed in each cartridge. Side by side with the photoreceptor terminals are the laminar post-synaptic processes, which synapse with terminals creating a region known as active zone. At the centre of the cartridge are located the laminar neurons L1 and L2. Yellow dotted circles highlight active zones between laminar neurons and photoreceptor terminals.

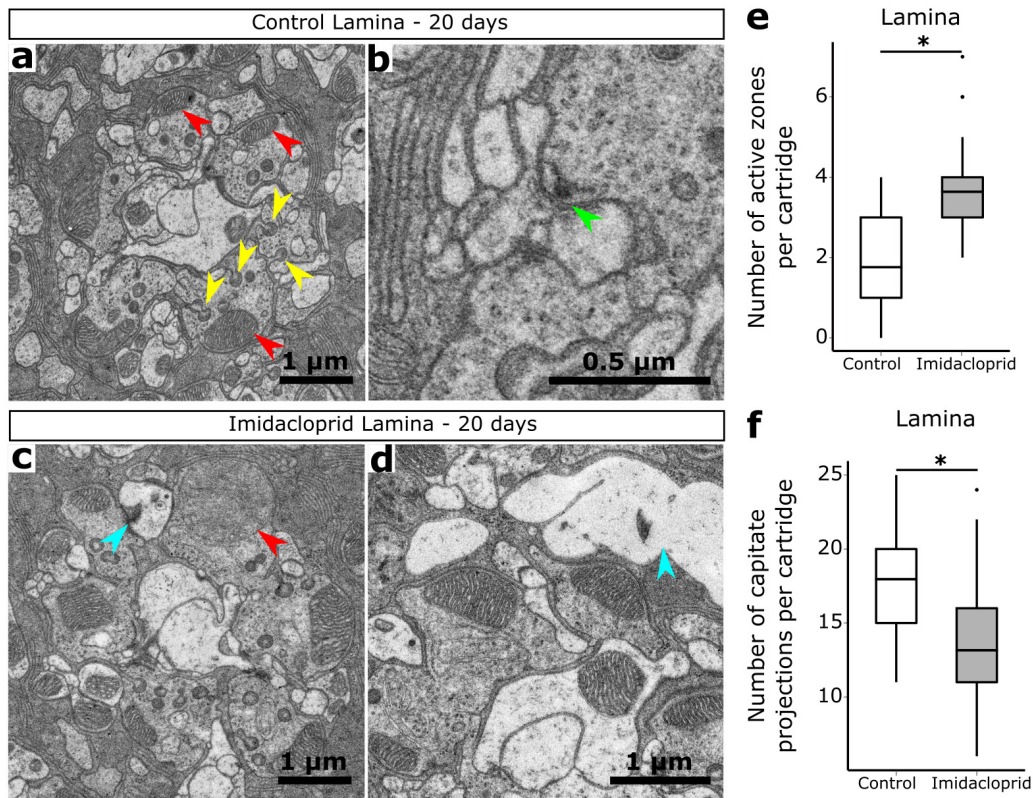


Figure 2.22. Chronic exposure to imidacloprid creates defects in the lamina. Electron microscopy of the lamina of flies exposed to 4 ppm of imidacloprid for 20 days. **a**, Lamina of a control animal showing a regular cartridge, red arrowheads indicate normal mitochondria, yellow arrowheads indicate capitate projections. **b**, Photoreceptor terminal, red arrowhead indicates an active zone. **c-d**, Lamina of imidacloprid exposed animal, red arrowhead indicates an abnormal mitochondrion, blue arrowhead indicates a vacuolated cell. **e**, Number of active zones per cartridge ($n = 3$ flies/ treatment; 16 cartridges/ fly). **f**, Number of capitate projections per cartridge ($n = 3$ flies/ treatment; 16 cartridges/ fly). (t-test; $*P < 0.01$).

Photoreceptors are very dependent on high mitochondrial activity and ATP production, due to the substantial energy consumption of the visual system (Niven et al. 2007). Morphological impacts in the visual system were conspicuous, but to test whether such lesions could functionally affect vision, electroretinograms (ERGs) were performed at regular intervals over the 20 days of exposure. A reduction in the ON transient was observed starting at day 5. A reduction in amplitude was observed from day 10 onward (Figure 2.31a-c). These defects correlate respectively with the impairment of synaptic transmission and phototransduction (Wang and Montell 2007), indicating the progressive loss of vision in chronically exposed flies.

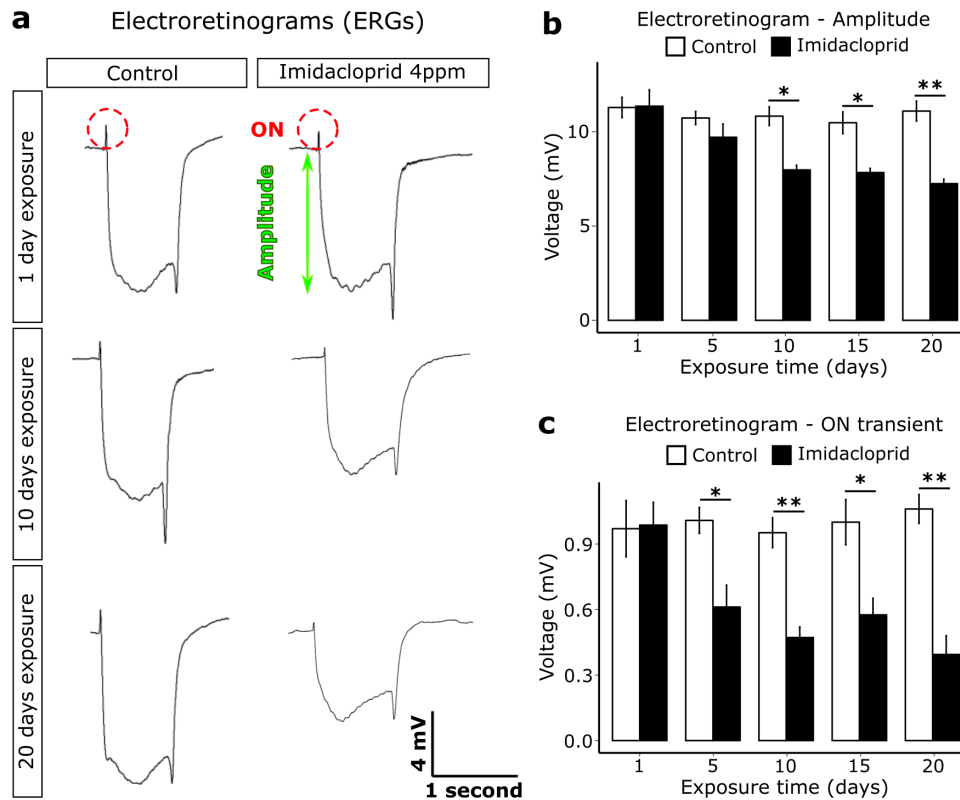


Figure 2.23. Chronic exposure to imidacloprid impairs vision. **a**, Characterization of electroretinograms (ERGs) of animals exposed to 4 ppm of imidacloprid for 1, 10 and 20 days. Red dotted circles indicate the ON transient signal and green arrow indicates the amplitude, (n = 8 to 10 adult flies/ time point/ treatment) **b**, Amplitude of ERGs after days 1, 5, 10, 15 and 20 of exposure to imidacloprid. **c**, On-transient signal of ERGs after days 1, 5, 10, 15 and 20 of exposure to imidacloprid. (t-test; *P < 0.05, **P < 0.01).

2.3.14 Impacts of low chronic exposures to imidacloprid may not be noticed if not investigated in depth

Finally, the Hematoxylin & Eosin stain (H&E) was used to check the overall level of neurodegeneration in chronically exposed flies. No conspicuous difference was found between controls and animals chronically exposed to imidacloprid for 20 days (**Figure 2.32**). The absence of obvious lesions when the brain is viewed on this scale provides a lesson. Low doses that contaminate environments exposing non-pest insects may generate morphological damage in the nervous system that would be easily missed if not investigated at the level that the visual system has been studied here. In this study the central brain region has not been investigated, so it cannot be determined whether there are defects there.

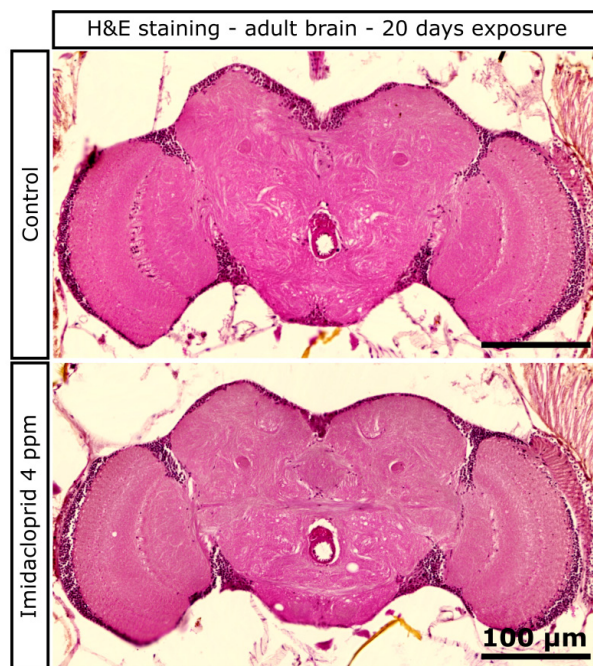


Figure 2.24. Chronic exposure to imidacloprid does not cause obvious impacts in brain anatomy. Midbrain frontal sections stained with hematoxylin and eosin (H&E). Flies exposed to 4 ppm of imidacloprid for 20 days (controls exposed to equivalent dose of DMSO) (n = 3 flies/ treatment). No conspicuous defects are observed in brains of exposed flies when compared to control brains.

2.3.15 A model for imidacloprid low dose exposure impacts on insects

In summary, acute exposure to low imidacloprid doses leads to the establishment of oxidative stress in the insect brain, which rapidly spreads to other tissues. The increase in Ca^{2+} flux into neurons caused by the persistent binding of imidacloprid to nAChRs, injuring mitochondria, is the likely cause of this stress. The generation of more stable oxidized products breaking free from the brain, such as lipid peroxides, could access the insect open circulatory system and reach the metabolic tissues, facilitating the cascade of oxidative damage. ROS would then precipitate the changes in the lipid environment in metabolic tissues and reduction of energy levels. The significance of ROS in the mode of action of imidacloprid at the doses used here is demonstrated by the significant amelioration of impacts on larval movement, LD accumulation in the larval fat body and climbing ability and longevity in adults treated with the antioxidant AD4. In adults the effects of chronic exposure were also reflected in the in the death of glial cells, progressive loss of vision and increased bang sensitivity. The results suggest a model

centred in the oxidative stress triggering a systemic collapse of insect biology: metabolic failure, neurodegeneration, fragilized immune system and energy loss (Figure 2.33).

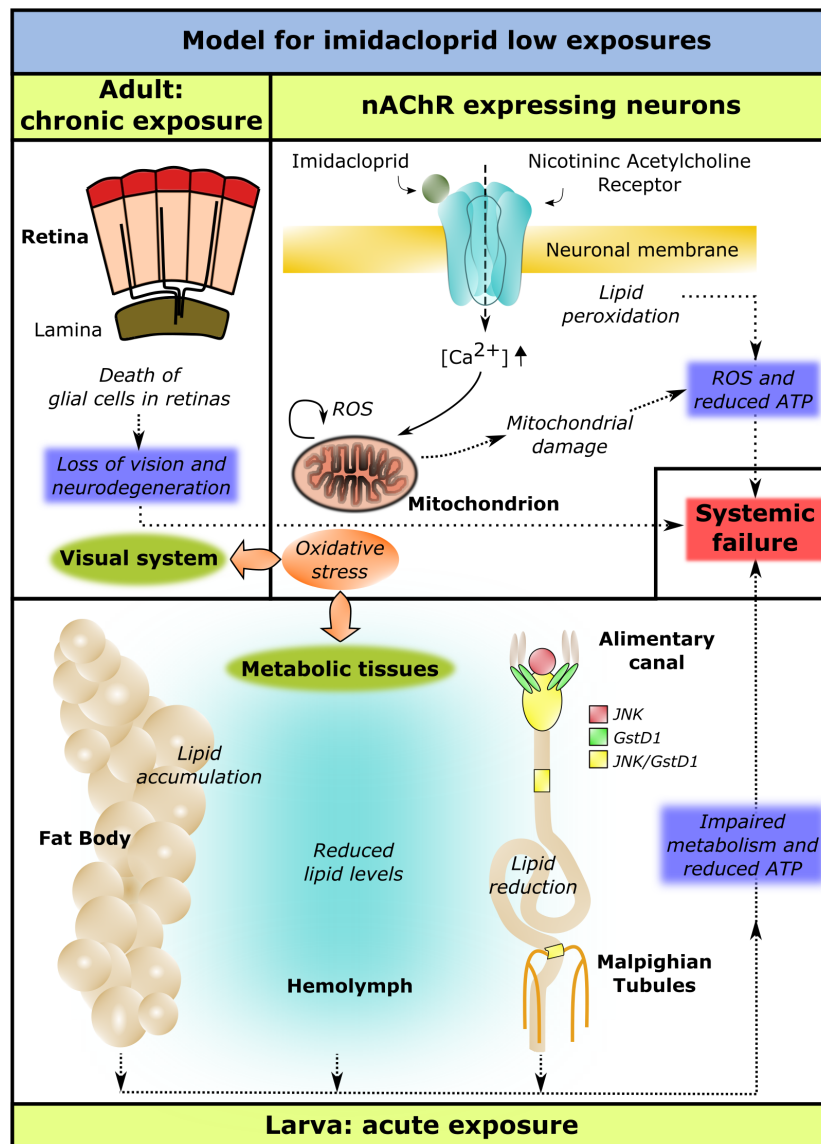


Figure 2.25. Proposed mechanism of action for low dose exposure to imidacloprid. Imidacloprid binds with high affinity to nicotinic acetylcholine receptors. The binding causes a low, but enduring, influx of Ca^{2+} into neuronal cells. Elevated cytosolic Ca^{2+} levels trigger mitochondrial stress, inducing oxidative stress. The effects of acute exposure involve reduction of ATP levels and increase of reactive oxygen species (ROS) in brains and anterior midgut. ROS trigger activation of stress pathways in midgut (JNK and GstD1) and cause redistribution of fat storages, which accumulate in fat body. Chronic exposures in adults to a dose that kills 50% of adults within 20-25 days causes neurodegeneration in the visual system.

Chapter Three

The impacts of low dose exposures to spinosad on insect biology

3.1 Introduction

Spinosad is an organic insecticide, composed of natural fermentation products made by the soil bacterium *Saccharopolyspora spinosa*. Spinosad has been assessed as having potent activity and lower environmental impact, compared to other insecticides (Kirst 2010; Mertz and Yao 1990). Spinosad is a mixture of two spinosyns, spinosyns A and D (**Figure 3.1**) that are present in an 85%:15% ratio in the final product. Spinosad is considered to be a “green” alternative to synthetic insecticides and occupies a small (3%), but growing share of the global insecticide market (Sparks et al. 2017). With an organic label spinosad is approved for use in organic agriculture by many nations. It is also being used to control fleas in domestic pets and head lice in humans (Kirst 2010).

Spinosad is registered for use in more than 80 countries and applied to over 200 crops to control numerous pest insects belonging to Lepidoptera, Diptera, Thysanoptera and Coleoptera orders (Biondi et al. 2012). Spinosad is also used in forests and domestic gardens. In agricultural and forest settings it is applied by directly spraying leaves, in ground broadcast foliar application or aerial foliar applications. Spinosad is not systemic in plants, but penetrates leaves (Biondi et al. 2012; Kirst 2010). Recommended dose rates vary greatly depending on the pest and crop, ranging from 96 ppm for Brassica crops to 480 ppm in apple fields (Biondi et al. 2012). Garden sprays containing spinosad as the active ingredient at a dose of 5000 ppm (Monterey Garden Insect Spray; Captain Jacks Dead Bug Brew® Conc; GREEN LIGHT Lawn & Garden Insect Killing Spray With SPINOSAD).

Levels of spinosad residues in the field vary depending on the formulation, the application mode and dose used, environmental conditions and proximity to the site of application. While residues for spinosad in sweet corn were detected at 0.32 ppm (Bailey et al. 2005), other estimates for spinosad residues can range up to 4 ppm (Christen et al. 2019). The half-life for spinosad can be short, ranging from 1.6 to 16 days depending on the level of exposure to sunlight. Protected from light, the half-life can be up to 200 days (Cleveland et al. 2002).

Although originally considered safe for non-target arthropods species due to its organic origins, studies show detrimental effects of spinosad exposure in beneficial

insects, with a focus on pollinators (Biondi et al. 2012). In honeybees 50% of exposed individual die within 72 hr if exposed to 96 ppm, whilst in bumblebees the same mortality ratios are obtained with exposure to 21 ppm (Biondi et al. 2012).

Spinosad is a hydrophobic compound belonging to a lipid class known as polyketide macrolactones, which target nAChRs, and possibly gamma amino butyric acid (GABA) receptors, in the insect nervous system (Perry et al. 2011; Salgado 1998). Spinosad, however does not target the same nAChR subunits as imidacloprid; only D α 6 subunit loss of function mutants show resistance to Spinosad (Perry et al. 2015). Exposure to this biopesticide at recommended field rates causes continuous activation of motor neurons leading to compulsory muscle contractions and tremors. Prolonged exposure leads to paralysis, an inability to feed and insect death (Biondi et al. 2012). It is believed that although it does not have an immediate effect on a newly hatched egg, it is not significantly metabolized so that when larvae develop into adults, spinosad is still present and able to exert its effect (Kirst 2010; Salgado 1998; Sparks, Crouse, and Durst 2001; Watson 2001). The precise mode of action of spinosad is still poorly understood (Perry et al. 2015).

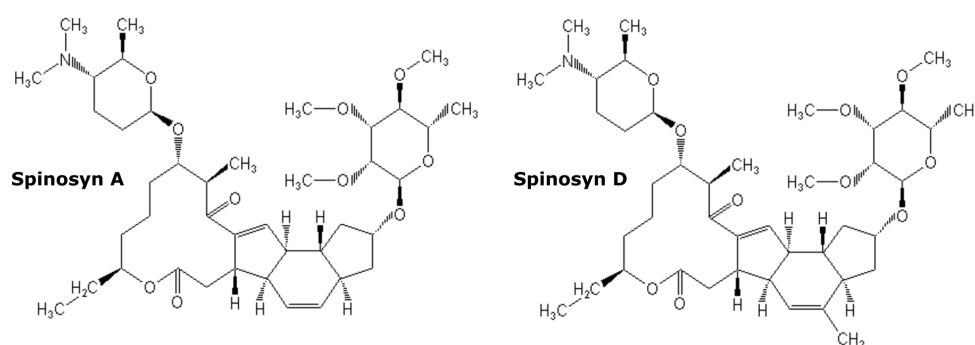


Figure 3.1. Spinosyn A and D chemical structures. Spinosad is composed of 85% spinosyn A $C_{41}H_{65}NO_{10}$ and 15% spinosyn D $C_{42}H_{67}NO_{10}$. Spinosad molar mass: 1477.9 g/mol.

3.2 Material & Methods

Experiments involving exposure to the insecticides spinosad and imidacloprid were performed at the same time, using sample groups of the same size, and compared against the same controls. A complete Material and Methods section for these experiments is described on Chapter Two. To avoid repeating content, here the Material and Methods section will present a simplified list of all experiments performed for spinosad exposures. Details are provided for any aspects of these experiments that were particular to spinosad. Experiments performed exclusively for spinosad exposures are described in full. All other procedures can be assumed identical to those described in Chapter Two.

3.2.1 Fly strains and rearing

The fly strains used for experiments involving imidacloprid exposure (section 2.2.1 - except for *Dα1* knockout mutants) were used to test the same phenotypes in response to spinosad exposure. In addition to that two mutants for the nAChR-*Dα6* gene, which confers resistance to spinosad (Perry et al 2015) and their background control lines were used to investigate the insecticide mode of action. The first of these is Line 14 *Dα6* KO strain, a mutant recovered following EMS mutagenesis in the Line 14 genetic background, with no detectable *Dα6* expression (Perry et al. 2015). The second mutant is a CRISPR knockout of *Dα6* generated in the CantonS genetic background. For experiments aiming to investigate the trafficking of *Dα6* nAChR in brains, UAS *Dα6* CFP tagged strain built in Line 14 *Dα6* KO background (obtained by CRISPR) was crossed to a Gal4-L driver in Line 14 *Dα6* KO background strain.

3.2.2 Collecting third instar larvae

The same procedure was used as presented in Chapter Two.

3.2.3 Insecticide dilution and exposure

The pure version of spinosad (Chem Service Inc) was used in assays involving acute exposure in larvae, whilst the pure version of spinosad (Sigma Aldrich®) was used in assays involving chronic exposures in adults. The chemical was diluted to create 1000 ppm stocks solution, using dimethyl sulfoxide (DMSO), and was kept on freezer (-20°C).

Before exposures, 5x stocks were generated for the dose being used by diluting the 1000 ppm stock in 5% Analytical Reagent Sucrose (Chem Supply) solution (or equivalent dose of DMSO for controls).

3.2.4 Larvae movement assay

Performed with larvae exposed to 2.5 ppm, 3 ppm and 6 ppm spinosad for 2 hr. GstD1 knockout larvae strain and its respective background strain were also exposed to 2.5 ppm for 2 hr. All respective controls were exposed to equivalent volume of DMSO.

3.2.5 Larvae viability and adult survival tests

Survival probability of larvae exposed to 2.5 ppm spinosad for 2 hr was analysed using Kaplan-Meier method and the Log-rank Mantel-Cox test. Correct percentage survival of larvae exposed to 0.5 ppm spinosad for 2 hr, or 0.1 ppm spinosad for 4 hr was analysed using Abbotts' correction. Survival probability of adults chronically exposed to 1 ppm, 0.5 ppm or 0.2 ppm spinosad was analysed using Kaplan-Meier method and the Log-rank Mantel-Cox test.

3.2.6 Tissue dissection

Besides the tissue dissections described in Chapter Two, *Drosophila* adult brains were also dissected for experiments described in Chapter Three. For adult brain dissections flies were first quickly immersed in ethanol and then transferred to a Sylgard plate, kept inside a PBS 1x drop and had the thoraxes pinned. Wings and legs were gently removed using a pair of forceps and proboscises pulled and severed. Tracheas within the head capsule were removed and while using one pair of forceps to hold the head capsule posteriorly, another pair of forceps was used to peel off the cuticle that covers the compound eyes. Using a pair of forceps, a gentle cut was done from the top of the head, behind the ocelli, moving along the compound eyes. The dorsal portion of the head cuticle was then gently removed revealing the adult brain underneath it, which is removed using a pair of forceps and mounted for microscopy.

3.2.7 Measurement of superoxide (O_2^-) levels

Performed with larvae exposed to 2.5 ppm spinosad for 1 hr and 2 hr (controls exposed to equivalent volume of DMSO for 1 hr 2 hr).

3.2.8 Evaluation of mitochondrial turnover

Performed with larvae exposed to 2.5 ppm spinosad for 2 hr (controls exposed to equivalent volume of DMSO for 2 hr).

3.2.9 Systemic mitochondrial aconitase activity

Performed with larvae exposed to 2.5 ppm spinosad for 2 hr (controls exposed to equivalent volume of DMSO for 2 hr).

3.2.10 Systemic ATP levels

Performed with larvae exposed to 2.5 ppm spinosad for 2 hr and assessing larvae immediately after the exposure or 12 hr after the exposure (controls exposed to equivalent volume of DMSO for 2 hr).

3.2.11 Evaluation of lipid environment of metabolic tissues in larvae

Performed with larvae exposed to 2.5 ppm spinosad (for fat bodies) for 2 hr, 0.5 ppm spinosad for 2 hr (for fat bodies, Malpighian tubules and midguts), or 0.1 ppm spinosad for 4 hr (for fat bodies, Malpighian tubules and midguts). Line 14 $\alpha 6$ knockout, CantonS $\alpha 6$ knockout and CantonS larvae also had fat bodies assessed after exposure to 2.5 ppm spinosad for 2 hr. All respective controls were exposed to equivalent volume of DMSO.

3.2.12 Lipid quantification in larvae hemolymph

Performed with larvae exposed to 2.5 ppm spinosad for 1 hr, or 2 hr (controls exposed to equivalent volume of DMSO). Lipid levels in hemolymph were also assessed in Line 14 $\alpha 6$ knockout, CantonS $\alpha 6$ knockout and CantonS larvae after exposure to 2.5 ppm spinosad for 2 hr. All respective controls were exposed to equivalent volume of DMSO.

3.2.13 Antioxidant treatment

Performed for Wiggle Index assay and larval survival probability. In both cases, larvae were treated with 300 µg/mL of AD4 for 5 hr prior to exposure to 2.5 ppm spinosad for 2 hr. Controls were exposed to spinosad only without AD4 pre-treatment.

3.2.14 Evaluation of JNK and GstD1 activity

Performed with larvae exposed to 2.5 ppm spinosad for 2 hr (controls exposed to equivalent volume of DMSO for 2 hr).

3.2.15 Lipid extraction and analysis for LC-MS

Performed with larvae exposed to 2.5 ppm spinosad for 2 hr (controls exposed to equivalent volume of DMSO for 2 hr).

3.2.16 RNA isolation, transcriptomics analysis, GO and KEGG pathway analysis

Performed with larvae exposed to 2.5 ppm spinosad for 2 hr (controls exposed to equivalent volume of DMSO for 2 hr).

3.2.17 GCaMP assay

Performed with adult brain neuronal cells exposed to 2.5 ppm or 25 ppm spinosad.

3.2.18 Investigating impacts on lysosomes

To investigate spinosad impacts on lysosomes the LysoTracker staining was used on larval fat bodies and larval brains dissected from 3rd instars. Larvae were dissected in PBS and tissue immediately transferred to PBS solution containing LysoTracker Red DND-99 (1:10,000) (Invitrogen). Fat bodies were maintained in this solution for 3 minutes and brains for 7 minutes, at room temperature. Tissues were then rinsed 3 times in PBS and slides were mounted for immediate microscopy 400x magnification (DsRed filter). For fat body analysis, larvae were exposed to 2.5 ppm spinosad or 2.5 ppm imidacloprid for 2 hr. A total of 6 fat body samples were assessed per group, with 3 random different

sections of 2500 μm^2 accounted per fat body. For brain analysis, larvae were exposed to 2.5 ppm spinosad for 1 hr or 2 hr, in the last case brains were assessed immediately after the 2 hr exposure or 6 hr after that. A total of 7 brain samples were assessed per group, with 3 random different sections of 900 μm^2 accounted per brain. To investigate the hypothesis of $\text{D}\alpha 6$ nAChRs being endocytosed and digested by lysosomes after exposure to 2.5 ppm spinosad for 2 hr, brains from larvae obtained by crossing UAS *D $\alpha 6$* CFP tagged in Line 14 *D $\alpha 6$* KO strain to Gal4-L driver in Line 14 *D $\alpha 6$* KO strain were also subjected to LysoTracker staining. Images were analysed using the software ImageJ and data were analysed using t-test.

3.2.19 Nile red staining of adult retinas

Performed with adults chronically exposed to 0.2 ppm spinosad for 1, 5, 10, 15 and 20 days. All respective controls were exposed to equivalent volume of DMSO. 1-, 5-, 10- and 20-days old, non -exposed, Line 14 $\text{D}\alpha 6$ knockout, CantonS $\text{D}\alpha 6$ knockout and CantonS flies also had their retinas assessed by confocal microscopy.

3.2.20 Electrophysiology of the retina

Performed with adults chronically exposed to 0.2 ppm spinosad for 1, 5, 10, 15 and 20 days. All respective controls were exposed to equivalent volume of DMSO. 5-, 10- and 20-days old, non -exposed, Line 14 $\text{D}\alpha 6$ knockout, CantonS $\text{D}\alpha 6$ knockout and CantonS flies also had their visual system response to light assessed by ERGs.

3.2.21 Spinosad crystals stain with Nile red

Spinosad (Chem Service Inc) diluted in DMSO, cholesterol (Sigma-Aldrich) diluted in DMSO (positive control) and pure DMSO (negative control) reaction to Nile red were tested. In a microscopy slide 5 μL of 1000 ppm solutions of either spinosad or cholesterol, or 5 μL of pure DMSO were incubated with 5 μL Nile red solution (1 $\mu\text{g}/\text{mL}$ in PBS) for 10 minutes. After incubation a cover slip was placed on the top of the final solution and slides were analysed in Leica SP5 Laser Scanning Confocal Microscope at 400x magnification. Red emission was observed with DsRed channel. Images were analysed in the software ImageJ.

3.2.22 Expression of $\alpha 6$ nAChRs in adult brains

The expression pattern of nAChR-D $\alpha 6$ gene in adult brains was assessed in the crossing between D $\alpha 6$ T2A Gal4 (BDSC #76137) and UAS-GFP.nls (BDSC #4775). Adult brains were dissected as described in 3.2.7 fixed in 4% paraformaldehyde (PFA-Electron Microscopy Science) in PBS for 20 minutes at room temperature. PFA was removed and tissues were washed 3 times in PBS. Samples were mounted in Vectashield (Vector Laboratories). Images were obtained with a Leica TCS SP8 (DM600 CS), software LAS X, 400x magnification, using GFP channel. Images were analysed using the software ImageJ.

3.2.23 Transmission Electron Microscopy (TEM)

Performed with adults chronically exposed to 0.2 ppm spinosad 20 days (controls exposed to equivalent volume of DMSO). Electron microscopy of retina and lamina of 20-day old CantonS and CantonS D $\alpha 6$ KO mutants aged in the absence of spinosad was also investigated.

3.2.24 Bang Sensitivity

Performed with adults chronically exposed to 0.2 ppm spinosad 1, 10 and 20 days (controls exposed to equivalent volume of DMSO).

3.2.25 Climbing assay

Performed with adults chronically exposed to 0.2 ppm spinosad 1, 10 and 20 days (controls exposed to equivalent volume of DMSO).

3.2.26 Adult brain histology (Hematoxylin & Eosin staining)

Performed with adults chronically exposed to 0.2 ppm spinosad 20 days (controls exposed to equivalent volume of DMSO).

3.2.27 Graphs and Statistical analysis

All graphs were created, and all statistical analysis were performed in the software R (v.3.4.3). Figures and schematics were designed using the free image software Inkscape (0.92.4).

3.3 Results & Discussion

3.3.1 Low doses of spinosad affect larvae motility and survival

As a starting point to study the systemic effects of low dose spinosad exposure, a dose that could reduce the movement of third instar larvae by 50% during a 2 hr exposure was determined using Wiggle Index movement assay. Among the doses tested (**Figure 3.2a**), 2.5 ppm was selected for matching this criterion (**Figure 3.2b**). This dose only showed impacts on larval movement after 90 min of exposure, indicating that it takes a relatively long time for spinosad to reach motor neurons expressing the targeted nAChRs. The majority of larvae exposed to spinosad under these conditions did not undergo metamorphosis and died over the course of the next 8 days (**Figure 3.2c**). Under the rearing conditions used, metamorphosis should be initiated within 24-36 hr. 17% of the larvae exposed to spinosad initiated metamorphosis, but died as pupae that were small and irregular in morphology (**Figure 3.3**). Such phenotype could be a consequence of starving for several days before starting metamorphosis. One single individual among 100 exposed larvae survived the exposure and emerged as an adult. The high mortality rate may suggest that once present in the CNS, spinosad is not easily metabolised and excreted.

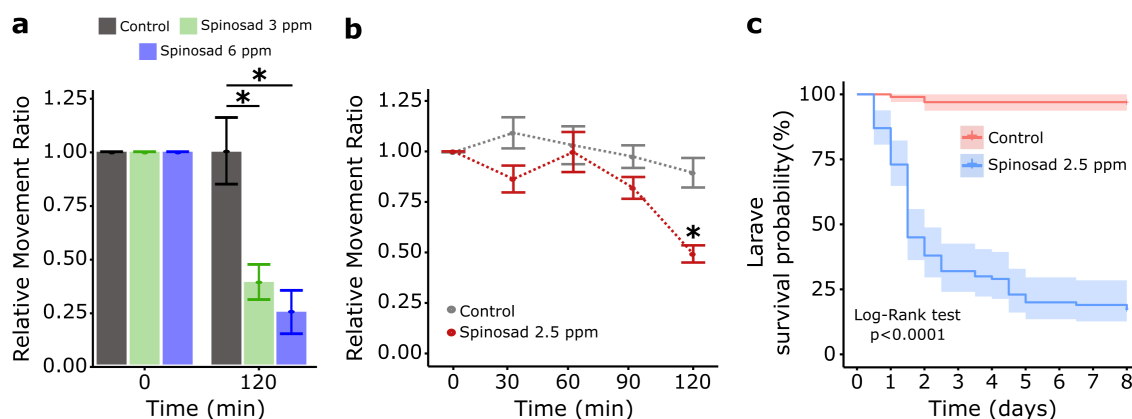


Figure 3.2. Toxicology and viability assays with larvae exposed to different doses of spinosad for 2 hr. **a**, Dose response to 3 ppm and 6 ppm of spinosad by an assay of larval movement, the Wiggle Index. Values are expressed in terms of Relative Movement Ratio (RMR) in response to the exposure time in minutes ($n = 25$ larvae/ replicate; 4 replicates/ treatment). **b**, Dose response to 2.5 ppm spinosad by Wiggle Index ($n = 100$ larvae/ treatment). **c**, Survival probability (%) of larvae submitted to a 2 hr exposure to 2.5 ppm spinosad, rinsed and placed back onto insecticide-free medium ($n = 25$ larvae/ replicate; 4 replicates/ treatment). Error bars in **a** and **b** represent standard error (t-test; * $P < 0.01$); shaded areas in **c** represent 95% confidence interval (Kaplan-Meier method and the Log-rank Mantel-Cox test; $P < 0.0001$).

Pupae appearance after larvae are exposed to 2.5 ppm spinosad for 2 hr

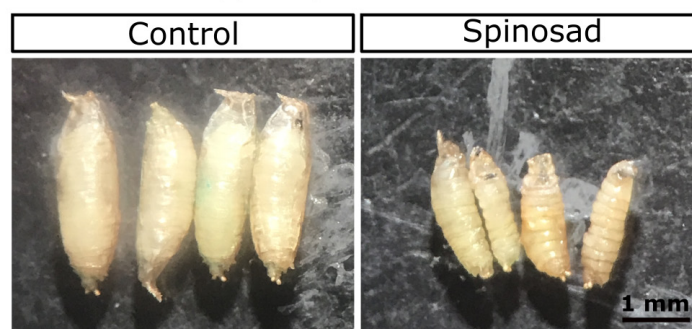


Figure 3.3. One day old pupae appearance of larvae exposed to spinosad. 17% of the larvae exposed to 2.5 ppm spinosad for 2 hr, rinsed and placed back onto insecticide-free media, initiated metamorphosis. Pupae were small and irregular in morphology and a single adult emerged among the exposed ($n = 25$ larvae/ replicate; 4 replicates/ treatment).

3.3.2 Spinosad creates oxidative stress and impairs mitochondria

Since other insecticides have been shown to create oxidative stress, the ability of spinosad to increase levels of ROS was investigated. The accumulation of the superoxide anion (O_2^-), was assessed using dihydroethidium (DHE) staining in larvae exposed to 2.5 ppm spinosad. After a 1 hr exposure there was a mean 89% increase in O_2^- accumulation in brains, but there was no significant change in the midgut (**Figure 3.4a, b**). After a 2 hr exposure there was a mean 28% increase in accumulation in the midgut and a mean 44% increase in O_2^- accumulation in the brain, compared to controls (**Figure 3.4a, b**). While there was decrease in the accumulation of O_2^- in brain after 2 hr compared to 1 hr, accumulation of ROS was observed in this organ before it was observed in the midgut. These data indicate that the spinosad triggers an increase in ROS in the central nervous system where the nAChRs are present. It subsequently reaches other organs.

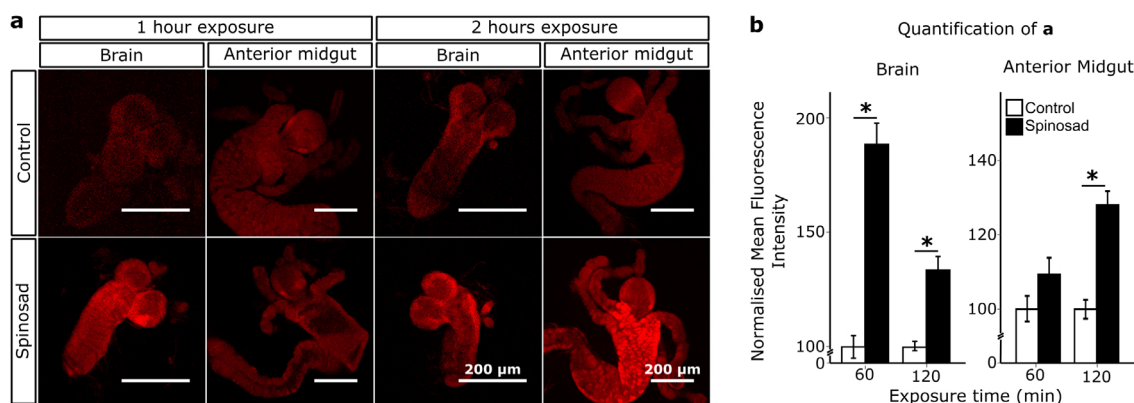


Figure 3.4. Spinosad induces the accumulation of ROS in the brain and midgut. a, Superoxide levels in brain and anterior midgut exposed to 2.5 ppm spinosad for either 1 hr or 2 hr. Images stained with DHE and obtained in Leica SP5 Laser Scanning Confocal Microscope, 200x magnification. **b,** Quantification of **a**, normalized mean fluorescence intensity ($n = 15$ larvae/ treatment; 3 image sections/ larva). Error bars represent mean $\pm$ s.e.m. (t-test; * $P < 0.01$).

After confirming the accumulation of ROS, mitochondrial turnover was assessed using the reporter line MitoTimer (Gottlieb and Stotland 2015) Spinosad exposure induced an increase of 31% and 36% for the green (healthy mitochondria) and red (stressed mitochondria) signals respectively, in the optical lobes of the larval brain (**Figure 3.5a, b**). For the digestive tract, a 19% and 32% increase was observed for green and red signal respectively after the 2 hr exposure (**Figure 3.5a, b**). The increase in the signal for stressed mitochondria is accompanied by an increase in the signal for healthy

mitochondria. This suggests that even though the exposure is inflicting mitochondrial damage, it also led to mitogenesis as a mechanism to compensate for an impairment in the energy metabolism (Chang et al. 2010).

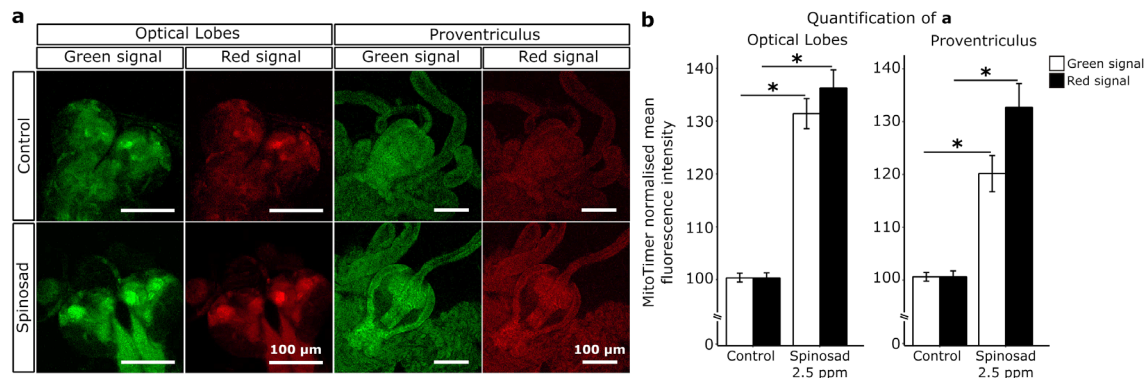


Figure 3.5. Mitochondrial turnover is increased in larvae exposed to 2.5 ppm of spinosad for 2 hr. MitoTimer reporter fly strain. **a**, Optic lobes in the larval brain and proventriculus in the larval alimentary canal. Insecticide exposure increased the signal for healthy (green) and unhealthy (red) mitochondria (n = 20 larvae/ treatment; 3 image sections/ larva). Images obtained in Leica SP5 Laser Scanning Confocal Microscope, 200x magnification. **b**, Quantification of **a** in terms of normalized mean fluorescence intensity. Error bars indicate standard error (t-test; *P < 0.01).

Two mitochondrial phenotypes were also investigated in larvae under the same exposure conditions: ATP levels and mitochondrial aconitase activity. Immediately after a 2 hr exposure to 2.5 ppm spinosad, there was a mean 36% increase in systemic ATP levels (**Figure 3.6a**). 12 hr after this 2 hr exposure, the systemic levels of ATP were reduced by 16.5% (**Figure 3.6b**). The initial increase in energy levels are consistent with the mitogenesis proposed based on the MitoTimer results. A mean 34% reduction in the activity of the ROS sensitive enzyme, mitochondrial aconitase (Yan et al. 1997), was measured at this same time-point (**Figure 3.6c**), indicating that ROS are accumulating in mitochondria during the 2 hr exposure. The reduction in ATP levels 12 hr after the exposure suggests that ROS accumulating in mitochondria could be impairing energy metabolism. This decrease in energy indicated that an increase in ROS is resulting in oxidative stress.

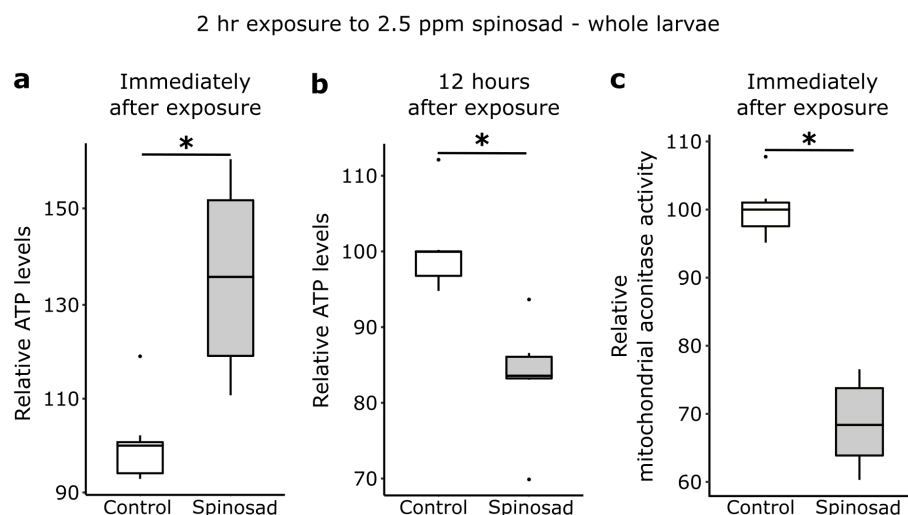


Figure 3.6. Spinosad impairs ATP levels and reduces mitochondrial aconitase activity. Larvae were exposed to 2.5 ppm spinosad for 2 hr. **a**, Relative systemic ATP levels immediately after spinosad exposure (n = 20 larvae/ replicate; 6 replicates/ treatment). **b**, Relative systemic ATP levels 12hr after the exposure to spinosad (n = 20 larvae/ replicate; 6 replicates/ treatment). **c**, Relative mitochondrial aconitase activity immediately after spinosad exposure (n = 25 larvae/ replicate; 6 replicates/ treatment). (t-test; *P < 0.01).

3.3.3 Knockout of *GstD1* increases spinosad susceptibility

The effects of *GstD1* activity were tested on motility of spinosad-exposed larvae. Besides the antioxidant role, GSTs are able to metabolize the major insecticide classes (Enayati et al. 2005) and Nuclear Magnetic Resonance has shown that *GstD1* binds spinosyn A (Chen & Gooley unpubl.). Even though the *Drosophila* genetic background control strain used for this experiment showed a greater tolerance to spinosad compared to Line 14, the *GstD1* knockout larvae were significantly more sensitive to spinosad exposure. At the 2 hr exposure time-point the relative movement ratio of the knockout flies was 40% lower than at time zero. In comparison, the relative movement ratio for the controls was 22% lower (**Figure 3.7**). The greater movement reduction in larvae with no *GstD1* expression points for the role of this enzyme in protecting flies against the damage inflicted by spinosad exposure either by directly binding to spinosad or by removing ROS and oxidized compounds produced by spinosad exposure.

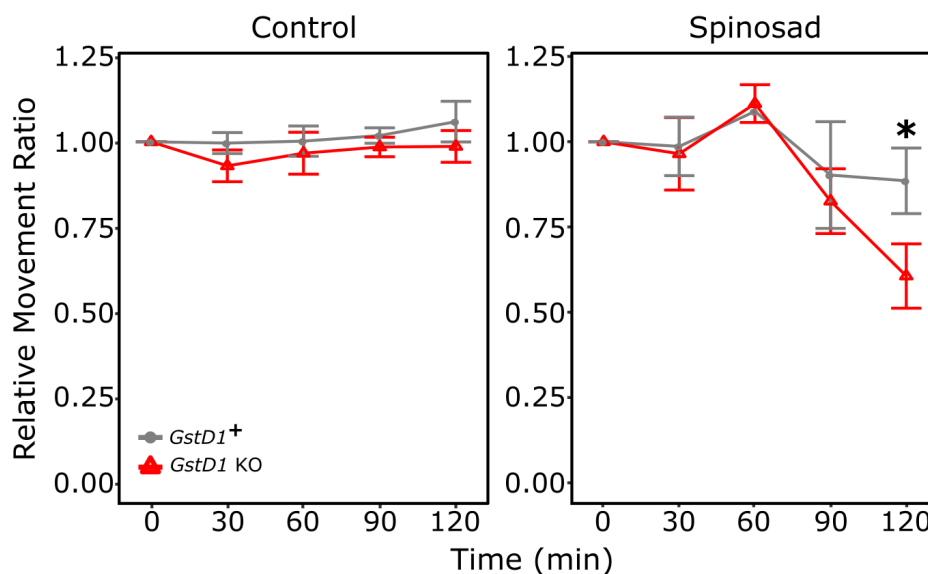


Figure 3.7. Knockout of the antioxidant enzyme GSTD1 increases sensitivity to spinosad. *GstD1* background control (*GstD1*⁺) larvae and *GstD1* knockout (*GstD1* KO) larvae exposed to 2.5 ppm spinosad for 2 hr. Dose response to insecticide measured using Wiggle Index analysis. Results are expressed in terms of Relative Movement Ratio (RMR) values in response to the exposure time in minutes (n = 25 flies/ replicate; 4 replicates/ treatment). All points correspond to the mean RMR values with standard error (t-test; *P < 0.01).

3.3.4 Oxidative stress created by spinosad affects the lipid environment, motility, and survival

Oxidative stress has the ability to affect the lipid environment of metabolic tissues, causing bulk redistribution of membrane lipids into LDs (Bailey et al. 2015). The possibility of impairment of the lipid environment in face of increased oxidative stress caused by spinosad exposure was therefore investigated. Larvae exposed to 2.5 ppm spinosad for 2 hr showed a 52% increase in the area covered by LDs in the fat body (**Figure 3.8a, b**). There was a 2.6-fold increase in the mean number of small LDs and a 4-fold decrease in the mean number of large LDs (**Figure 3.8c, d**). Pre-treatment with the antioxidant AD4 significantly reduced the impacts of spinosad exposure on this phenotype. Even though still significant, the area occupied by LDs in fat bodies increase only 20% with AD4 pre-treatment (**Figure 3.8a, b**). Antioxidant pre-treatment also improved movement of larvae exposed to spinosad. At the 2 hr exposure time-point, the larval movement ratio for pre-treated larvae was 33% lower than at time zero. For larvae that were not pre-treated, it was 65% lower (**Figure 3.9**). Antioxidant pre-treatment also

improved survival. The adult emergence of exposed larvae increased from 4% to 15% in individuals pre-treated with AD4 compared to those treated with spinosad only (**Figure 3.10**). These data demonstrate that oxidative stress triggered by spinosad exposure is a causal factor in impacting the lipid environment in the fat body, larval movement, and the capacity to complete development.

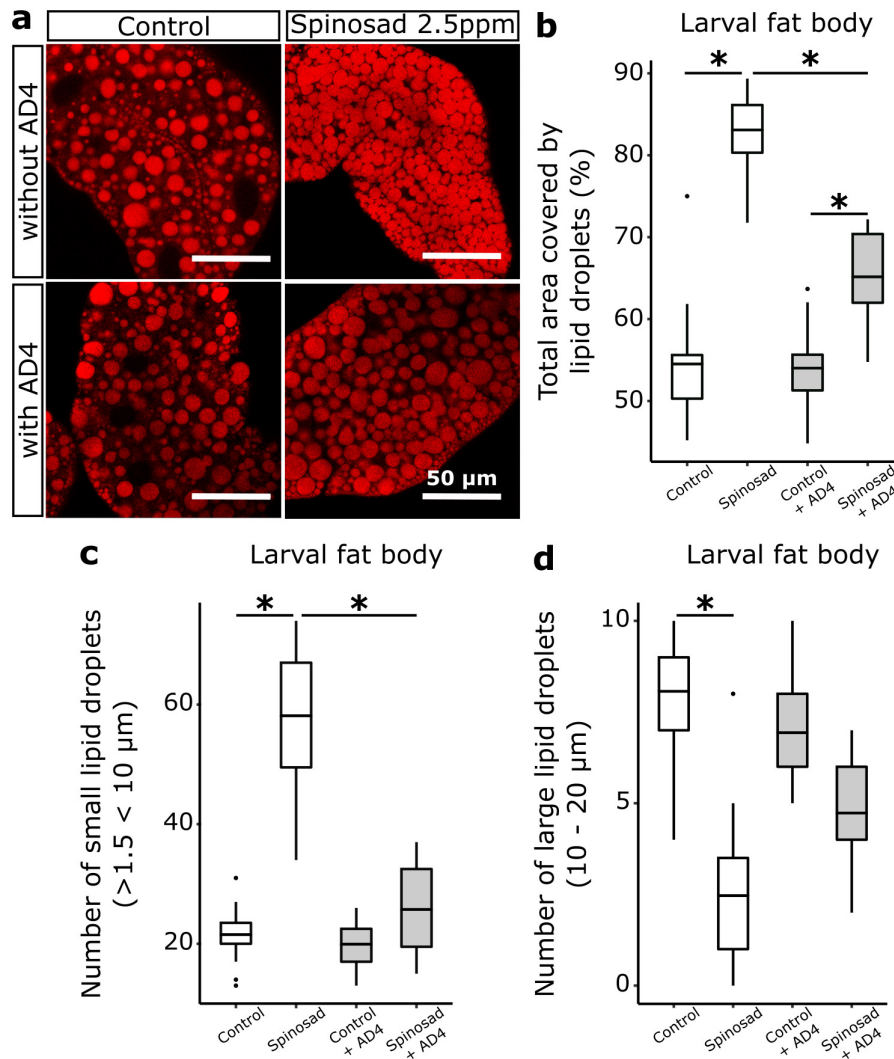


Figure 3.8. Spinosad increases lipid storage in fat body and antioxidant pre-treatment reduces this accumulation. **a**, Larvae treated with 300 μ g/mL of antioxidant N-acetylcysteine amide (AD4) for 5 hrs prior to a 2 hr exposure to 2.5 ppm spinosad showed a reduced accumulation of LDs in the fat body. Nile red staining. Images obtained in Leica SP5 Laser Scanning Confocal Microscope, 400x magnification (n = 3 larvae/treatment; 5 image sections/ larva). **b**, Percentage of area occupied by LD in fat body (sections of 50 μ m x 50 μ m). **c**, Number of small LD (> 1.5 μ m < 10 μ m) in larval fat body (sections of 50 μ m x 50 μ m). **d**, Number of large LD (10 μ m - 20 μ m) in larval fat body (sections of 50 μ m x 50 μ m). (t-test; *P < 0.01).

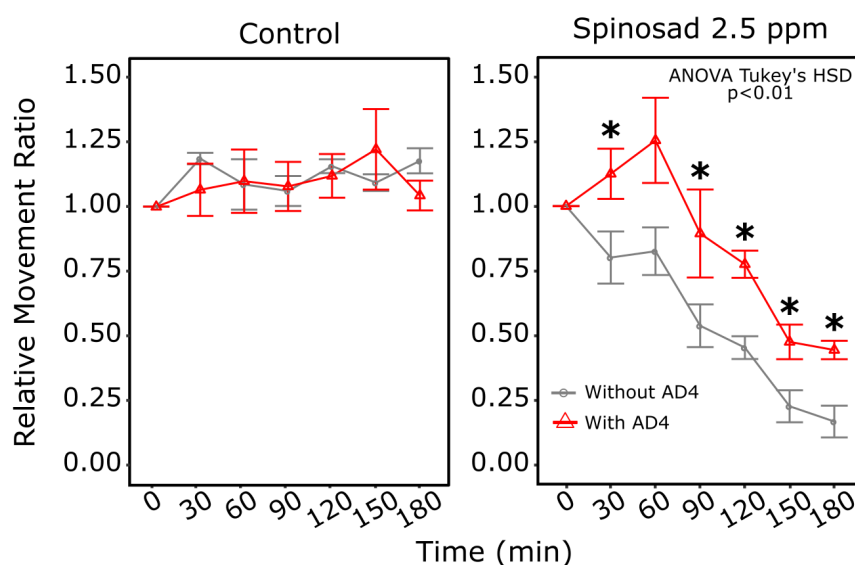


Figure 3.4. Antioxidant pre-treatment improves the movement of spinosad exposed larvae. Larvae treated with 300 $\mu\text{g/mL}$ of antioxidant N-acetylcysteine amide (AD4) for 5 hrs prior to the exposure to 2.5 ppm spinosad. Dose response to insecticide by Wiggle Index analysis. Results are expressed in terms of Relative Movement Ratio (RMR) values in response to the exposure time in minutes ($n = 25$ larvae/ replicate; 4 replicates/ treatment). All points correspond to the mean RMR values with s.e.m. (t-test; * $P < 0.01$).

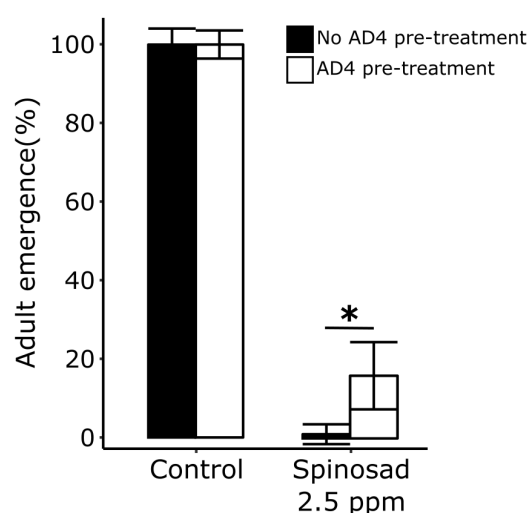


Figure 3.5. Antioxidant pre-treatment improves survival of larvae exposed to spinosad. Larvae treated with 300 $\mu\text{g/mL}$ of antioxidant N-acetylcysteine amide (AD4) for 5 hrs prior to the exposure to 2.5 ppm spinosad show increased survival ($n = 100$ larvae/ treatment). Corrected adult emergence (%) after larvae exposed to 2.5 ppm spinosad for 2 hr are rinsed and placed back onto insecticide-free medium and allowed to develop to adulthood ($n = 25$ larvae/ replicate; 4 replicates/ treatment). Bars indicate corrected percentage survival (Abbott's correction); error bars indicate 95% confidence interval (t-test; * $P < 0.01$).

3.3.5 Spinosad doses that do not affect survival also impair the lipid environment

The next issue to be addressed was whether doses of spinosad that do not impact survival could also trigger changes on the lipid environment of metabolic tissues in larvae. Survival assays were performed to find the highest acute doses of spinosad that do not interfere with adult emergence. 3rd instar larvae were exposed to different doses for a 2 hr or 4 hr period, rinsed and placed back onto insecticide-free media and allowed to develop to adulthood. Among the doses tested 0.1 ppm for 4 hr and 0.5 ppm for 2 hr were selected for matching the criteria established (**Figure 3.11**). Both doses caused on average a 29% increase in the area occupied by LDs in fat bodies (**Figure 3.12a, b**). These doses caused a smaller impact than the dose of 2.5 ppm of spinosad for 2 hr, showing that this phenotype is dose dependent. Once again an increase in the number of small LDs and reduction in the number of large LDs was observed (**Figure 3.12c, d**). The changes in the numbers of small and large LDs is greater than the changes in the area covered by LDs, suggesting that the oxidative stress triggered by insecticide is not only causing redistribution of membrane lipids to LDs, but also causing the fission of large LDs into smaller ones.

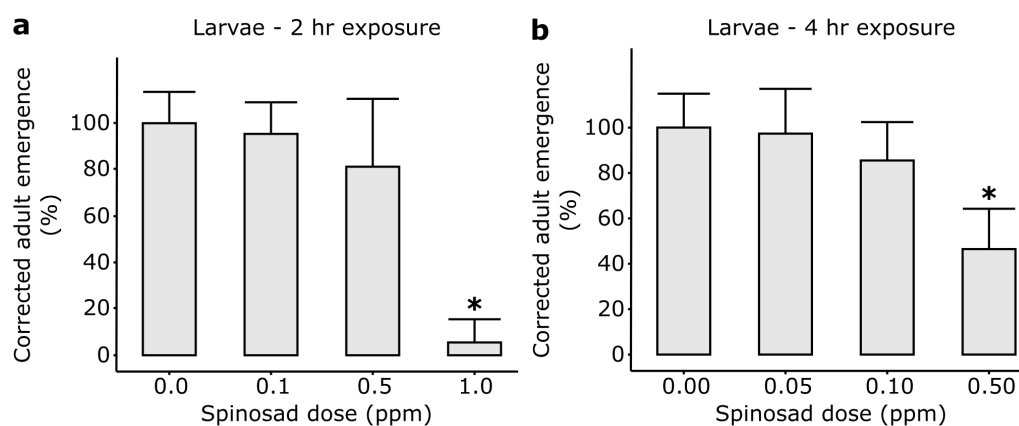


Figure 3.6. Impacts on adult emergence after larval exposure to different spinosad doses. Exposed larvae were rinsed in 5% sucrose and placed back onto insecticide-free media for quantification of adult emergence. **a**, Larvae exposed to 0.1, 0.5 or 1 ppm spinosad for 2 hrs. Exposure to 0.5 ppm spinosad for 2 hrs was selected as the highest dose that does not interfere with adult emergence. **b**, Larvae exposed to 0.05, 0.1 or 0.5 ppm spinosad for 2 hrs. Exposure to 0.1 ppm spinosad for 4 hrs was selected as the highest dose that does not interfere with adult emergence. Grey bars indicate corrected percentage survival (Abbott's correction) ($n = 60$ animals/ treatment). Error bars indicate 95% confidence interval (One-way ANOVA, Turkey's HSD; * $P < 0.05$).

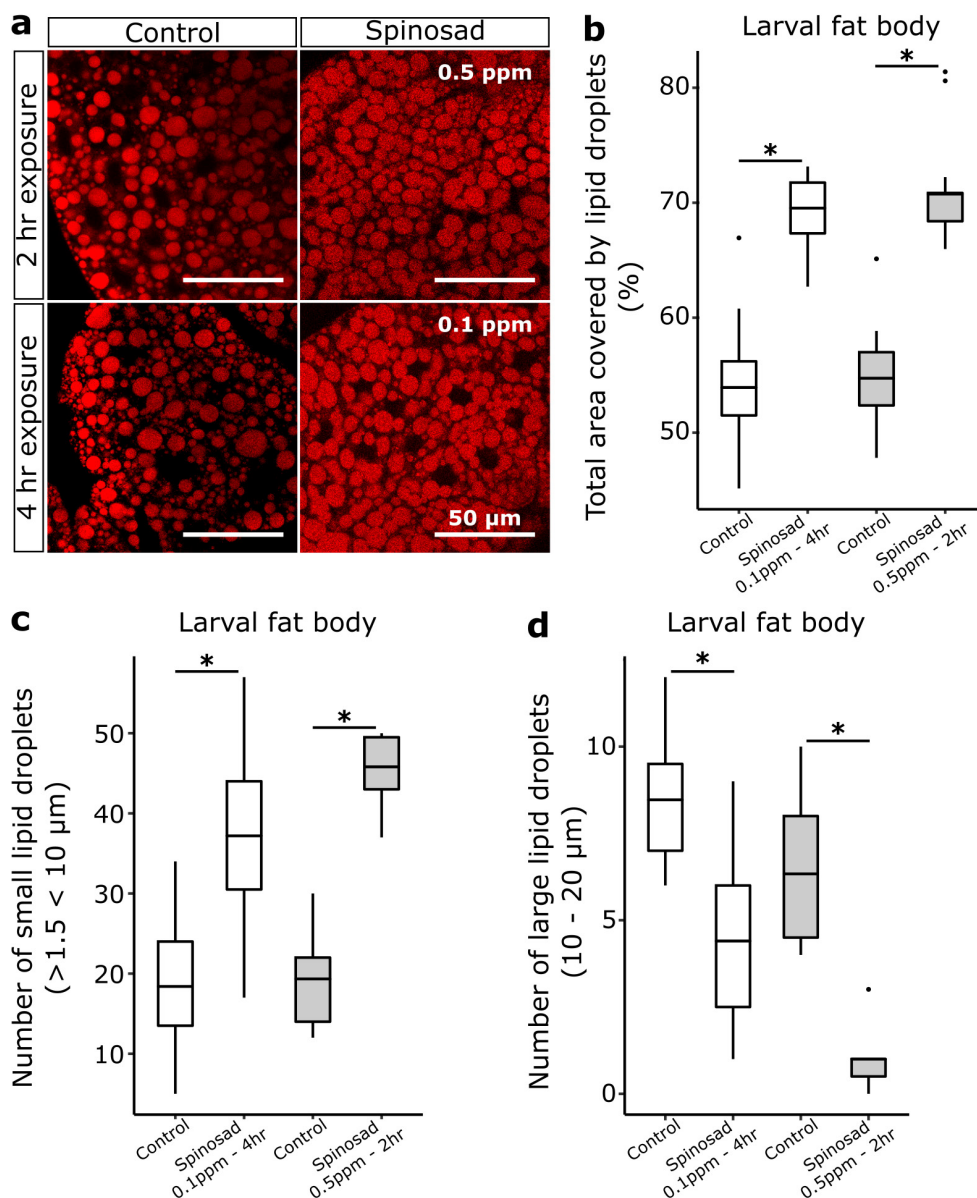


Figure 3.7. Higher doses of spinosad cause greater accumulation of lipid storage in fat body. **a**, Larvae exposed to either 0.1 ppm for 4 hr or 0.5 ppm for 2 hr. Nile red staining. Images obtained in Leica SP5 Laser Scanning Confocal Microscope, 400x magnification (n = 3 larvae/ treatment; 5 image sections/ larva). **b**, Percentage of area occupied by LD in fat body (sections of 50 μ m x 50 μ m). **c**, Number of small LD (> 1.5 μ m < 10 μ m) in larval fat bodies (sections of 50 μ m x 50 μ m). **d**, Number of large LD (10 μ m - 20 μ m) in larval fat bodies (sections of 50 μ m x 50 μ m). (t-test; *P < 0.01).

The highest doses of spinosad that do not impact survival also affected the lipid environment of the Malpighian tubules and the midgut. The doses of 0.1 ppm for 4 hr and 0.5 ppm for 2 hr caused a mean 72% and 73% reduction in the total number of LDs in the Malpighian Tubules, respectively (**Figure 3.13a, b**). There was also a reduction in LD numbers in the midgut. In all control replicates zones of LDs accumulation were

identified. Such zones were not present, or were smaller, in the midgut of larvae exposed to spinosad (**Figure 3.13c**).

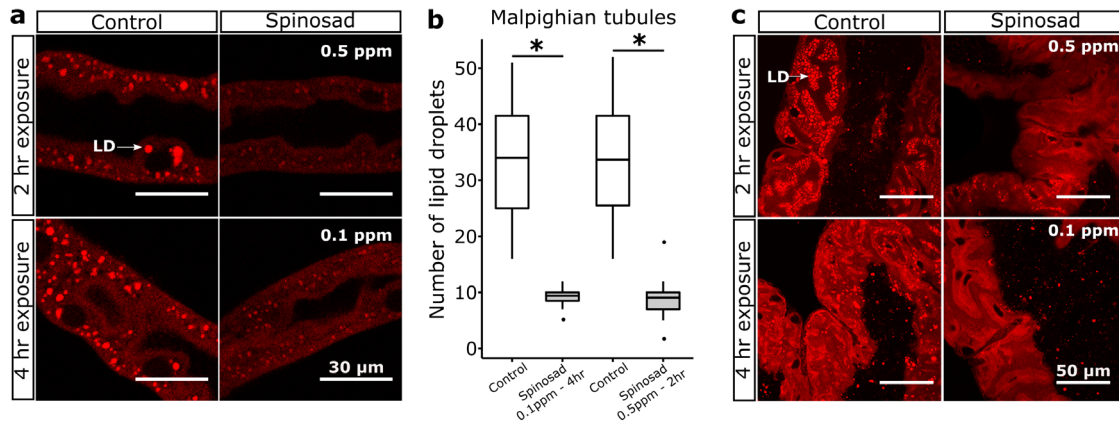


Figure 3.8. Spinosad reduces lipid levels in the Malpighian tubules and midgut. Larvae exposed to either 0.5 ppm spinosad for 2 hr, or 0.1 ppm for 4 hr. Nile red staining. Images obtained in Leica SP5 Laser Scanning Confocal Microscope, 400x magnification ($n = 3$ larvae/ treatment; 5 image sections/ larva). **a**, Malpighian Tubules main segment microscopy. White arrow indicates a LD. **b**, Number of lipid droplets per Malpighian Tubule (sections of 30 μm x 30 μm) ($n = 3$ larvae/ treatment; 5 sections/ larva). **c**, Posterior midgut. White arrow indicates LDs. Zones with LD accumulation were only found in non-exposed animals ($n = 3$ larvae/ treatment). (t-test; * $P < 0.01$).

The levels of lipids in the hemolymph were measured in response to exposure to 2.5 ppm spinosad, using the sulfo-phospho-vanillin method. No change was observed at 1 hr exposure, but at the 2 hr time point a significant 10% mean increase in lipid levels detected (**Figure 3.14**). Considered together, the differential impacts on the lipid environment of the different metabolic tissues raised the possibility that spinosad induced lipid mobilization between them. This possibility is discussed in more detail along with the tissue specific transcriptomic analysis, later in section 3.3.9.

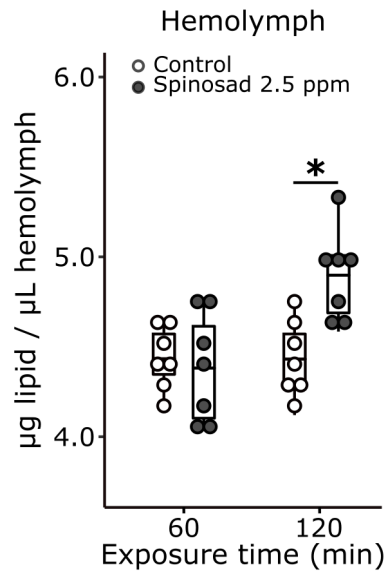


Figure 3.9. Spinosad increases lipid levels in the hemolymph. Amount of lipid in the hemolymph ($\mu\text{g}/\mu\text{L}$) of larvae exposed to 2.5 ppm spinosad for 1 hr or 2 hr. Measured using the colorimetric vanillin assay ($n = 7$ replicates/ treatment/ time point; 30 larvae/ replicate). (t-test; * $P < 0.01$).

3.3.6 A brain signal triggers the impacts of spinosad on metabolic tissues

Once inside the insect body, spinosad could theoretically access any tissue via the open circulatory system. That elevated levels of ROS were observed in the brain earlier than in metabolic tissues (**Figure 3.4**) and that the spinosad target, the nAChR subunit $D\alpha 6$ subunit, is localized in the brain (Perry et al., 2015; Somers et al., 2015) prompts a significant question. Could the interaction between spinosad and $D\alpha 6$ in the brain provide the signal that ultimately leads to the observed disturbance of the lipid environment in the metabolic tissues? *Dα6* knockout flies are viable, allowing this question to be addressed. Two different *Dα6* knockout mutants (CantonS *Dα6* KO and Line 14 *Dα6* KO) and their respective genetic background control lines (CantonS and Line 14) were tested. Larvae were exposed to 2.5 ppm of either spinosad or imidacloprid for 2 hr. Line 14 *Dα6* KO mutant larvae showed no impact on LD dynamics after spinosad exposure, but showed a significant mean increase of 23% in the area occupied by LDs after imidacloprid exposure (**Figure 3.15a, b**). CantonS wildtype flies showed a significant mean increase of 25.6% and 44.6% in the area occupied by LDs in response to imidacloprid and spinosad exposure, respectively (**Figure 3.15a, c**). CantonS *Dα6* KO mutant larvae, however showed a significant mean increase of 13.7% in the area occupied by LDs after imidacloprid exposure, but no changes after spinosad exposure (**Figure 3.15a, d**).

Insecticide impacts on lipid levels in hemolymph were also assessed in the mutant *Drosophila* strains. In the same way it was observed for Line 14, CantonS wildtype larvae showed a significant 5% reduction in the mean lipid levels when exposed to imidacloprid and a significant 13% average increase in lipid levels when exposed to spinosad (**Figure 3.15e**). Line 14 *Dα6* KO mutants and CantonS *Dα6* KO mutants, however, showed no increase in lipid levels when exposed to spinosad, but still a significant 23% decrease in the mean lipid levels after imidacloprid exposure (**Figure 3.15e**). Considered together these data support the hypothesis that the changes to the lipid environment in the fat body and hemolymph are triggered by a signal initiated in the brain: the binding of spinosad binding to its neuronal target, Dα6. It should be noted that in the absence of insecticide exposure the Line 14 *Dα6* KO and CantonS *Dα6* KO mutants had a greater area of the fat body occupied by LDs (17% and 20%, respectively) and higher levels of lipid in the hemolymph (10% and 20%, respectively) than their respective background control lines. These observations suggest that despite their apparently overall health, the knockout of nAChR Dα6 subunit is triggering impacts on the lipid environment. A Genome-Wide Association Study of oxidative stress resistance in *Drosophila* pointed to an unexpected role for nAChR Dα6 subunit in responding to oxidative stress (Weber et al. 2012), which could be involved with generation of the phenotypes in *Dα6* KO flies.

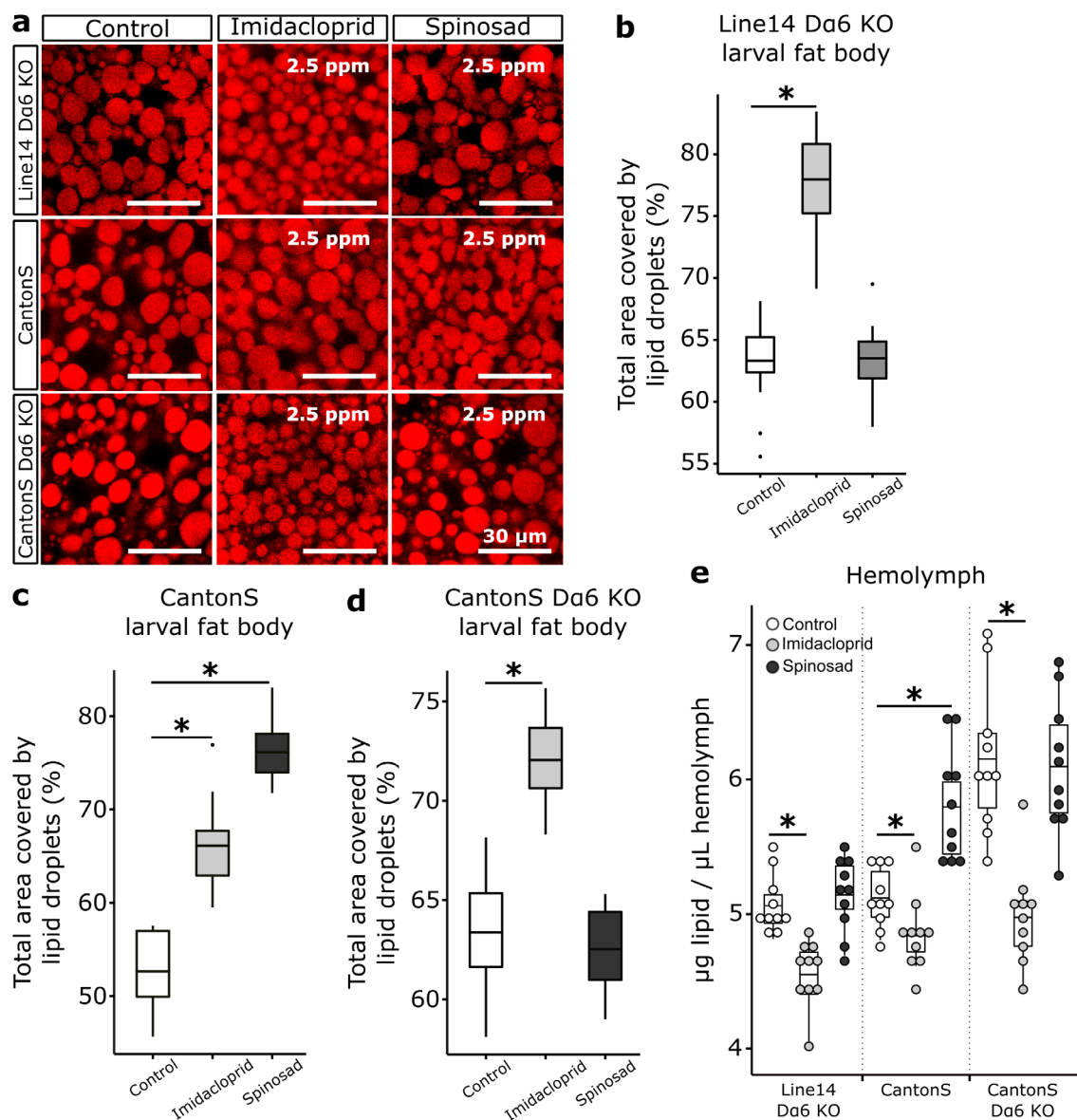


Figure 3.10. *Da6* knockout (KO) resistant larvae show a decreased impact on lipid environment in response to spinosad exposures. **a**, Larvae exposed to 2.5 ppm of either imidacloprid or spinosad for 2 hr. Nile red staining. Images obtained in Leica SP5 Laser Scanning Confocal Microscope, 400x magnification (n = 3 larvae/ treatment; 5 image sections/ larva). **b**, Percentage of area occupied by LDs in fat body (sections of 50 µm x 50 µm) of Line 14 *Da6* KO larvae. **c**, Percentage of area occupied by LDs in fat body (sections of 50 µm x 50 µm) of CantonS larvae. **d**, Percentage of area occupied by LDs in fat body (sections of 50 µm x 50 µm) of CantonS *Da6* KO larvae. **e**, Amount of lipids in hemolymph (µg/µL) of Line 14 *Da6* KO, CantonS and CantonS *Da6* KO larvae exposed to insecticides for 2 hr. Measured using the colorimetric vanillin assay (n = 10 replicates/ treatment/ time-point; 30 larvae/ replicate). (t-test; *P < 0.01).

3.3.7 Spinosad induces the activation of major stress response pathways

The dose of 2.5 ppm spinosad also increased the expression of JNK stress signalling pathway and *GstD1* expression in the midgut after a 2 hr exposure. The mean *GstD1* signal increased by 63%, while the mean JNK increased by 66% compared to non-exposed larvae (**Figure 3.16a, b**). The activation of JNK pathway and *GstD1* expression suggests that spinosad increases the expression of genes involved in antioxidant response and other stress response pathways in *D. melanogaster*, a result that was further investigated with tissue specific transcriptomic analysis discussed later in this Chapter (section 3.3.9).

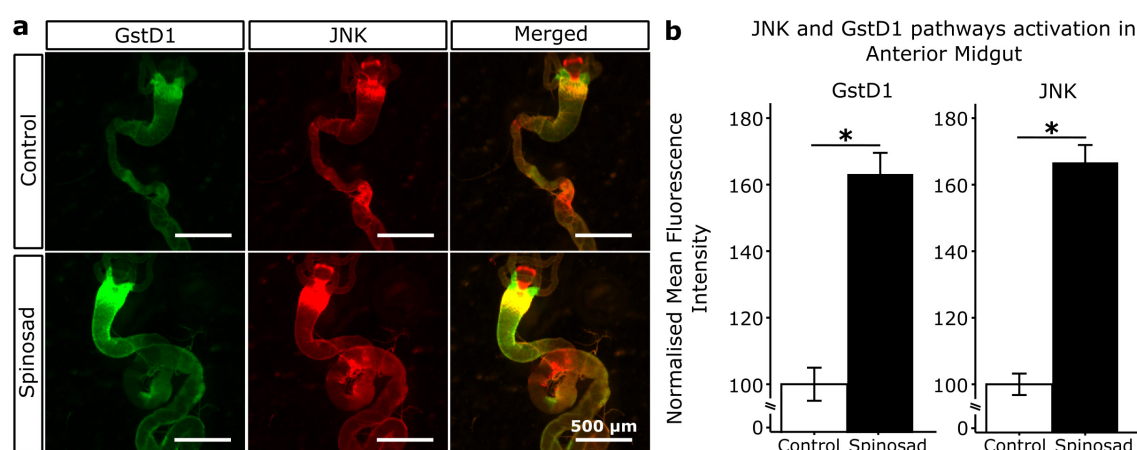


Figure 3.11. Spinosad increases the expression of GstD1 and the JNK pathway in the anterior midgut. Double reporter (*GstD1*-GFP; TRE-DsRed) larvae exposed to 2.5 ppm spinosad for 2 hr ($n = 40$ larvae/ treatment). **a**, Activation of *GstD1* and the JNK pathway in the anterior midgut. Images were acquired in Leica M205 FA microscope with Leica DFC7000T camera, using the Leica Application Suite X (LAS X) software at 30x magnification. **b**, Mean Fluorescence Intensity of the *GstD1* and JNK reporter signals in the anterior midgut, gastric caeca and proventriculus. Images obtained in Leica SP5 Laser Scanning Confocal Microscope, 400x magnification. (t-test; * $P < 0.01$).

3.3.8 Spinosad triggers major alterations in the lipidome

The lipidomics profiles of whole larvae were then assessed using MS-LC. The results reflected the changes observed in the lipid environment of metabolic tissues and the impairment of mitochondria activity in larvae exposed to 2.5 ppm spinosad for 2 hr. 18 major lipid classes were identified: triacylglycerols (TAG), phosphatidylcholines (PC), diacylglycerols (DAG), phosphatidylethanolamines (PE), odd chain phosphatidylcholines (oddPC), phosphatidylinositols (PI), lysophosphatidylcholines

(LPC), lysophosphatidylethanolamines (LPE), phosphoethanolamines alkyl ether (PE(O)), phosphoethanolamines 1Z-alkenyl ether (PE(P)), phosphatidylserines (PS), phosphatidylcholines alkyl ether (PC(O)), phosphatidylglycerols (PG), cholesterol esters (CE), lysophosphatidylcholines alkyl ether (LPC(O)), cardiolipins (CL), dihydroceramides (dhCer) and modified phosphatidylcholines (modPC) plus three major classes of oxidized compounds (oxidized phosphatidylcholines, oxidized phosphatidylethanolamines and oxidized phosphatidylglycerols). 80% of total lipid quantified was from the TAG, PC, DAG, and PE classes.

Significant changes were observed in the levels of 88 lipids out of 378 detected by mass spectrometry (**Figure 3.17a**; **Supplementary Table I**). The main lipid classes affected were PE, PC, TAG, and CL. The multivariate analysis (**Figure 3.17b**) indicates that the overall lipidomics profile of exposed larvae sit tightly together and are distinct from controls. The reduction in the levels of PE, PC and TAG species correlate with the impacts on lipid droplet dynamics observed in metabolic tissues (section 2.3.10) of larvae exposed to spinosad. The use of whole larvae for lipidomic analysis diluted some of the tissue-specific responses, such as the accumulation of LD in fat body and concomitant reduction in Malpighian tubules and midguts. The use of whole larvae did allow the identification of broader impacts on larval biology, such as 65% reduction in the levels of identified CLs (**Figure 3.17c**). This corroborates the observed impacts on mitochondria, since CLs are vital for mitochondrial energy metabolism (Kagan et al. 2014; Ren et al. 2014) and their reduction is associated with increased oxidative stress (Quintana et al. 2010). A significant increase in 5 oxidized lipid species was also measured in larvae exposed to spinosad (HPOD keto 36:5-PE-/18:3 neg; HPOT keto 34:3-PG-/16:0 neg; 2HPOT keto 36:4-PE-/18:1 neg; 2HPOT keto 36:5-PG-/18:2 neg and HPOT keto 34:2-PG-/16:0 neg), besides a decrease in six other oxidized compounds (HOD 34:3-PC-/16:0; oPDA 34:3-PC-/18:3; HOT 34:3-PC-/16:0; M36:4-PC-/18:1; HPOT keto 36:4-PE-/18:1 neg and HOT 34:2-PC-/16:0), suggesting that spinosad has a broader impact on lipid peroxidation.

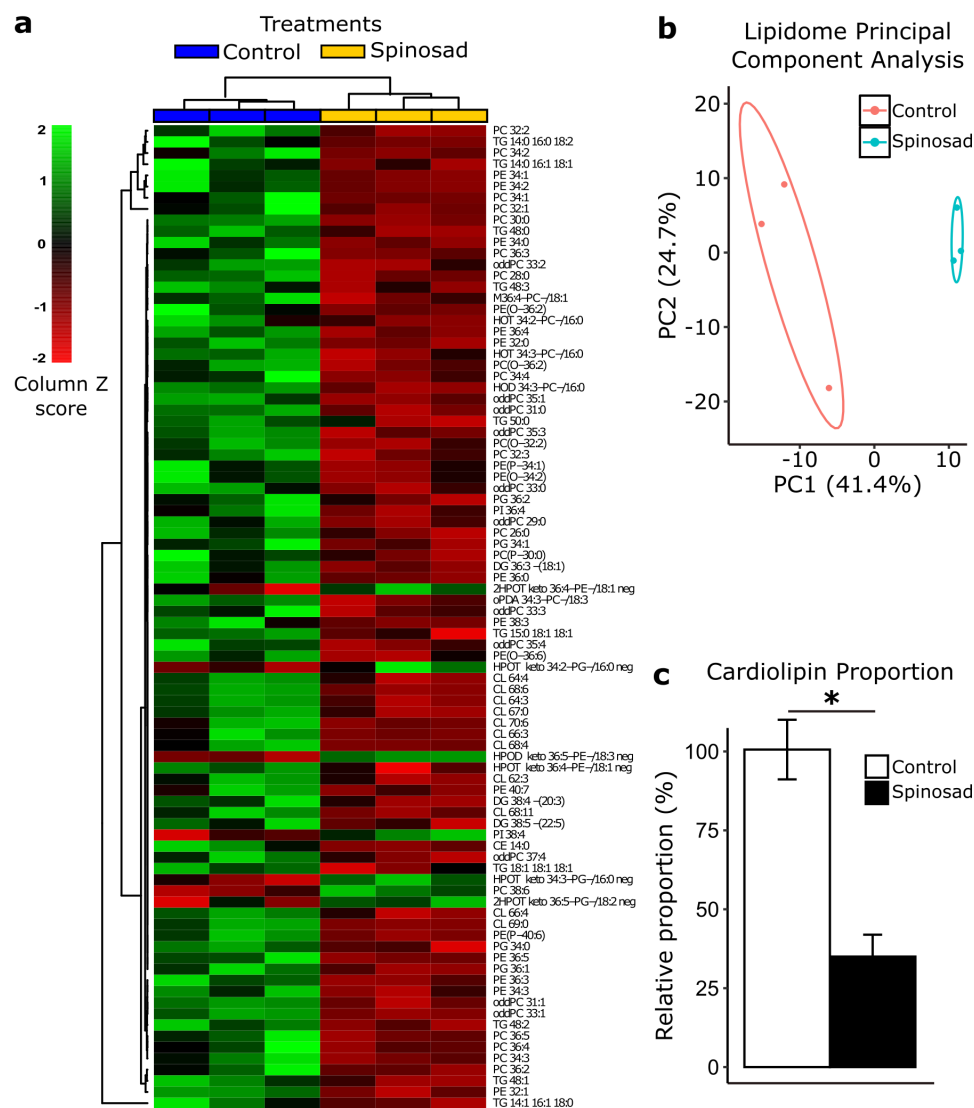


Figure 3.12. Spinosad disturbs the lipid profile of exposed larvae. Lipidomic profile of larvae exposed to 2.5 ppm spinosad for 2 hr (n = 10 larvae/ replicate; 3 replicates/ treatment). **a**, 88 lipid species out of the 378 identified were significantly affected by insecticide treatment (One-way ANOVA, Turkey's HSD, $P < 0.05$). The column Z score is calculated subtracting from each value within a row the mean of the row and then dividing the resulting values by the standard deviation of the row. The features are colour coded by row with red indicating low intensity and green indicating high intensity. **b**, Principal Component Analysis of 378 lipid species. Each dot represents the lipidome data sum of each sample. First component explains 41.4% of variance and second component explains 24.7% of variance. **c**, Relative proportion of cardiolipins in exposed animals versus control. Error bars in c represent mean $\pm$ s.e.m. (t-test; * $P < 0.01$).

3.3.9 Acute exposures to spinosad cause major perturbations in gene expression

Larvae exposed to 2.5 ppm spinosad for 2 hr were also submitted to a tissue specific (brain and fat body) transcriptomic analysis (**Supplementary Table II**). The transcriptomic analysis identified 9,640 transcripts expressed in brains. 380 of these transcripts were downregulated in spinosad-exposed samples (foldchange $>|2|$, P adj < 0.05), whereas 1125 were upregulated (foldchange $>|2|$, P adj < 0.05) (**Figure 3.18a**). The lists of differentially expressed genes (DEGs) were used to generate Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses, using the software DAVID (6.8). GO and KEGG terms were considered based on P-value ($P < 0.01$). The GO and KEGG terms upregulated in brains of spinosad-exposed group included xenobiotics metabolism by cytochrome P450s, metabolism of amino acids, immune response, transmembrane transport, and oxidation-reduction processes. The GO and KEGG terms downregulated in the brains of spinosad-exposed group included neuroactive ligand-receptor interaction and carbohydrate metabolism (**Figure 3.18a**). The transcriptomics analysis of fat body identified 9,564 transcripts expressed in this tissue of which 298 were downregulated (foldchange $>|2|$, P adj < 0.05), whereas 1109 were upregulated in spinosad-exposed samples (foldchange $>|2|$, P adj < 0.05) (**Figure 3.18b**). The GO and KEGG terms upregulated in the fat body under conditions of spinosad exposure included metabolism of amino acids and sugars, energy derivation by compound oxidation and transmembrane transport. The GO and KEGG terms downregulated in fat body in the spinosad-exposed group included xenobiotics metabolism by cytochrome P450s and immune response.

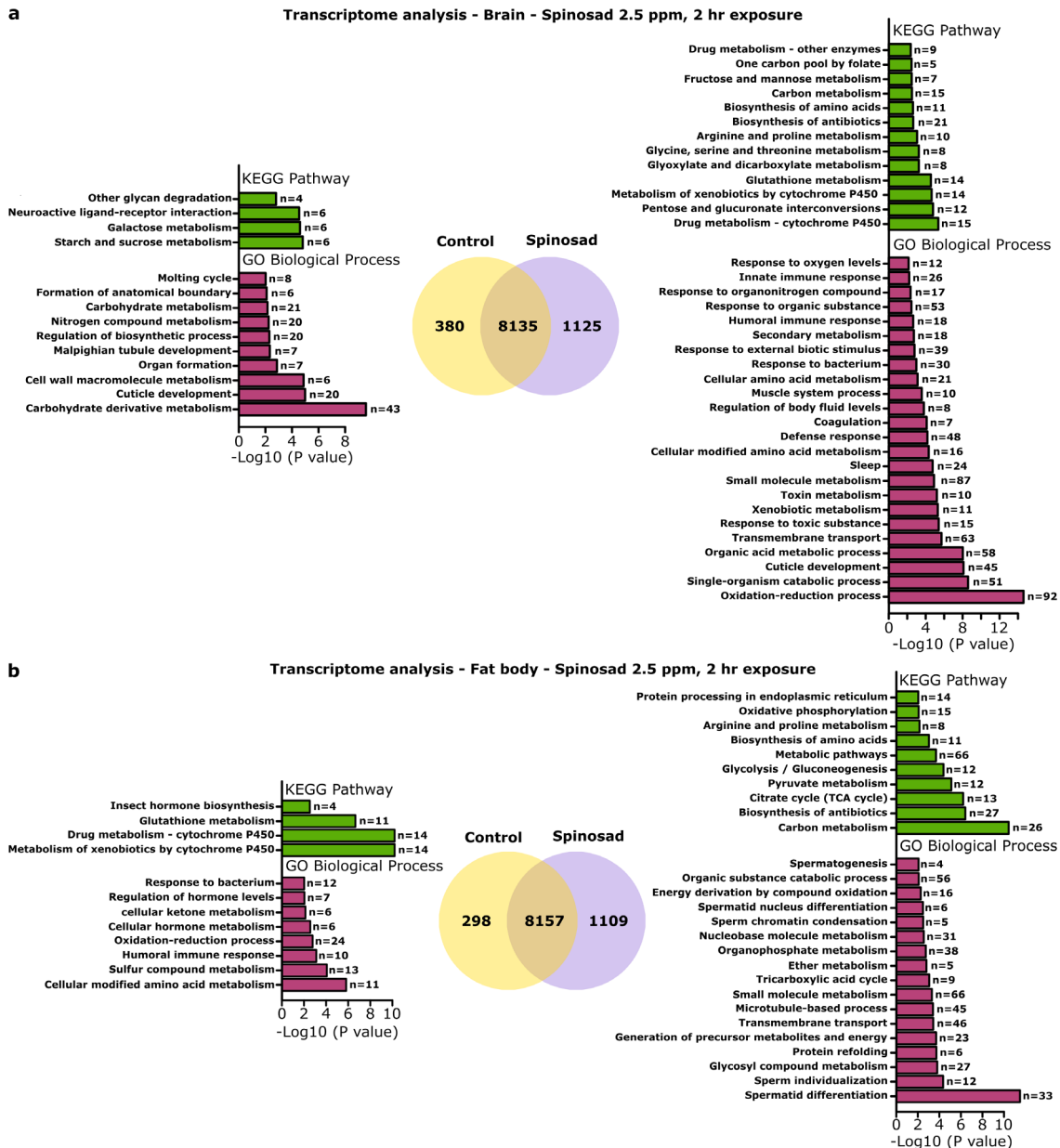


Figure 3.13. Transcriptomic analysis of the brain and fat body of larvae acutely exposed to spinosad. Third instar larvae exposed to 2.5 ppm spinosad for 2 hr (n = 40 larvae/ sample; 3 samples/ treatment). **a**, Transcriptomic analysis of the brain, 380 genes downregulated and 1125 genes upregulated in spinosad-exposed samples (foldchange >|2|, adj p-value < 0.05). **b**, Transcriptomic analysis of the fat body, 289 genes downregulated and 1109 genes upregulated in spinosad-exposed sample (foldchange >|2|, adj p-value < 0.05). KEGG and Gene Ontology analysis were performed in DAVID (Bioinformatics Resources 6.8), the Modified Fisher Exact P-value was transformed using -log (10) scale. The “n” values in front of the bars indicate the number of genes associated to each KEGG Pathway or GO Biological Process in the dataset.

The GO and KEGG analysis corroborated the impacts of spinosad exposure on metabolism and the rising levels of oxidative stress as evidenced in earlier experiments. A group of GO terms with a contrasting response in the two tissues was the one related to the metabolism of xenobiotics by cytochrome P450s, which fulfill roles in development and detoxification, including insecticide metabolism (Feyereisen 1999; Rane et al. 2019). Of the 86 P450 genes present in *Drosophila* (Chung et al. 2009), 24 had the expression perturbed by spinosad (**Table 2.1**). 20 genes were upregulated in the brain, while 3 were downregulated. While some of the upregulated genes such as *Cyp6g1*, *Cyp6g2* and *Cyp6w1* have been associated with resistance to other insecticides (Denecke et al. 2017; Schmidt et al. 2017), none have been associated with spinosad resistance. Indeed, evidence of P450 mediated resistance to spinosad in any insect species has been limited. It is possible that such resistance has not been detected in many insect pest species due to the prevalence of high-level resistance mediated by loss of function alleles of *Dα6*. The upregulation of so many P450 genes suggests that the capacity of the encoded enzymes to detoxify this insecticide should be evaluated. It should be noted that genes such as the P450s may be upregulated as a part of a general xenobiotic response, which is unlikely to be customized for xenobiotics that are not regularly encountered by this species. It is not clear that *Drosophila* encounters xenobiotics compounds that are structurally similar to the spinosyns. Under these circumstances it not necessary to assume that every gene upregulated here contributes to minimizing the impact of spinosad. Clearly the transcriptomic data can reflect both control and chaos, changes in expression that ameliorate the impact of spinosad and those that contribute to it and yet others that are neutral.

Changes in P450 expression in the fat body were few in number, small in magnitude and showed downregulation. The Malpighian tubules and midgut were not investigated here with transcriptomic analyses. These tissues may be the primary sites of insecticide metabolism by P450s (Harrop et al. 2014).

GST genes were also investigated in further detail (**Table 2.1**) since they are major components of xenobiotics metabolism and antioxidant response (section 3.3.3 and 3.3.7). Significant changes in expression were observed for 26 of the 35 genes encoding for GSTs enzymes in *Drosophila*. Most GST genes also had an increased expression in the brain and decreased expression in the fat body of exposed larvae. The increased expression of GSTs in brains is expected since this tissue harbors the target of spinosad

and the accumulation of oxidative stress was first observed there (**Figure 3.4**). The primary activation of GSTs in brains and reduced expression in fat bodies could be reflecting the crosstalk between the two organs, a biological circuit between brain and fat body is responsible for coordinating several aspects of insect life (Li et al. 2019). Again, the involvement of the other metabolic tissues (Malpighian tubules and midgut) could be significant; there is evidence of elevated *GstD1* expression in the anterior midgut after a 2hr exposure to 2.5ppm spinosad (**Figure 3.16**).

Table 3.1. Spinosad perturbs the expression of P450s and GTSs. List of genes of interest affected by 2.5 ppm spinosad exposure for 2 hr. Brain and fat body transcriptomic analysis (↓ gene downregulated; ↑ gene upregulated).

Flybase ID	Gene	Brain - Spinosad			Fat body – Spinosad		
		response	fold change	adj p-value	response	fold change	adj p-value
FBgn0028382	Cyp33	↓	-0.83	0.000594	--	--	--
FBgn0031126	Cyp6v1	↓	-0.76	0.000004	--	--	--
FBgn0031182	Cyp6t1	↓	-0.19	0.033435	↓	-0.06	0.000090
FBgn0033065	Cyp6w1	↑	6.88	0.000000	--	--	--
FBgn0000473	Cyp6a2	↑	6.23	0.000000	--	--	--
FBgn0013773	Cyp6a22	↑	5.85	0.000000	--	--	--
FBgn0013772	Cyp6a8	↑	5.25	0.000000	--	--	--
FBgn0039519	Cyp6a18	↑	4.73	0.000000	--	--	--
FBgn0015038	Cyp9b1	↑	3.81	0.000000	↓	-0.45	0.000093
FBgn0030369	Cyp318a1	↑	2.76	0.004874	--	--	--
FBgn0033697	Cyp6t3	↑	2.62	0.000086	--	--	--
FBgn0038076	Cyp313a4	↑	2.40	0.000004	--	--	--
FBgn0038194	Cyp6d5	↑	2.29	0.000000	↓	-0.28	0.047680
FBgn0025454	Cyp6g1	↑	2.26	0.000000	--	--	--
FBgn0013771	Cyp6a9	↑	2.18	0.000000	--	--	--
FBgn0033981	Cyp6a21	↑	2.07	0.003534	--	--	--
FBgn0033696	Cyp6g2	↑	2.00	0.000000	--	--	--
FBgn0037601	Cyp313b1	↑	1.94	0.000006	--	--	--
FBgn0033775	Cyp9h1	↑	1.88	0.000000	--	--	--
FBgn0039006	Cyp6d4	↑	1.59	0.000000	↓	-0.39	0.008030
FBgn0038037	Cyp9f2	↑	1.36	0.000000	--	--	--
FBgn0033978	Cyp6a23	↑	1.29	0.000000	↓	-0.27	0.036793
FBgn0015039	Cyp9b2	↑	1.25	0.035155	--	--	--
FBgn0034756	Cyp6d2	--	--	--	↓	-0.26	0.004145
FBgn0001149	GstD1	↑	1.22	0.000000	↓	-2.18	0.000225
FBgn0010038	GstD2	↑	3.65	0.000000	--	--	--
FBgn0010039	GstD3	↑	2.17	0.000000	↓	-2.01	0.032846
FBgn0010040	GstD4	--	--	--	↓	-5.81	0.000000
FBgn0010041	GstD5	↑	2.54	0.000925	↓	-5.80	0.000000
FBgn0010043	GstD7	↑	8.51	0.000000	--	--	--
FBgn0038020	GstD9	--	--	--	↓	-3.43	0.000114
FBgn0042206	GstD10	↑	1.86	0.000000	↓	-2.62	0.026692
FBgn0034335	GstE1	↑	1.24	0.000462	--	--	--
FBgn0063497	GstE3	↑	3.09	0.000000	--	--	--
FBgn0063496	GstE4	↑	3.91	0.001305	--	--	--
FBgn0063495	GstE5	--	--	--	↓	-2.18	0.004557
FBgn0063494	GstE6	↑	1.50	0.000000	↓	-3.25	0.000000
FBgn0063493	GstE7	↑	2.08	0.000000	↓	-4.61	0.001086
FBgn0063492	GstE8	↑	1.96	0.001727	↓	-2.22	0.040955
FBgn0063491	GstE9	↑	6.42	0.000000	--	--	--
FBgn0063499	GstE10	↑	2.80	0.000001	--	--	--
FBgn0034354	GstE11	↓	-1.54	0.000000	↓	-1.83	0.003657
FBgn0027590	GstE12	↑	1.34	0.000000	--	--	--
FBgn0035907	GstO1	--	--	--	↑	2.06	0.001416
FBgn0010226	GstS1	↓	-1.57	0.000000	--	--	--
FBgn0050000	GstT1	↑	1.11	0.031736	↓	1.63	0.011898
FBgn0050005	GstT2	↑	1.23	0.019749	--	--	--
FBgn0031117	GstT3	↑	1.73	0.000000	--	--	--
FBgn0030484	GstT4	↑	1.40	0.000000	↑	1.89	0.001190
FBgn0037697	GstZ2	↓	-1.14	0.000000	--	--	--

Curiously, the GO and KEGG did not identify terms directly related to lipid metabolism, which would have been expected after observing the effects of spinosad on the lipid environment of metabolic tissues. Thus, the expression profiles of genes involved on lipid metabolism in *Drosophila* and presented in **Table 1.1** were investigated in more detail. Genes involved in LD accumulation in the brain and neurodegeneration, as well as genes related to oxidative stress response were investigated. Spinosad exposure did not impact the expression of any of the lipid synthesis genes in the fat body, except for the upregulation of *Lipin*, a phosphatase involved in energy metabolism, the insulin pathway and lipid anabolism (**Table 3.2**). The expression of none of the key lipid synthesis genes was affected: *Dgat2* and *mdy* (TAG synthesis), *ACC* (catalyses malonyl-CoA, the rate-limiting substrate for fatty acid synthesis), and *FASN1*, *FASN2* and *FASN3* (fatty acid synthases). However, *fabp* (lipid binding protein) and CG6295 (a lipase) were downregulated, whereas *LRPI* (cell receptor for lipoproteins transported on hemolymph) expression was upregulated (**Table 3.2**). These data, along with the lipid levels measured in the hemolymph, suggest that even though some lipid synthesis might be occurring in the fat body, the main process responsible for the accumulation of lipids in this organ is lipid mobilization. Lipids are likely mobilized from midguts and Malpighian tubules, which showed a reduction in lipid droplet storage, and then transported through hemolymph to fat body.

Considering the brain, under conditions of spinosad exposure, there were major impacts on the expression of genes involved in lipid metabolism **Table 3.2**. These included the activation of the transcription factors SREBP and Mef2. SREBP coordinates fatty acid synthesis and is inhibited by PE production (Liu and Huang 2013). Lipidomic analysis showed that the levels of PE are reduced under these conditions of spinosad exposure – **Figure 3.17**. Mef2 promotes expression of lipogenic and glycogenic enzymes and upon infection promotes the expression of antimicrobial peptides (Azeez et al. 2014). Several of these genes involved in lipid metabolism are also known to be involved in the onset of neurodegeneration (Liu et al. 2017): *Glial lazaro*; *pdha*; *pdhb*; *ldh*; *kdn*; *Silmoon*; *outsiders*; *Basigin*; and *Shakin B* (discussed in section 2.3.11). Liu et al. (2017) showed that knockdown of these genes reduces the accumulation of LDs in glial cells in *Drosophila*, which reduces the neurodegeneration (Liu et al. 2015). All of these genes were upregulated in the brain of larvae exposed to spinosad, suggesting the activation of a genetic pathways that would lead to neurodegeneration. Later in this Chapter, the

prospect of neurodegeneration will be investigated in adults chronically exposed to low doses of spinosad.

Genes involved in stress, mainly oxidative stress, were also further investigated using the transcriptomic data (**Table 3.2**). The expression of the antioxidant enzymes *Sod1* and *Sod2* (superoxide dismutases - responsible for the detoxifying superoxide radicals) and *Cat* (catalase - clears hydrogen peroxide) were upregulated in the brain of exposed larvae. This might be expected given the elevated levels of ROS observed in this tissue at this time point. Experiments involving the double reporter line had already pointed for the increased expression of JNK signalling pathways and *GstD1* in the midgut of exposed insects (**Figure 3.16**). The transcriptomic analysis also revealed the upregulation of *Hep*, *Jra* and *kay* (involved in JNK stress response pathway – see section 2.3.11) and *Keap1* and *cnc* (regulators in a major xenobiotic response pathway in insects – see section 2.3.11) in the brain of spinosad-exposed group. The upregulation of both *Keap1* and *cnc* is interesting as the expression of these genes is autoregulated. Therefore, it is possible that this xenobiotic response pathway is activated in the brain. This could explain the upregulation of many P450 and GST genes in this tissue (Misra et al. 2011). In contrast, the downregulation of *Keap1* also in fat body should lead to the activation of Cnc, leading to increased levels of many of the genes regulated by Cnc (Misra et al. 2011). The lack of upregulation for many P450 and GST genes in this tissue may suggest that other regulators are modulating the expression of these genes.

Table 3.2. Spinosad affects the expression of genes involved in lipid metabolism, neurodegeneration, and oxidative stress. List of genes of interest affected by 2.5 ppm spinosad exposure for 2 hr. Brain and fat body transcriptomic analysis (↓ gene downregulated; ↑ gene upregulated).

Flybase ID	Gene	Brain - Spinosad			Fat body – Spinosad		
		response	fold change	adj p-value	response	fold change	adj p-value
FBgn0037913	<i>fabp</i>	↓	-1.48	0.016005	↓	-1.76	0.003111
FBgn0039471	<i>CG6295</i>	↓	-1.80	0.000024	↓	-9.82	0.001525
FBgn0053087	<i>LRP1</i>	↑	1.10	0.041773	↑	1.72	0.003504
FBgn0263593	<i>Lipin</i>	↑	1.14	0.000000	↑	1.58	0.019488
FBgn0004797	<i>mdy</i>	↑	1.85	0.000000	--	--	--
FBgn0087002	<i>apolpp</i>	↑	8.22	0.000000	--	--	--
FBgn0032136	<i>Apoltp</i>	↑	10.17	0.000000	--	--	--
FBgn0029994	<i>Ldsdh1</i>	↑	3.05	0.000000	--	--	--
FBgn0066101	<i>LpR1</i>	↑	1.14	0.000042	--	--	--
FBgn0026718	<i>Agpat2</i>	↑	1.73	0.000000	--	--	--
FBgn0039114	<i>Lsd-1</i>	↑	4.97	0.000000	--	--	--
FBgn0030608	<i>Lsd-2</i>	↑	1.54	0.000000	--	--	--
FBgn0040212	<i>Dhap-at</i>	↑	1.15	0.029109	--	--	--
FBgn0033246	<i>ACC</i>	↑	1.32	0.000000	--	--	--
FBgn0042627	<i>FASN2</i>	↑	1.44	0.000005	--	--	--
FBgn0036996	<i>mag</i>	↑	1.56	0.000031	--	--	--
FBgn0036449	<i>bmm</i>	↑	1.65	0.000000	--	--	--
FBgn0000568	<i>Eip75B</i>	↑	1.30	0.000000	--	--	--
FBgn0261283	<i>SREBP</i>	↑	1.18	0.000000	--	--	--
FBgn0011656	<i>Mef2</i>	↑	1.48	0.000000	--	--	--
FBgn0033799	<i>Glial Iazarillo</i>	↑	1.33	0.001141	--	--	--
FBgn0028325	<i>Pdha</i>	↑	1.75	0.000000	--	--	--
FBgn0039635	<i>Pdhb</i>	↑	1.45	0.000000	--	--	--
FBgn0001258	<i>Ldh</i>	↑	1.60	0.000000	--	--	--
FBgn0261955	<i>kdn</i>	↑	1.72	0.000000	--	--	--
FBgn0033657	<i>Silnoon</i>	↑	1.74	0.000000	--	--	--
FBgn0259834	<i>Outsiders</i>	↑	2.44	0.000000	--	--	--
FBgn0261822	<i>Basigin</i>	↑	1.21	0.000000	--	--	--
FBgn0085387	<i>Shaking B</i>	↑	1.49	0.000000	--	--	--
FBgn0010303	<i>Hep</i>	↑	1.13	0.000169	--	--	--
FBgn0001291	<i>Jra (c-Jun)</i>	↑	1.50	0.000000	--	--	--
FBgn0001297	<i>kay (Fos)</i>	↑	2.11	0.000000	--	--	--
FBgn0262975	<i>cnc (Nrf2)</i>	↑	1.21	0.000000	--	--	--
FBgn0038475	<i>Keap1</i>	↑	1.46	0.000000	↑	2.81	0.000000
FBgn0003462	<i>Sod1</i>	↑	1.06	0.004721	--	--	--
FBgn0010213	<i>Sod2</i>	↑	1.14	0.000014	--	--	--
FBgn0000261	<i>Cat</i>	↑	1.23	0.000000	--	--	--

Finally, the transcriptomic analysis revealed the impact of spinosad exposures in several genes involved in immune response (**Table 3.3**). The anti-microbial peptides (AMPs) are among the genes most highly impacted by spinosad exposure. The expression of *drosomycin-like 2* showed more than a 70-fold reduction in the brain, whilst *diptericin A* and *diptericin B* showed more than a 260-fold and 100-fold reduction respectively, in the fat body. Several other components of the innate immune response were also affected including the transcription factors *Relish*, *cactus* and *dorsal* (**Table 3.3**). These data suggest that the immune system is being compromised by insecticide exposure. This would render the insect more susceptible to microbial pathogens and parasites. In summary, the transcriptomic analysis reflected the severe impacts of spinosad exposure in larvae. Even after an acute 2 hr exposure to a relatively low dose compared to the doses used in the field, metabolism and the xenobiotics and immune responses appear to be impacted. There is also evidence that pathways that would lead to neurodegeneration are activated.

Table 3.3. Spinosad affects the expression of genes involved in immune response.
List of genes of interest affected by 2.5 ppm spinosad exposure for 2 hr. Brain and fat body transcriptomic analysis (↓ gene downregulated; ↑ gene upregulated).

Flybase ID	Gene	Brain - Spinosad			Fat body - Spinosad		
		Response	fold change	adj p-value	response	fold change	adj p-value
FBgn0010385	<i>Defensin</i>	--	--	--	↓	-5.50	0.002095
FBgn0004240	<i>Diptericin A</i>	↓	-27.81	0.000000	↓	-263.09	0.000095
FBgn0034407	<i>Diptericin B</i>	--	--	--	↓	-102.38	0.000036
FBgn0010388	<i>Drosocin</i>	↓	-5.77	0.001500	↓	-32.55	0.000471
FBgn0014865	<i>Metchnikowin</i>	--	--	--	↓	-16.48	0.000323
FBgn0052279	<i>Drosomycin-like 2</i>	↓	-73.57	0.000000	--	--	--
FBgn0035434	<i>Drosomycin-like 5</i>	--	--	--	↓	-11.40	0.000000
FBgn0012042	<i>Attacin-A</i>	↑	7.88	0.000021	--	--	--
FBgn0041581	<i>Attacin-B</i>	↑	1.85	0.013529	--	--	--
FBgn0041579	<i>Attacin-C</i>	--	--	--	↓	-6.40	0.011792
FBgn0000278	<i>Cecropin B</i>	↑	8.42	0.000274	--	--	--
FBgn0000279	<i>Cecropin C</i>	↑	2.02	0.017672	--	--	--
FBgn0000250	<i>cactus</i>	↑	1.26	0.000000	--	--	--
FBgn0014018	<i>Relish</i>	↑	1.78	0.000000	--	--	--
FBgn0260632	<i>Dorsal</i>	↑	1.52	0.000000	--	--	--
FBgn0033367	<i>Prophenoloxidase 2</i>	↑	1.74	0.000000	--	--	--
FBgn0004425	<i>Lysozyme B</i>	↓	-14.36	0.000000	--	--	--
FBgn0004426	<i>Lysozyme C</i>	↓	-13.59	0.000000	--	--	--
FBgn0004427	<i>Lysozyme D</i>	↓	-15.60	0.000000	--	--	--
FBgn0004428	<i>Lysozyme E</i>	↓	-12.04	0.007018	--	--	--
FBgn0004429	<i>Lysozyme P</i>	↑	11.74	0.000025	--	--	--
FBgn0004430	<i>Lysozyme S</i>	↓	-3.30	0.000000	--	--	--
FBgn0004431	<i>Lysozyme -</i>	↑	13.00	0.002416	--	--	--
FBgn0002930	<i>necrotic</i>	↑	5.87	0.000000	--	--	--
FBgn0043841	<i>vir-1</i>	↑	3.05	0.000000	--	--	--
FBgn0261560	<i>Thor</i>	↑	3.20	0.000000	--	--	--
FBgn0026760	<i>Tehao</i>	↓	-2.58	0.013444	--	--	--
FBgn0041182	<i>Tep2</i>	↑	2.70	0.000000	--	--	--
FBgn0030925	<i>Hayan</i>	↑	3.23	0.000000	--	--	--
FBgn0030310	<i>PGRP-SA</i>	↑	2.81	0.000000	--	--	--
FBgn0043578	<i>PGRP-SB1</i>	↑	2.89	0.000004	↓	-4.79	0.000000
FBgn0043576	<i>PGRP-SC1a</i>	↓	-5.74	0.006075	↓	-10.85	0.000105
FBgn0033327	<i>PGRP-SC1b</i>	↓	-6.69	0.000203	--	--	--
FBgn0035806	<i>PGRP-SD</i>	--	--	--	↓	-4.05	0.000086
FBgn0014380	<i>RhoL</i>	↑	2.14	0.000000	--	--	--
FBgn0039102	<i>SPE</i>	↑	8.76	0.000000	--	--	--
FBgn0053329	<i>Sp212</i>	↑	3.77	0.000182	↑	3.27	0.00312
FBgn0040653	<i>IM4</i>	↑	5.14	0.038506	--	--	--
FBgn0031561	<i>IM33</i>	↑	12.22	0.000000	--	--	--
FBgn0016675	<i>Lectin-galC1</i>	↑	2.34	0.000000	--	--	--
FBgn0033593	<i>Listericin</i>	↓	-5.22	0.000612	--	--	--
FBgn0030051	<i>spirit</i>	↑	12.00	0.000000	--	--	--
FBgn0014000	<i>Helical Factor</i>	↑	9.97	0.000000	--	--	--
FBgn0031055	<i>eye transformer</i>	↑	2.73	0.000000	--	--	--
FBgn0052185	<i>edin</i>	--	--	--	↓	-105.18	0.000140
FBgn0267339	<i>p38c</i>	--	--	--	↓	-6.16	0.000000
FBgn0040321	<i>GNBP3</i>	↑	2.32	0.000000	--	--	--
FBgn0005660	<i>Ets21C</i>	↑	3.29	0.000000	--	--	--
FBgn0039593	<i>Sid</i>	↑	3.20	0.000005	--	--	--
FBgn0034366	<i>Atg7</i>	--	--	--	↓	-2.05	0.004967
FBgn0243514	<i>eater</i>	↑	1.51	0.000016	↓	-2.31	0.028515
FBgn0039494	<i>grass</i>	↑	2.41	0.000000	--	--	--
FBgn0037515	<i>Serine protease 7</i>	↑	2.63	0.000000	--	--	--
FBgn0032638	<i>SPH93</i>	↑	10.04	0.000000	--	--	--

3.3.10 Spinosad is not a nAChR agonist

Since spinosad targets nAChRs in the insect CNS, the effect of spinosad exposure on Ca^{2+} influx was investigated. Ca^{2+} flux into neurons expressing the nAChR $\text{D}\alpha 6$ was measured using the GCaMP assay. Cultured brain cells from larvae, obtained by crossing $\text{D}\alpha 6$ T2A Gal4 with UAS GCaMP, were exposed to either 2.5 ppm or 25 ppm spinosad. In both cases, no Ca^{2+} flux into neuronal cells was recorded. A subsequent exposure to carbachol, an acetylcholine analogue, showed that spinosad-exposed neurons had a very large decrease in cholinergic response, evidenced by a large reduction in Ca^{2+} influx, when compared with the controls (**Figure 3.19**). The decreased cholinergic response shows that the nAChRs are being inhibited by spinosad; the insecticide is showing an antagonistic effect. Finally, cells were also exposed to thapsigargin, a drug that causes Ca^{2+} release from the endoplasmic reticulum. The purpose of this was to test whether the neuronal cells remained alive throughout the assay. The GCaMP results observed in response to thapsigargin indicated a normal physiological response from both control and spinosad-treated cells (**Figure 3.19**).

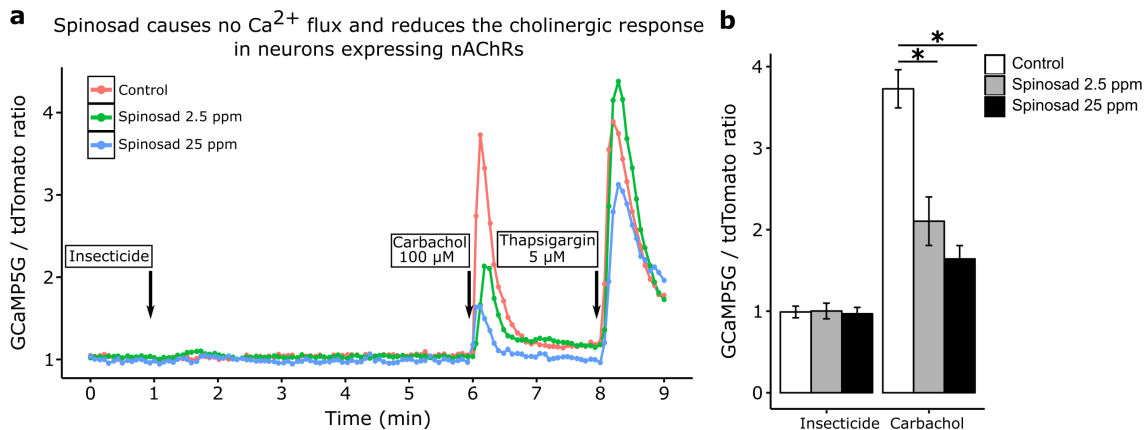


Figure 3.14. Spinosad blocks Ca^{2+} flux into neurons and impairs the cholinergic response. **a**, Ca^{2+} flux measured by GCaMP in neuronal cells expressing nAChRs. Measurement is expressed in terms of GCaMP5G signal divided by tdTomato constitutive expression. At 1 min spinosad groups only were exposed to either 2.5 ppm or 25 ppm of this insecticide and at 6 min and 8 min insecticide and control groups were exposed to carbachol 100 μM and thapsigargin 5 μM , respectively. Each point represents the average of at least 50 cells. **b**, Ca^{2+} influx peak response to spinosad and carbachol. Error bars represent mean $\pm$ s.e.m. (t-test; * $P < 0.01$).

In the face of these data a hypothesis was elaborated to explain spinosad mode of action (**Figure 3.20**). Spinosyn molecules bind to $\alpha 6$ subunits in nAChRs, blocking receptor activity and then triggering its recycling from cell membranes by endocytosis, ferrying spinosad into the cell. Endocytosis is a mechanism by which macromolecules present in the extracellular medium or in the cell membrane can be taken up by a living cell (Silverstein et al. 1977). In neurons, endocytosis allows the intracellular recovery of fragments of cell membrane which are brought back to the cell surface during the exocytosis of presynaptic vesicles (Saheki and De Camilli 2012). The endocytosis of spinosyn-blocked nAChRs would then start with the invagination of the cell membrane surrounding the receptor, forming the endocytic vesicles. The endocytic vesicles migrate into the cytoplasm where they merge into multivesicular bodies and then may fuse with lysosomes for digestion. Lysosomes are membrane-bound acidic organelles rich in hydrolytic enzymes which act as the cell waste disposal system, recycling a large number of cell components (Darios and Stevanin 2020).

Spinosad-blocked nAChRs could increase the trafficking of endocytic vesicles and thus, increase lysosome digestion in neurons (**Figure 3.20**). This in turn could trigger (1) the establishment of a lysosomal storage disease (LSD), and (2) oxidative stress. LSD is a metabolic disorder that emerges from the inability of lysosomes to digest their content. Non-functional lysosomes are enlarged since they accumulate undigested material in their lumen, causing a recycling problem for the neuronal cells which would eventually die, leading to the onset of neurodegeneration (Darios and Stevanin 2020). Spinosyns are highly hydrophobic and complex lipid compounds (polyketide macrolactones), probably undigestible by lysosomal acidic hydrolytic enzymes. Non-digested spinosyn molecules could accumulate in lysosomes, which would cause them to enlarge and gradually lose the ability to function normally, leading to LSD. There is extensive evidence for crosstalk between mitochondria and lysosomes. Defects in lysosomes are known to impact other organelles, but especially mitochondria (Deus et al. 2020). In parallel, the increased vesicle trafficking caused by blocking nAChRs that must increase recycling, ceramide production and oxidative stress. In *Drosophila*, in comparison to other cell types, neuronal cell membranes are composed of a high proportion of a lipid known as ceramide phosphoethanolamine (CerPE). Inside lysosomes, CerPE is converted into ceramide (Cer) by acidic sphingomyelinase in the sphingolipid metabolism pathway (Kogot-Levin and Saada 2014). Cer accumulation inhibit cardiolipin

production by stimulating activity of phospholipases which in turn causes mitochondrial membrane permeabilization, leading to ROS (Kogot-Levin and Saada 2014; Zachman et al. 2010). These mechanisms could explain the accumulation the rise of oxidative stress in brains of larvae exposed to 2.5 ppm spinosad (**Figure 3.4**).

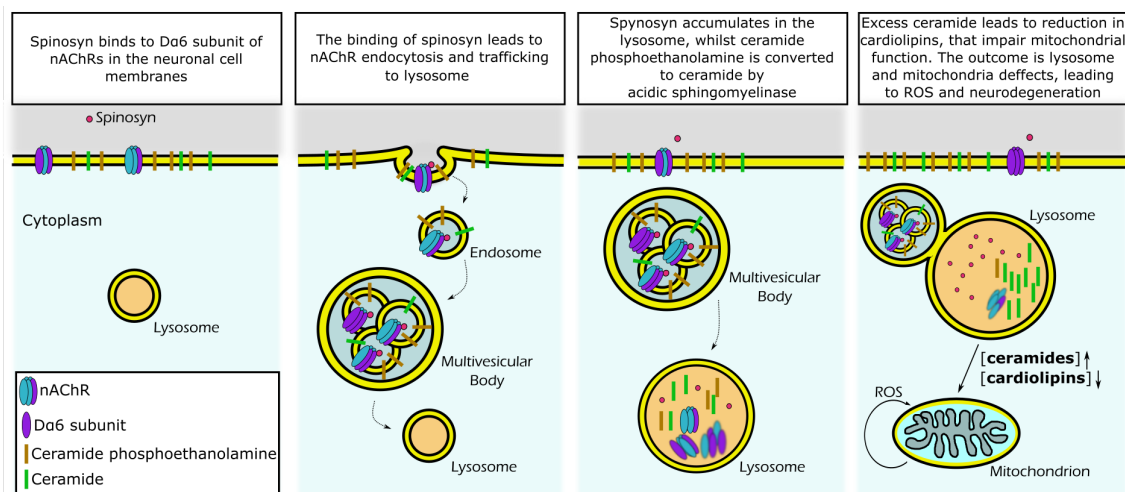


Figure 3.15. Proposed mechanism for internalization of spinosad after binding to the Dα6 nAChR target. Spinosyn A/D binds to Dα6 subunits, blocking nAChR activity and leading to its endocytosis and trafficking to lysosomes. Spinosyn molecules are not properly digested accumulating in lysosomes causing lysosomal storage disease, triggering oxidative stress and neuronal damage.

The hypothesis was tested with an experiment involving LysoTracker, a stain used to detect acidic organelles, i.e. lysosomes. A 2 hr exposure to 2.5 ppm spinosad caused an 8-fold increase in the area occupied by lysosomes in brains, while no impact was found in imidacloprid-exposed larvae (**Figure 3.21**). This result strongly supports the hypothesis of LSD in neurons. To test if this was a time dependent phenotype, brains of larvae exposed to 2.5 ppm spinosad were also stained after 1 hr exposure (**Figure 3.22**), and no differences were observed in comparison to controls. On the other hand, 6 hr after larvae were submitted to the 2 hr exposure the area occupied by lysosomes in brains was 24-fold greater than in controls (**Figure 3.22**). This significant worsening on the lysosome phenotype may indicate that spinosad is not easily metabolized and could accumulate in neuronal cells leading to neurodegeneration. Finally, the LysoTracker was applied to the brains of flies expressing fluorescently (CFP) tagged Dα6 nAChR subunits. These flies were generated by crossing the L driver strain (to drive expression of nAChRs) with UAS nAChR Dα6 CFP, in Line 14 Dα6 KO background. Hence, the only Dα6 expressed

contains the CFP tag. Exposure to 2.5 ppm spinosad for 2 hr showed that the Dα6 CFP signal faded away from neuronal membranes and colocalized with lysosomes (**Figure 3.23**), demonstrating that spinosad is causing endocytosis of nAChRs and digestion by these acidic organelles.

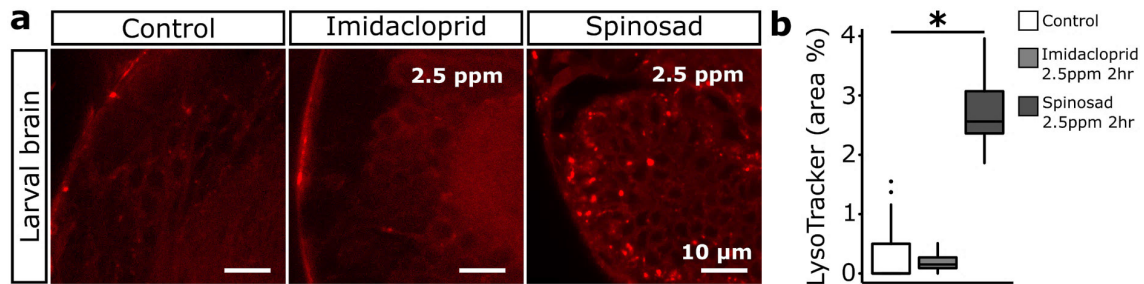


Figure 3.16. Spinosad leads to the accumulation of lysosomes in brain. Larvae exposed to 2.5 ppm of either imidacloprid or spinosad for 2 hr. **a**, Brain (optic lobes) of larvae exposed to spinosad show an increase in the number of lysosomes, no differences are observed between controls and imidacloprid exposed groups. LysoTracker staining. Images obtained in Leica SP8 Laser Scanning Confocal Microscope, 400 x magnification. **b**, Quantification of the area occupied by lysosomes in optical lobe sections of 30 μ m x 30 μ m (n = 7 larvae / treatment, 3 optical lobe sections/ larva) (t-test; *P < 0.01).

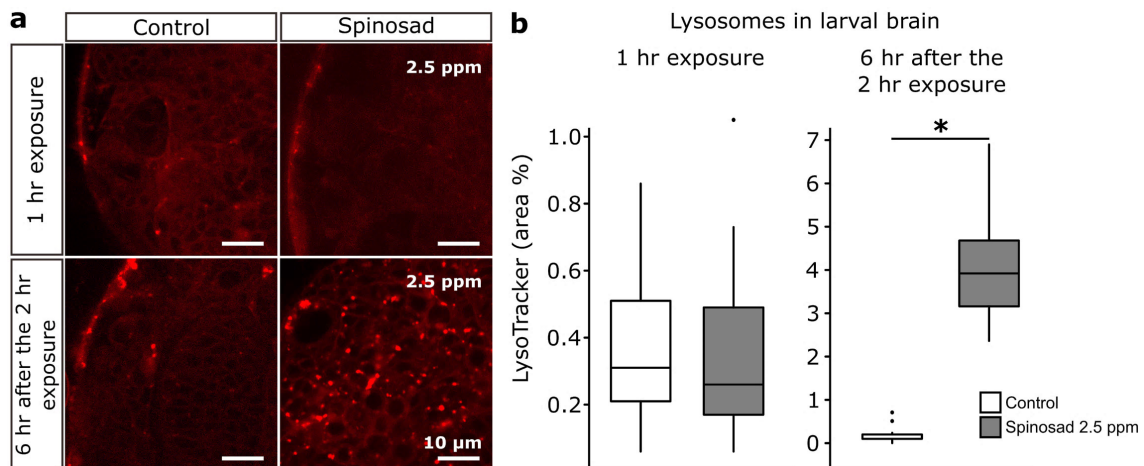


Figure 3.17. The accumulation of lysosomes in brain is time dependent. Larvae stained after a 1 hr exposure to 2.5 ppm spinosad or 6 hr after a 2hr exposure to 2.5 ppm spinosad. **a**, No difference between control and exposed groups is observed after 1 hr. The accumulation of lysosomes is greater 6 hr after the 2 hr exposure than immediately after the 2 hr exposure. LysoTracker staining. Images obtained in Leica SP8 Laser Scanning Confocal Microscope, 400 x magnification. **b**, Quantification of the area occupied by lysosomes in optical lobe sections of 30 μ m x 30 μ m (n = 7 larvae / treatment, 3 optical lobe sections/ larva) (t-test; *P < 0.01).

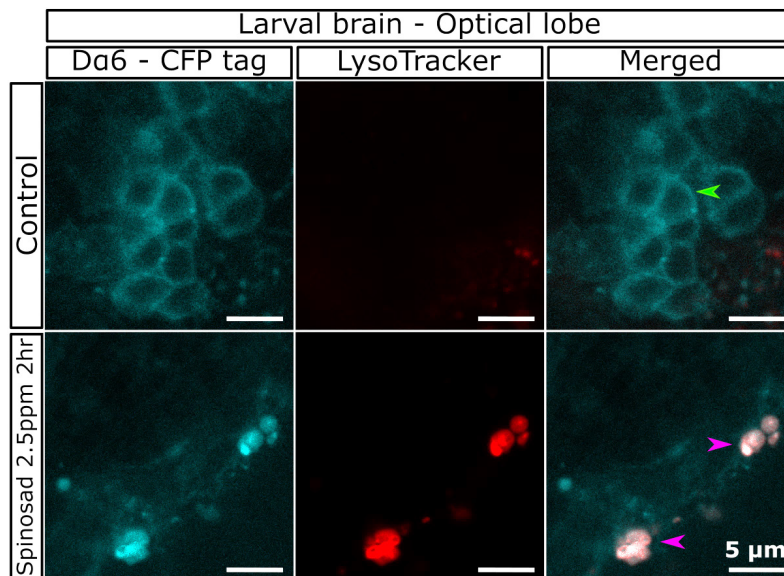


Figure 3.18. nAChRs are removed from cell membrane and transferred to lysosomes in response to spinosad exposure. Larvae expressing Dα6 tagged with CFP exposed to 2.5 ppm spinosad for 2 hr show the colocalization of Dα6 and lysosomal signals. Green arrowhead indicates Dα6 CFP signal in neuronal membranes of non-exposed larvae. Pink arrowheads indicate Dα6 CFP signal colocalizing with lysosomes. Brain optic lobes. Lysosomes stained with LysoTracker staining. Images obtained in Leica SP5 Laser Scanning Confocal Microscope, 600 x magnification.

KEGG pathway analysis of the brain transcriptome also indicated perturbations in lysosomes and sphingolipid metabolism under conditions of spinosad exposure. In total 17 genes were downregulated whereas 32 were upregulated (out of 93 genes known to participate in lysosome dynamics, either in *Drosophila* or other model organisms) by spinosad exposure (**Figure 3.24**). For sphingolipid metabolism which produces ceramides, a total of 4 genes were downregulated whereas 17 were upregulated (out of 28 genes known to participate in sphingolipid metabolism, in *Drosophila* or other model organisms) by spinosad exposure (**Figure 3.25**). These data further support the hypothesis of spinosad exposures leading to LSD and oxidative stress (**Figure 3.20**).

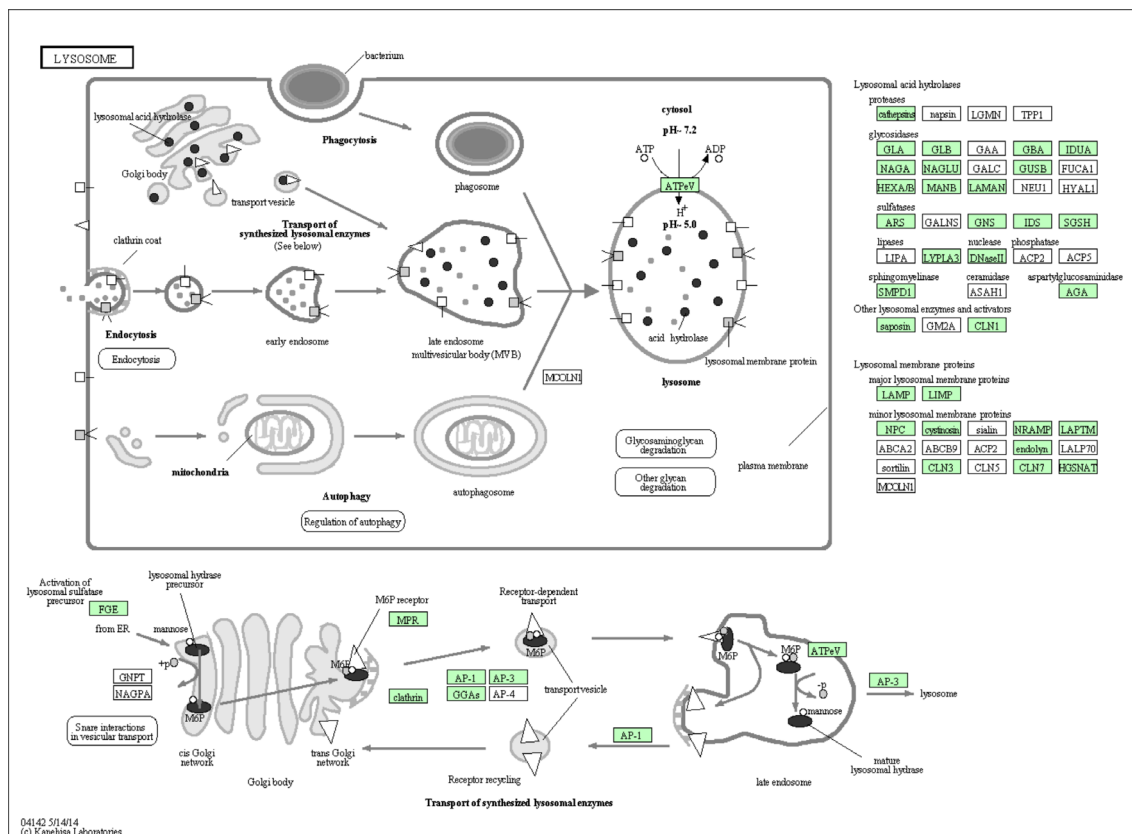


Figure 3.19. KEGG pathway analysis reveals perturbation of lysosome dynamics in brains. Brain transcriptomics of larvae exposed to 2.5 ppm spinosad for 2 hr showed significant perturbation in the expression levels of 49 genes involved in lysosome dynamics (adj p-value < 0.05).

Survival probability of adults chronically exposed to spinosad

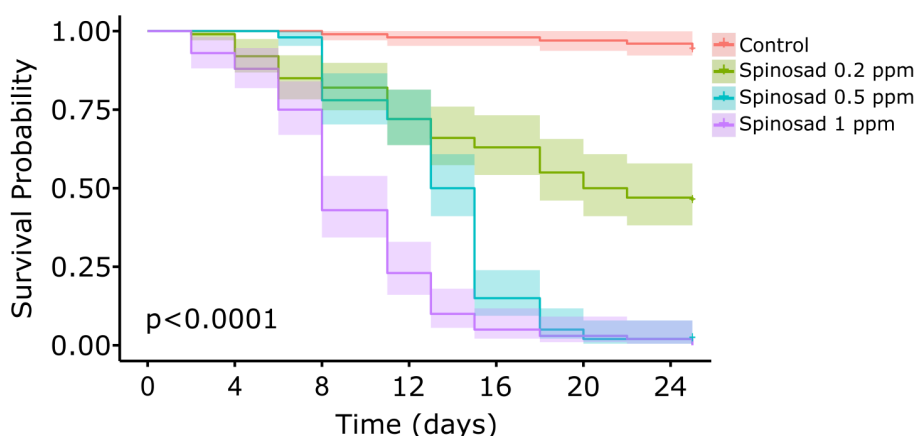


Figure 3.21. Adult chronic exposures to spinosad. Females adults (2-5 days old) were exposed to different concentrations of spinosad for 25 days (n = 25 flies/ replicate; 4 replicates/ treatment). The dose of 0.2 ppm, which killed 50% of flies within 20 to 25 days was selected to be used in experiments involving adult chronic exposures. Shaded areas represent 95% confidence interval (Kaplan-Meier method and the Log-rank Mantel-Cox test).

3.3.12 Chronic exposures to spinosad cause a diffuse lipid accumulation in fly retinas, progressive loss of vision, and neurodegeneration

Adult female flies chronically exposed to 0.2 ppm spinosad had their retinas checked in search for signs of degeneration, such as the accumulation of LDs in glial cells (Liu et al. 2015). Even though no LDs were observed in retinas throughout the 20 days of exposure, a diffuse progressive lipid accumulation in the photoreceptor cells surrounding the rhabdomeres was detected (**Figure 3.27a, b**). This lipid accumulation might include a buildup of spinosad in the retina over time. Spinosad is a lipid and can be detected with Nile Red staining (**Figure 3.28**). An accumulation of spinosad could be explained by the hypothesis that spinosyn molecules are shuttled into neuronal cells when promoting the endocytosis of nAChRs, and could be accumulating if lysosomes fail to digest them.

Even though nAChR D α 6 is not expressed in the retina, it is widely expressed in the adult brain, notably the medulla, a part of the visual system that is adjacent to the retina (**Figure 3.29a, b**). If spinosad is not eliminated by metabolism, spinosad or spinosad metabolites would slowly accumulate in the brain when flies are subjected to a

chronic low exposure. The high expression of nAChR D α 6 subunits in the medulla suggest this tissue as a likely target of this insecticide. Accumulation there could prove access to the retina due to its lipophilic nature (Tan et al. 2017).

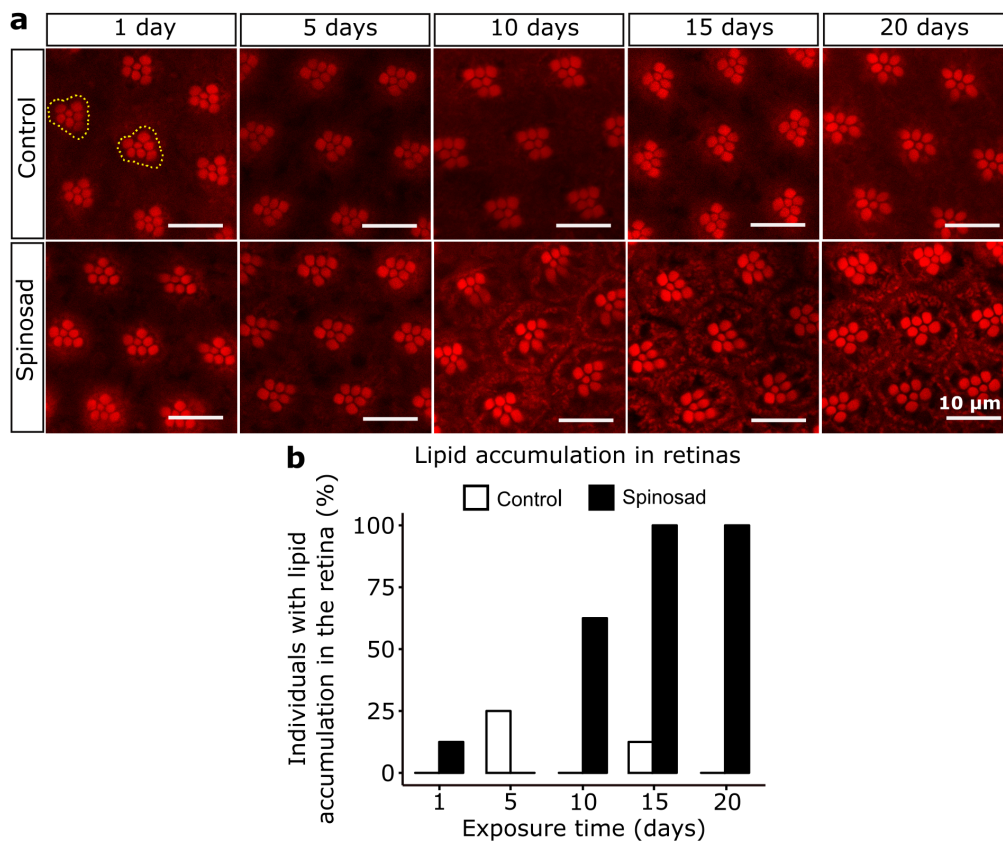


Figure 3.22. Chronic exposure to 0.2 ppm spinosad causes the accumulation of lipids in the retina. **a**, Clusters of rhabdomeres (light trapping structures) in the retina. In day 1 – control, two clusters of rhabdomeres are delimited with yellow dotted-lines. A diffuse lipid accumulation is observed from day 10 onwards. Nile red staining. Images obtained in Leica SP8 Laser Scanning Confocal Microscope, 600 x magnification. **b**, Percentage of animals that presented accumulation of lipids in retinas (n = 8 flies/ treatment/ time point).

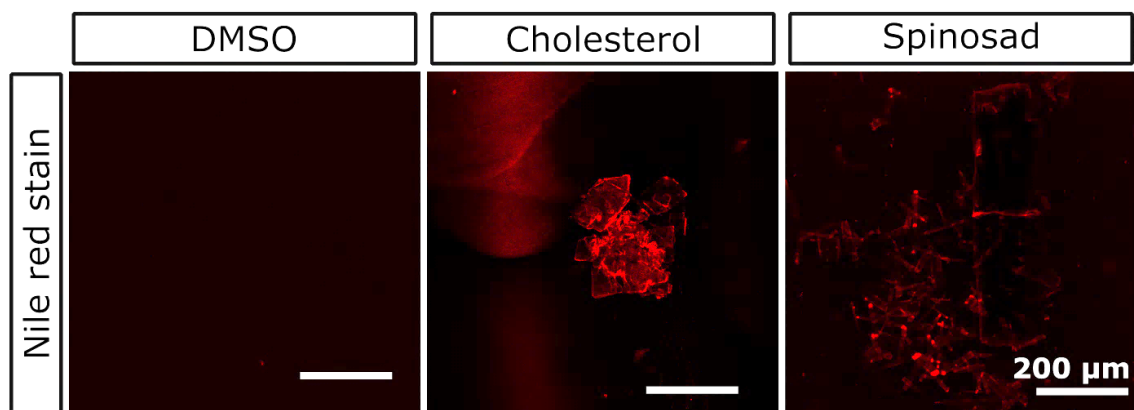


Figure 3.23. Spinosad crystals are Nile red positive. DMSO (negative control), cholesterol (positive control) and spinosad were incubated with the lipid stain Nile red. No signal is obtained with DMSO, which was used to dilute cholesterol and spinosad. Both cholesterol and spinosad stained red. Images obtained in Leica SP5 Laser Scanning Confocal Microscope, 400x magnification.

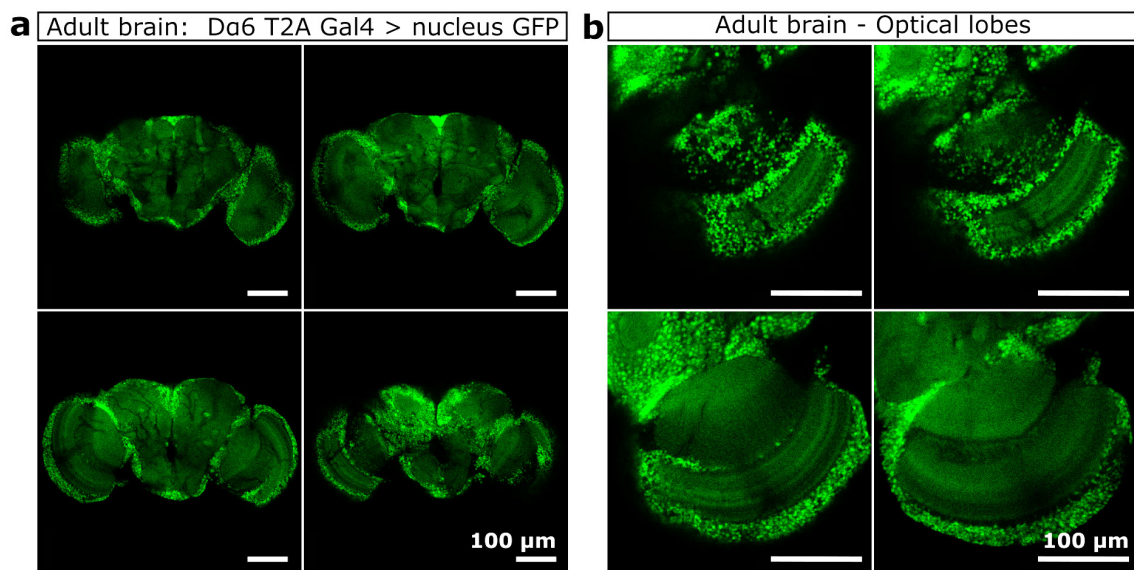


Figure 3.24. nAChR subunit Dα6 expression in Drosophila adult brain. Dα6 T2A Gal4 crossed with UAS nucleus GFP, showing expression pattern of nAChR subunit Dα6 in the adult brain. **a**, broad view of adult brain, showing different cross sections through anterior-posterior axis, 400 x magnification. **b**, Optic lobes showing detail of nAChR subunit Dα6 expression in medulla through different sections of anterior-posterior axis, 800 x magnification. Images obtained in Leica SP8 Laser Scanning Confocal Microscope.

Transmission Electron Microscopy (TEM) was used to investigate the fly visual system in more detail. Transverse sections of the retinas of flies subjected to 20 days of chronic exposure to 0.2 ppm spinosad were examined. TEM analysis indicated no conspicuous structural changes in the retina. The ommatidium presented a regular morphology. There were no signs of neurodegeneration or an increased number of pigment granules (**Figure 3.30a-c**). The only alteration detected in the retina of exposed flies was an increased number of mitochondria (**Figure 3.30d**). Such an increase correlates with the results obtained with the MitoTimer reporter fly strain performed in larvae (**Figure 3.5**). Together, these results corroborate the hypothesis that in face of the oxidative stress (**Figure 3.4**) created by spinosad exposure, mitogenesis could be activated to compensate for mitochondrial damage.

Severe morphological alterations were detected in transverse sections of lamina of flies exposed for 20 days (**Figure 3.31a-g**). Large endosomes were observed inside photoreceptor terminals, indicating an increase in endocytosis and digestion, correlating with the evidence of lysosomal storage disease in spinosad exposed larvae. Large amorphous masses were also detected. These do not correspond to any normal structure found in healthy lamina and could be the remains of dead glial cells or photoreceptor terminals. A severe vacuolation of cells in lamina was also detected. On average 70% of images from lamina of exposed animals showed some degree of vacuolation (**Figure 3.31d, e**). The lamina of exposed flies also showed a mean 34% increase in the number of mitochondria (**Figure 3.31f**). Furthermore, a mean 92% increase in the number of active zones was observed (**Figure 3.31f**), suggesting that the photoreceptor terminals are establishing more synapses with laminar neurons, perhaps to maintain synaptic transmissions in the face of the structural damage. The vacuolation of lamina is a particularly noteworthy phenotype. It is an incontestable marker of neurodegeneration and a robust indicator of the loss of vision.

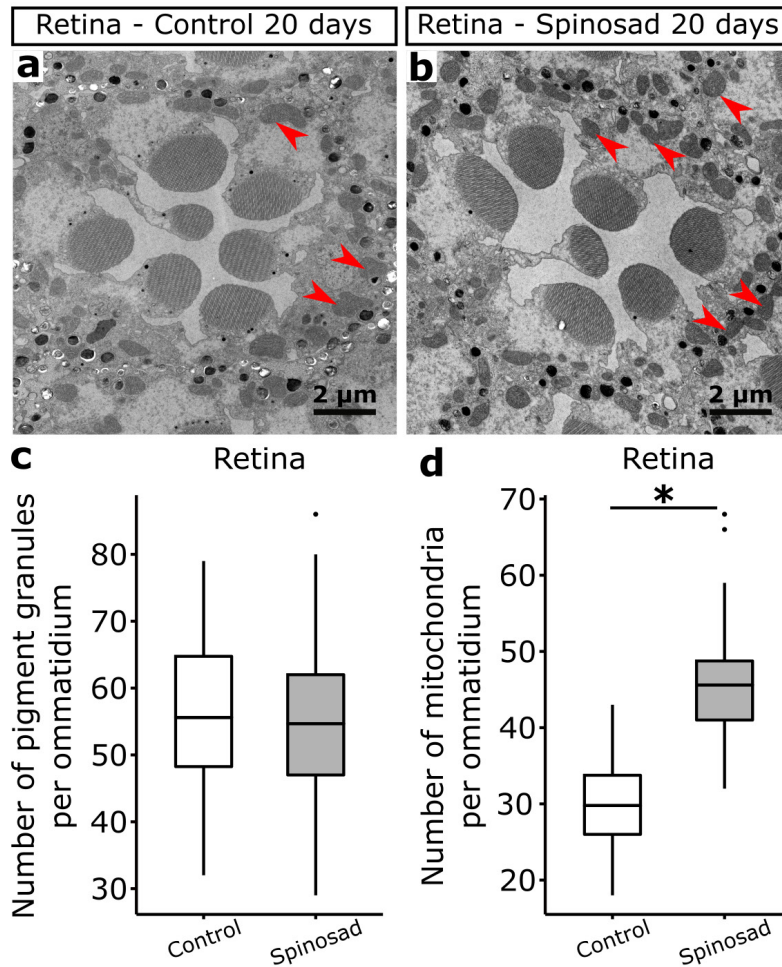


Figure 3.25. Chronic exposure to spinosad increases the number of mitochondria in retina. Electron microscopy of the retina of flies exposed to 0.2 ppm spinosad for 20 days. **a**, The retina of a control fly showing a typical ommatidium, red arrowheads point to mitochondria. **b**, The retinas of exposed flies show no conspicuous alteration, but an increased number of mitochondria (red arrowheads). **c**, Number of pigment granules per ommatidium (16 ommatidia/ fly; 3 flies/ treatment). **d**, Number of mitochondria per ommatidium (16 ommatidia/ fly; 3 flies/ treatment). (t-test; *P < 0.05, **P < 0.01).

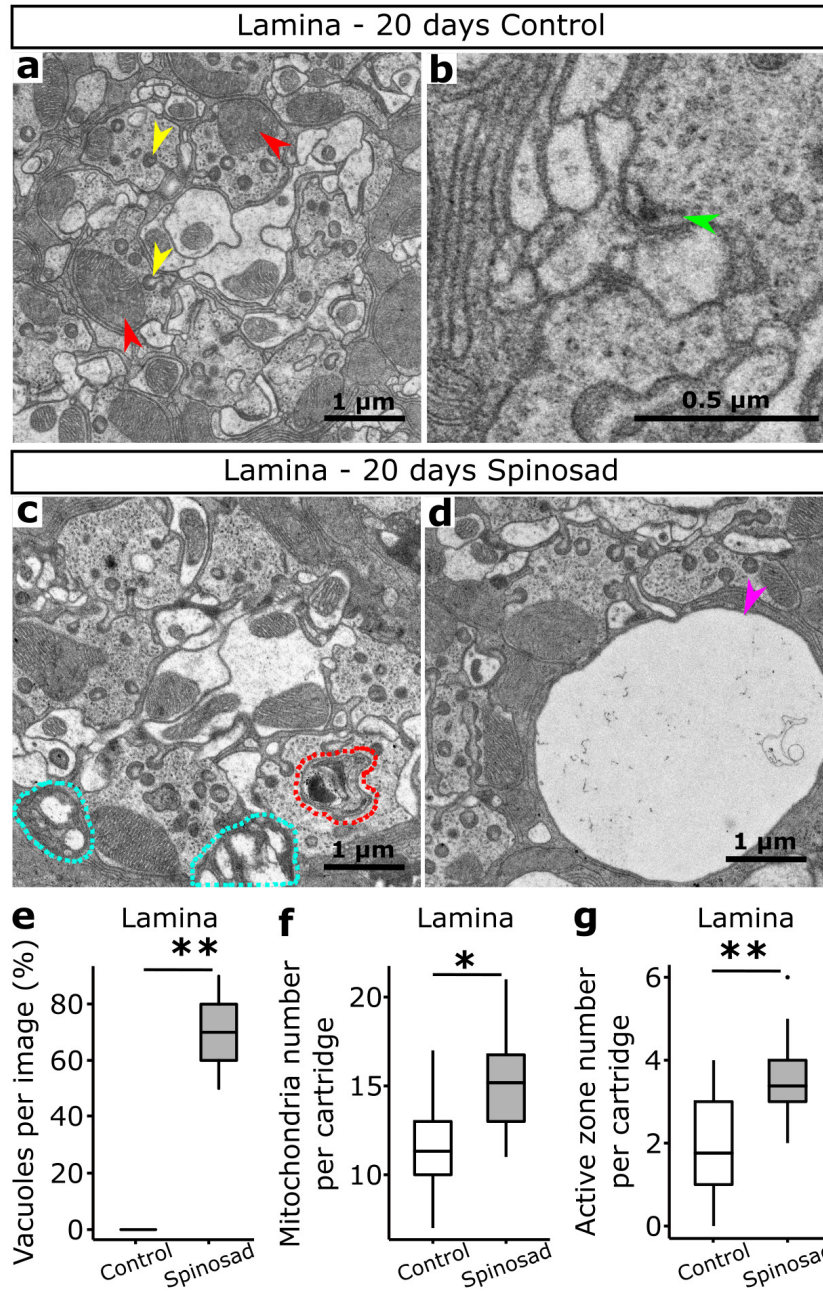


Figure 3.26. Chronic exposure to spinosad creates defects in the lamina. Electron microscopy of lamina of flies exposed to 0.2 ppm spinosad for 20 days. **a**, Lamina of a control animal showing a regular cartridge, red arrowheads indicate normal mitochondria, yellow arrowheads indicate capitate projections. **b**, Photoreceptor terminal, green arrowhead indicates an active zone. **c**, Lamina of spinosad exposed animal, red dotted-line delimits an enlarged digestive vacuole inside a photoreceptor terminal; blue dotted-lines delimit abnormal accumulations in the cartridge. **d**, Lamina of exposed animal, pink arrowhead points to a vacuole almost as large as a cartridge. **e**, Percentage of images with vacuoles in lamina (10 images/ fly; 3 flies/ treatment). **f**, Number of mitochondria per cartridge (n = 3 flies/ group; 16 cartridges/ fly). **g**, Number of active zones per cartridge (n = 3 flies/ group; 16 cartridges/ fly). (t-test; *P < 0.01).

To quantify the impact of these lesions on the response of the visual system to light, electroretinograms (ERGs) were performed at regular intervals over the 20 days of spinosad exposure (**Figure 3.32a-c**). A large reduction in the ON transient was observed from day 1 of exposure. The amplitude of exposed flies was only significantly impacted after 20 days of exposure, indicating a mild impact in phototransduction (Wang and Montell 2007). These reduction in the ON transient is evidence of a rapid loss of post-synaptic transmission in laminar neurons (Wang and Montell 2007) and consequent impaired vision in chronically exposed flies.

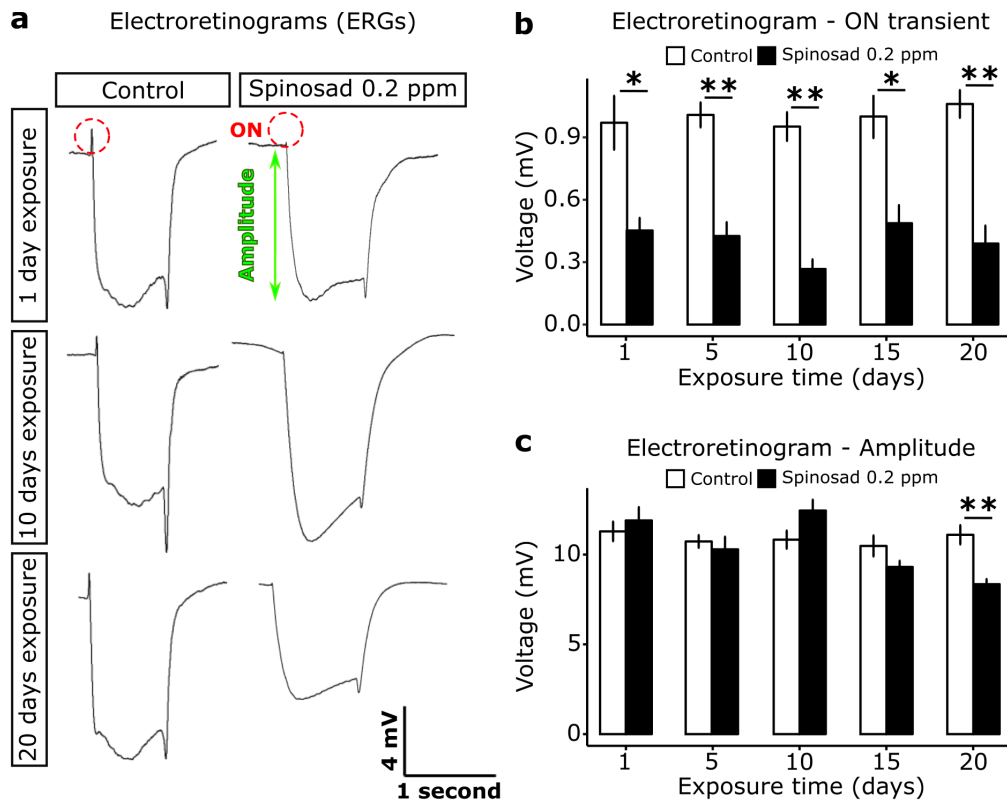


Figure 3.27. Chronic exposure to spinosad impairs vision. **a**, Electoretinograms (ERGs) of animals exposed to 0.2 ppm spinosad for 1, 10 and 20 days. Red dotted circles indicate the ON transient signal and green arrow indicates the amplitude, (n = 8 to 10 adult flies/ time point/ treatment) **b**, On-transient signal of ERGs after days 1, 5, 10, 15 and 20 of exposure to spinosad. **c**, Amplitude of ERGs after days 1, 5, 10, 15 and 20 of exposure to spinosad. (t-test; *P < 0.05, **P < 0.01).

3.3.13 nAChR $D\alpha 6$ knockout mutant flies have defective ERGs and photoreceptors

It is proposed that spinosad exposure leads to a loss of $D\alpha 6$ -containing nAChRs from the cell membranes of neurons, internalizing the insecticide. Through the lysosomal disorder the insecticide may cause oxidative stress within neurons, but the ROS generated could possibly spread to other cells in the brain. $D\alpha 6$ KO mutants lack $D\alpha 6$ on neuronal membranes and, in the absence of spinosad exposure, do not have the internalized insecticide. Given that $D\alpha 6$ has a role in responding to oxidative stress (Weber et al. 2012), it is possible that levels of oxidative stress are elevated in loss of function mutants, but this has not been demonstrated.

In examining the visual system of a $D\alpha 6$ KO mutant the impacts of the loss of $D\alpha 6$ function and the internalization of spinosad may be partitioned. The visual system of $D\alpha 6$ KO mutant was examined in flies allowed to age in the absence of spinosad. ERGs were recorded for the CantonS and CantonS $D\alpha 6$ KO mutants and, also Line14 (section 3.3.12) and Line 14 $D\alpha 6$ KO mutants aged for 5, 10 and 20 days. A decrease in the ON transients was observed for all mutants in comparison to their respective background controls at all time-points, except for the comparison between 10-day old Line 14 $D\alpha 6$ KO and Line 14 (**Figure 3.33a, b**). An impact on amplitude however was only observed for the comparison between 5-day old Line 14 $D\alpha 6$ KO and Line 14, and 5- and 20-day old CantonS $D\alpha 6$ KO and CantonS wildtype (**Figure 3.33c**). When observing retinas through confocal microscopy swollen and/or degenerated rhabdomeres were also identified in CantonS $D\alpha 6$ KO mutants (**Figure 3.34**). A diffuse lipid accumulation was also found surrounding rhabdomeres in the mutants (**Figure 3.34**).

The visual system defects described here are the first nervous system defects to be found in a $D\alpha 6$ KO mutant. Mutants have only been previously characterized for impacts on spinosyn resistance (Perry et al. 2015). That a decrease in ON-transient is observed in both the $D\alpha 6$ KO mutants and wild type flies exposed to spinosad, suggests that this phenotype that may arise in the exposed flies due to a loss of $D\alpha 6$ function. While the amplitude defect observed in the $D\alpha 6$ KO mutant did not become more severe over time after 20 days of spinosad exposure but in wild type flies it did. This is, thus, more likely to be a response to spinosad instead of the absence of $D\alpha 6$. And whereas the presence of swollen and degenerated rhabdomeres seems to be a defect found only in $D\alpha 6$

mutants, the defects in lamina appear to be a direct response to spinosad exposure. Since 20-day old CantonS *Da6* KO mutants show no conspicuous defects in lamina in comparison to their background (**Figure 3.35**). The diffuse lipid accumulation in retinas, found both in mutants and exposed flies, also seems to be a phenotype that arose in the exposed flies due to a loss of *Da6* function, which was further increased due to the likely accumulation of spinosad in this tissue.

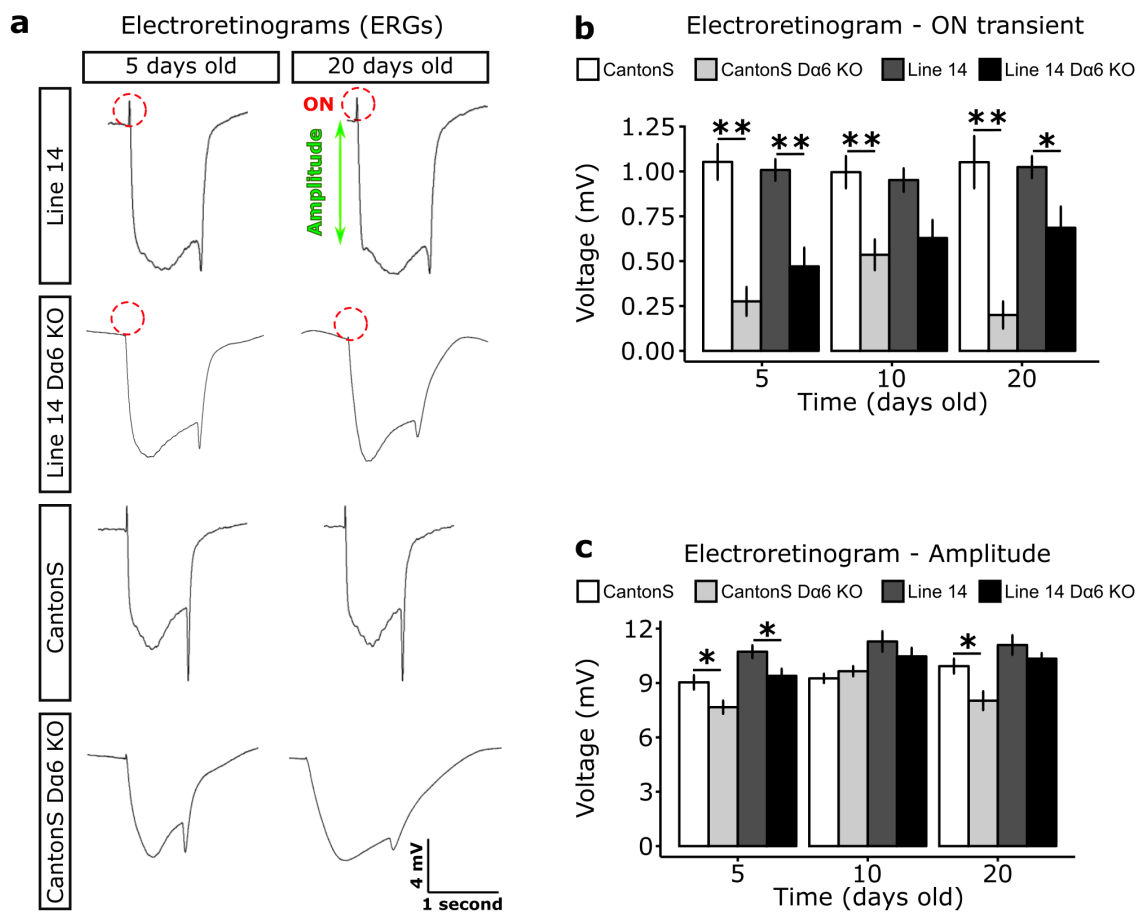


Figure 3.28. nAChR *Da6* knockout mutants show impaired vision. a, Electrophoretograms (ERGs) of 5- and 20-days old females from Line 14, Line 14 *Da6* KO mutant, CantonS and CantonS *Da6* KO mutant. Red dotted circles indicate the ON transient signal and green arrow indicates the amplitude (n = 8 to 10 adult flies/ strain/ time point) **b,** On-transient signal of ERGs of 5-, 10- and 20-days old flies. **c,** Amplitude of ERGs of 5-, 10- and 20-days old flies. (t-test; *P < 0.05, **P < 0.01).

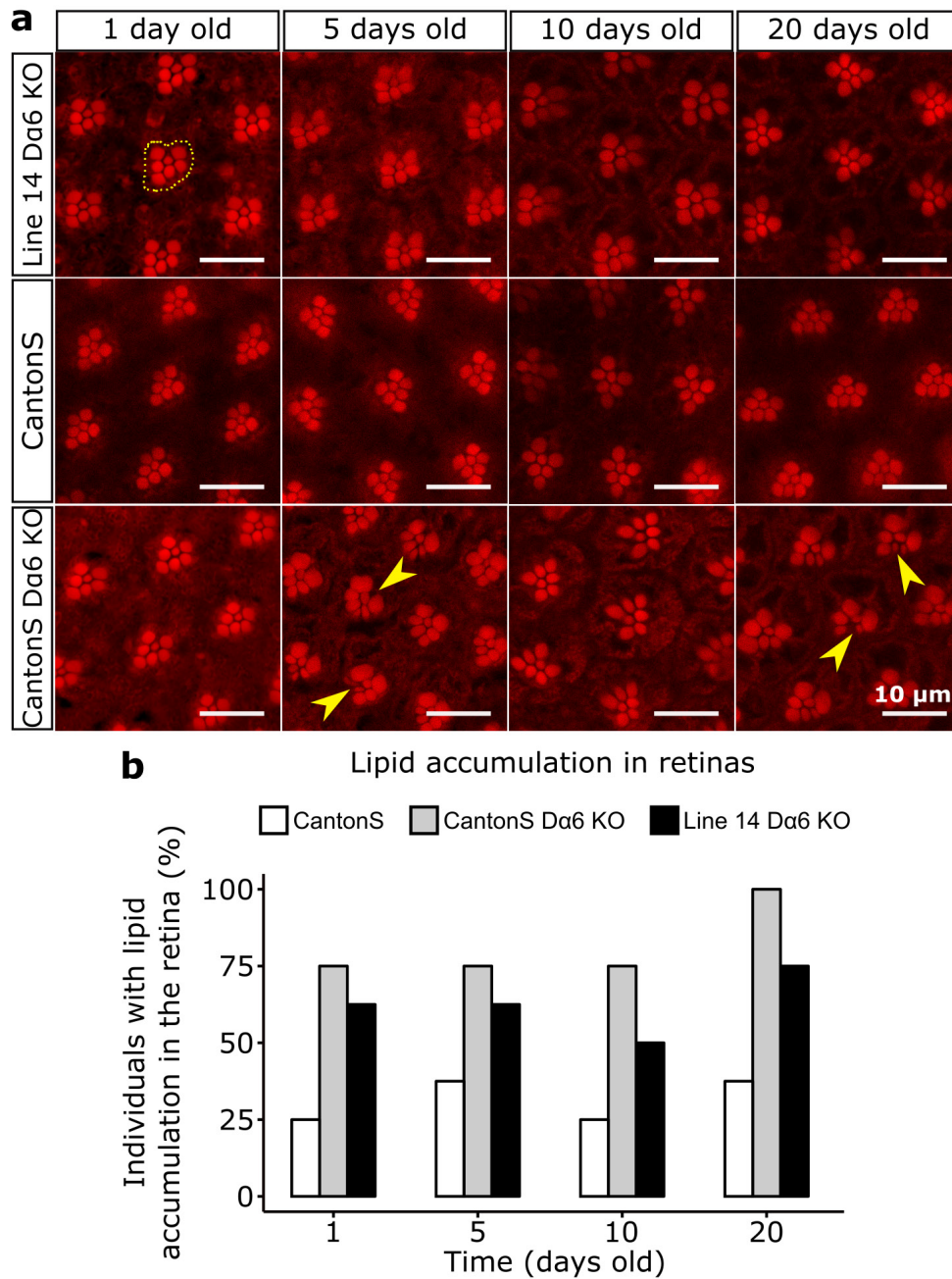


Figure 3.29. nAChR Dα6 knockout mutants show defective photoreceptors and lipid accumulation in retinas. **a**, Fly retinas showing the clusters of rhabdomeres (light trapping structures) in Line 14 Dα6 KO mutant, CantonS and CantonS Dα6 KO mutant for 1-, 5-, 10- and 20-days old flies. Yellow arrowheads indicate swollen or degenerated rhabdomeres. A diffuse lipid accumulation is found surrounding rhabdomeres. Nile red staining. Images obtained in Leica SP8 Laser Scanning Confocal Microscope, 600 x magnification. **b**, Percentage of animals that presented accumulation of lipids in retinas (n = 8 flies/ treatment/ time point).

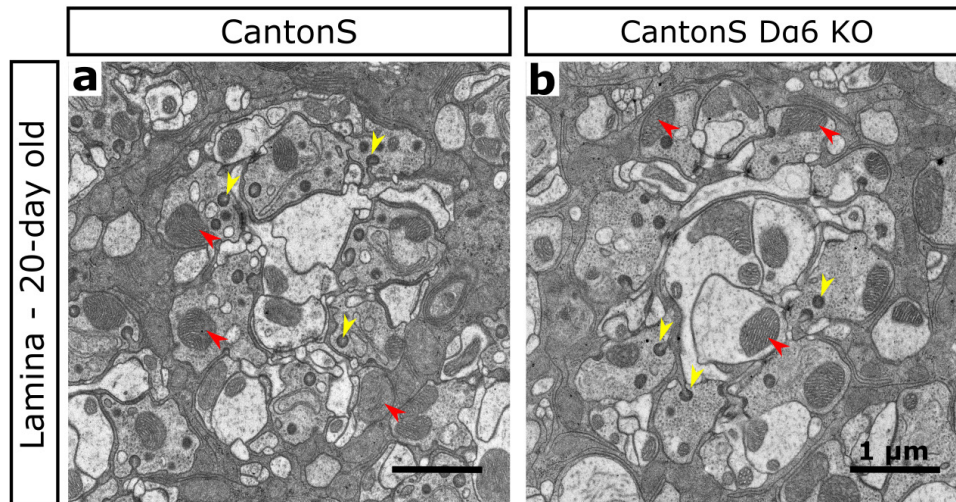


Figure 3.30. CantonS $\Delta\alpha 6$ KO mutants show no impacts in the lamina. **a**, Electron microscopy of the lamina of 20-day old CantonS flies aged in the absence of spinosad. **b**, Electron microscopy of the lamina of 20-day old CantonS $\Delta\alpha 6$ KO mutant flies aged in the absence of spinosad. Red arrowheads indicate normal mitochondria, yellow arrowheads indicate capitate projections. No conspicuous difference was noticed between mutant and background strains (10 images/ fly; 3 flies/ genotype).

3.3.14 Flies chronically exposed also show behavioural and histological signs of neurodegeneration

Two different behavioural assays were used to further investigate the neurodegeneration in flies chronically exposed to spinosad: bang sensitivity and climbing. Exposure to 0.2 ppm spinosad for 10 and 20 days increased the bang sensitivity phenotype, which is associated with mitochondrial defects (McGurk et al. 2015) (**Figure 3.36a**). 16%, 73% and 84% of flies failed to climb after 1, 10 and 20 days of exposure, respectively (**Figure 3.36b**), suggesting a progressive neurodegeneration in chronically exposed individuals (McGurk et al. 2015).

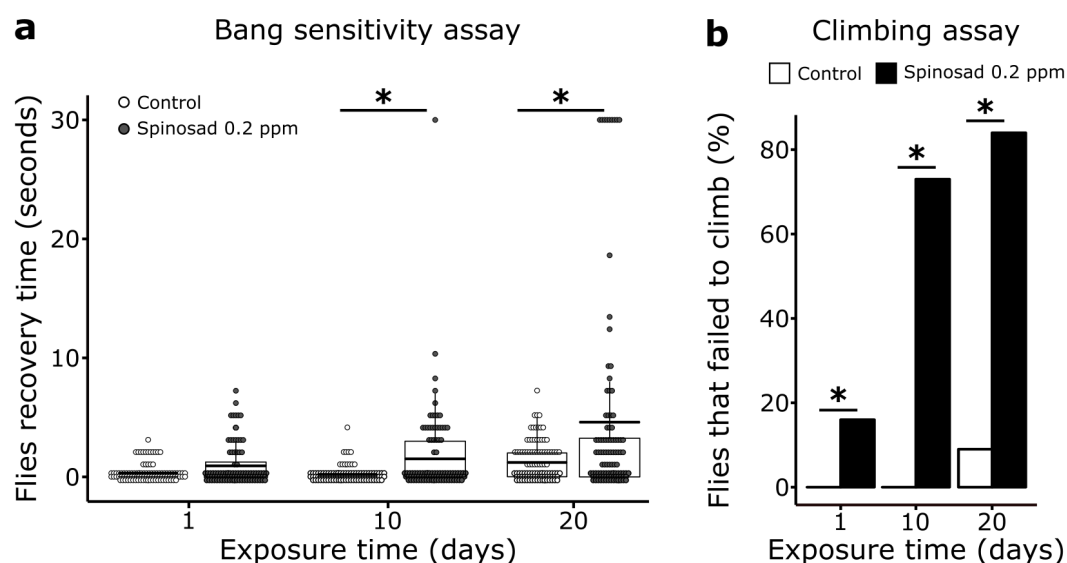
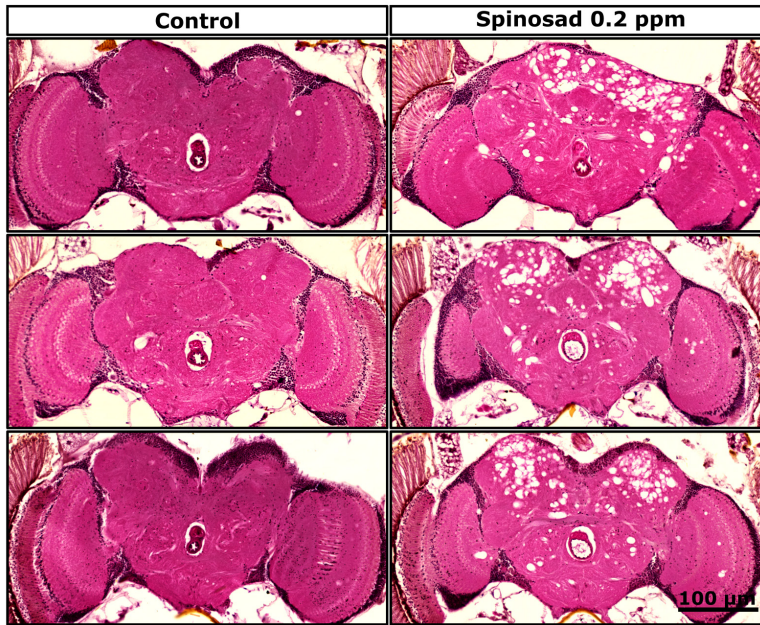


Figure 3.31. Chronic exposure to spinosad increases bang sensitivity and reduces climbing ability. Adult flies exposed to 0.2 ppm spinosad for 20 days. **a**, Bang sensitivity assay of adults at days 1, 10 and 20 of exposure. Groups of 5 flies are vortexed in a clear vial for 10 seconds at maximum speed and recovery time (time regain normal standing posture) for each fly is recorded ($n = 100$ flies/ time point/ treatment). **b**, Climbing assay. Percentage of adult flies that failed to climb at days 1, 10 and 20 of exposure ($n = 100$ flies/ time point/ treatment). (Wilcoxon test; $*P < 0.01$).

Finally, the Hematoxylin & Eosin stain (H&E) of adult fly brain provided proof of neurodegeneration supporting the data from behavioural assays and electron microscopy. All flies exposed to spinosad for 20 days showed extensive vacuolation in the midbrain region (**Figure 3.37a**). On average, 17% of the total midbrain area was consumed by vacuoles in exposed flies (**Figure 3.37b**). The regions impacted are similar in all flies examined. The neurodegeneration seems to be largely contained to the functional regions known as the optic tubercle, mushroom body, crepine, ellipsoid body, fan-shaped body, superior lateral protocerebrum and superior medial protocerebrum (**Figure 3.38**). Optic tubercle and mushroom body are important centres respectively for vision and memory, and learning and cognition in flies (Schürmann 2016). The superior medial protocerebrum is a shapeless neuropil highly connected with the superior lateral protocerebrum and the fan-shaped body (Namiki and Kanzaki 2016). Neuron populations innervating these regions are involved in memory retention, visual pattern memory and sensory stimulation. Most of the output neurons from the mushroom body also innervate these regions (Namiki and Kanzaki 2016). Neurodegeneration in these regions indicate that a wide range of behavioural paradigms would be critically compromised in exposed flies.

a H&E staining of adult brains - flies exposed for 20 days



b Quantification of **a**

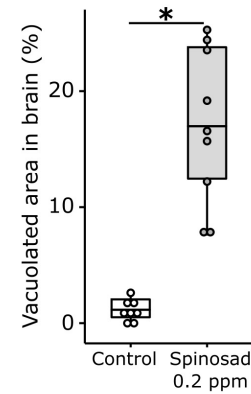


Figure 3.32. Chronic exposure to spinosad causes vacuolation of the adult brain. a, Flies exposed to 0.2 ppm spinosad for 20 days (controls exposed to the equivalent dose of the solvent used, DMSO). **a,** Midbrain frontal sections stained with hematoxylin and eosin (H&E) showing vacuoles in exposed animals. **b,** Quantification of neurodegeneration in terms of percentage of brain area vacuolated (n = 3 flies/ treatment, 3 sections/ fly) (t-test; *P < 0.01).

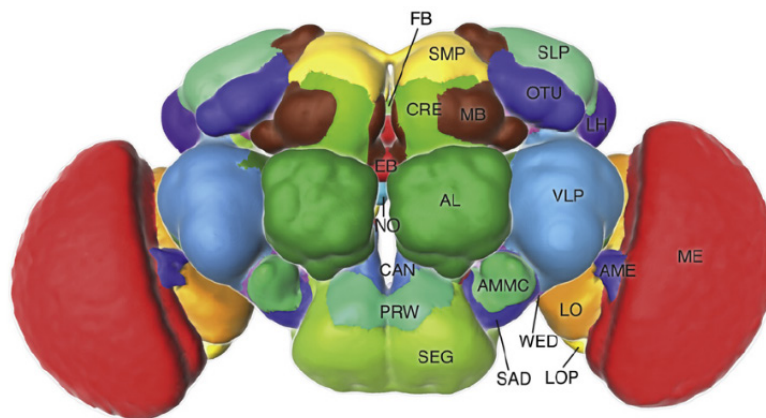


Figure 3.33. Adult *Drosophila* brain morpho-functional areas. AL, antennal lobe; AME, accessory medulla; AMMC, antenna-mechanosensory and motor center; CAN, cantle; CRE, crepine; EB, ellipsoid body; FB, fan-shaped body; LH, lateral horn; LO, lobula; LOP, lobula plate; MB, mushroom body; ME, medulla; NO, noduli; OTU, optic tubercle; PRW, prow; SAD, saddle; SLP, superior lateral protocerebrum; SMP, superior medial protocerebrum; SEG, subesophageal ganglion; VLP, ventrolateral protocerebrum; WED, wedge. Image published in (Jenett et al. 2012).

3.3.15 A model for spinosad low dose exposure impacts on flies

The impacts observed in acutely exposed larvae and chronically exposed adults has allowed a model to be developed of the mechanism by which low dose exposures to spinosad impact flies (**Figure 3.39**). Spinosad binds to its target, the nAChRs D α 6 (Perry et al. 2015), which is present in over 66% of adult brain neurons (Croset et al. 2018). Spinosad is an nAChR antagonist, blocking receptors and provoking their recycling from neuronal membranes. An increase in endocytic recycling pathway leads to lysosomal storage disease and the consequent rise of oxidative stress in the central nervous system. The increased levels of ROS in the CNS are not confined to this tissue. A systemic reduction in ATP levels and perturbation of the lipid environment of metabolic tissues occur under conditions of acute exposure. Under conditions of chronic exposure, lysosomal storage disorder and oxidative stress trigger the progressive loss of vision and severe neurodegeneration (**Figure 3.39**). The model presented here is centred on the production of oxidative stress. The administration of the antioxidant (AD4) improved the survival and motility of larvae acutely exposed, besides reducing the accumulation of lipid stores in the fat body. It is clear that low doses of spinosad can severely compromise insect biology. The data presented here raise great concern over the use of spinosad. In the public domain organic insecticides are often assumed to be safer than synthetic ones for the environment and non-target insect species. This is clearly not true for spinosad.

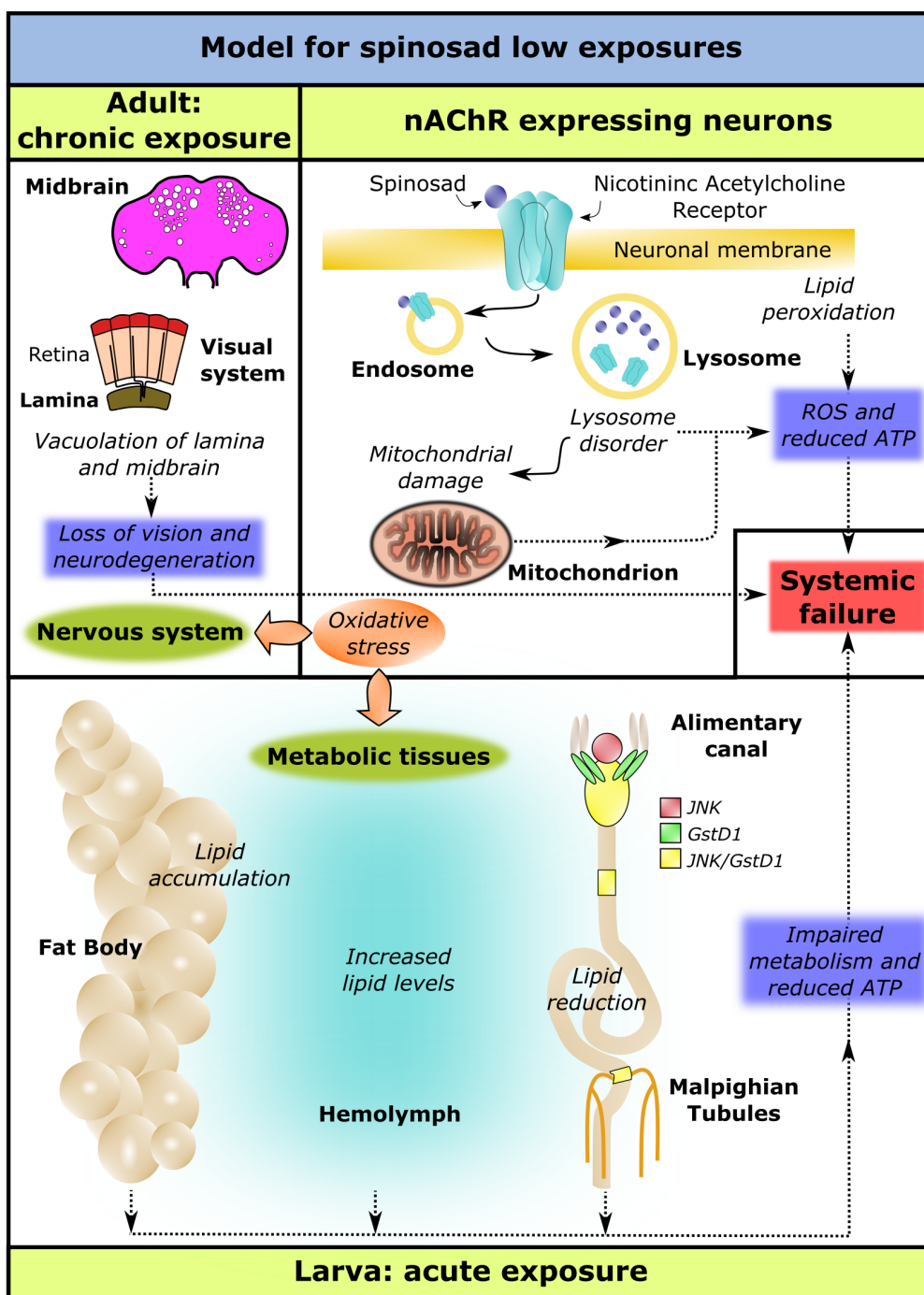


Figure 3.34. Proposed mechanism of action for low doses of spinosad. Spinosad binds to target nAChRs, blocking receptors and inducing their recycling from neuronal membranes. The endocytic recycling pathway leads to lysosome disorder, which damages mitochondria causing neurodegeneration and oxidative stress. The increased levels of ROS generate in the CNS are not confined to this tissue, causing a systemic reduction in ATP levels and impairing the lipid environment of metabolic tissues under acute exposure conditions. Under chronic exposure, lysosome disorder and ROS cause a progressive loss of vision.

Chapter Four

Discussion

Imidacloprid and spinosad both target nAChRs but have very different modes of action and yet low doses of these insecticides trigger a very similar cascade of downstream impacts in *Drosophila*. These similarities emerged from the early generation of oxidative stress in the central nervous system, which proves to be the key to precipitating the systemic impacts observed. Oxidative stress drives the activation of stress response pathways, the perturbation of transcriptomic and lipidomic profiles and, finally, the neurodegeneration and progressive loss of vision in adults. The overall effect is decreased energy levels and a systemic impact on insect biology. This Chapter will discuss the similarities and differences between the mechanism of action of these two chemicals, as well as the consequences that emerge from the impacts caused.

4.1 The insecticides

That insects are key to maintenance of life on the planet is not debated. Even though our understanding of insect diversity and its ecological significance remains poorly understood, the scientific community agrees that insects are vital for ecosystems and agriculture (Aizen et al. 2009; Montgomery et al. 2020; Ollerton et al. 2011). That insect numbers are plummeting is also not debated, even though there is discussion on the number of species affected, the regions impacted and the rates of decline (van Klink et al. 2020; Montgomery et al. 2020; Sánchez-Bayo and Wyckhuys 2019). It is generally agreed that the “Insect Apocalypse” is a multifactorial phenomenon. The extent to which each factor identified (i.e. climate change, parasites, pathogens, habitat loss, and insecticides) contributes to this phenomenon and the mechanisms by which they could synergise, remains largely unknown and is likely to vary between insects and regions (Cardoso et al. 2020; Montgomery et al. 2020; Sánchez-Bayo and Wyckhuys 2019). Insecticides are vital for pest control to maximize crop yield (Klein et al. 2007), but the collateral damage they may inflict on beneficial insect populations could ultimately reverse this positive impact, reducing global agricultural productivity. It is therefore vital that the impacts of low doses of insecticides on insect biology be systematically investigated.

4.2 The agonistic action of imidacloprid on nAChRs

Neonicotinoids act by interfering with neural transmission in the central nervous system. As agonists of nAChRs in postsynaptic neurons, neonicotinoids show a much higher affinity than nicotine or the natural ligand ACh (Anderson et al. 2015; Thany 2010). Their effects start with excitation and a lack of coordination, which rapidly results in partial paralysis as the extended stimulation of nAChRs leads to a block of synaptic excitability. This blockage causes ACh to accumulate in the synaptic cleft causing neurological toxicity (Gibbons et al. 2015). The stimulation of nAChRs also induces Ca^{2+} influx and studies from insect nAChRs described an increase of intracellular Ca^{2+} concentration after nicotine application in the cockroach *Periplaneta americana* (Thany 2010). In this study the GCaMP assay showed that imidacloprid caused a low and enduring Ca^{2+} flux into neuronal cells expressing nAChRs (**Figure 2.4**). Ca^{2+} is a ubiquitous intracellular messenger, responsible for controlling a variety of cellular processes. Variation in intracellular Ca^{2+} levels are detected by a range of calcium-sensing proteins, which control several aspects of cell biology, such as calmodulin and protein kinases A and C (PKA and PKC) (Endo 2006). The excessive Ca^{2+} influx caused by cholinergic stimulation, impairs many intracellular processes, such as energy metabolism (Milatovic et al. 2006). This phenomenon is known as excitotoxicity, the pathological process by which nerve cells are damaged or killed by excessive stimulation by neurotransmitters or similar substances (Bano and Ankarcrona 2018).

The activation of calmodulin, by elevation of intracellular Ca^{2+} levels, leads to the activation of nitric oxide synthase, which in turn produces nitric oxide (NO). NO is an important cell messenger but also an oxidizing agent, which can react with superoxide anions (O_2^-) to create peroxynitrite (OONO^-), precipitating neurotoxicity. Both NO and excess intracellular Ca^{2+} can directly interfere with mitochondrial activity: NO, by inhibiting the activity of key enzymes involved in electron transport chain, particularly cytochrome c oxidase and complex IV, and Ca^{2+} by overloading the mitochondrial matrix, creating membrane instability and mitochondrial damage (Brookes et al. 2004; Milatovic et al. 2006). The outcome of elevated levels of Ca^{2+} caused by prolonged cholinergic stimulation is the overproduction of ROS and the rise of oxidative stress. This leads to mitochondrial instability, the depletion of high-energy phosphates (HEP) and a reduction in the levels of ATP and phosphocreatine (PCr) (Brookes et al. 2004; Milatovic et al.

2006). The dose of imidacloprid tested in *Drosophila* neurons did not cause a peak of Ca^{2+} influx. It produced however, a Ca^{2+} influx that lasted for several minutes before the assay was terminated with the addition of carbachol, which revealed an impaired cholinergic response (**Figure 2.4**). An increased cytosolic level of Ca^{2+} is probably the responsible for the mitochondrial impacts observed in acutely exposed larvae (**Figures 2.6**), the accumulation of O_2^- in the brain and midgut (**Figure 2.5**), the reduction in mitochondrial aconitase activity (**Figure 2.7a**) and the depletion of ATP levels (**Figure 2.7b**).

4.3 The antagonistic action of spinosad on nAChRs

Spinosad has been used for over two decades, but its mechanism of action has remained unclear. It is currently accepted that spinosad causes an increased sensitivity to ACh in certain nAChRs (the ones expressing subunits $\text{D}\alpha 6$) and an enhanced response at some GABAergic synapses, causing involuntary muscle contractions, paralysis and death (Biondi et al. 2012; Perry et al. 2011; Salgado 1998). Salgado (1998) measured nerve impulses in cockroaches with electromyograms and found an increased electric response to spinosad, concluding that spinosad promoted an excitatory motor neuron effect. Salgado and Saar (2004) found that spinosad allosterically activates non-desensitized nAChRs, but that small doses were also capable of antagonizing the desensitized nAChRs. Orr et al. (2009) found that spinosad failed to displace several radioligands that identify binding sites at neuronal receptors, concluding that this chemical has neither positive nor negative activity on receptors. Subsequent studies provide evidence that the spinosad binding site is in the C terminal region of $\text{D}\alpha 6$ (Puinean et al. 2013; Somers et al. 2015), well removed from the ligand binding domain, These studies create a confusing picture and fall short of defining spinosad as an agonist or an antagonist.

Here, for the first time, the capacity of spinosad to interact with its target nAChRs to stimulate the flux of Ca^{2+} flux into neurons was quantified (**Figure 3.19**). The results obtained with GCaMP assay clearly showed that spinosad caused no Ca^{2+} flux into $\text{D}\alpha 6$ nAChRs expressing neurons. It also reduced the cholinergic response to carbachol. These data demonstrate an antagonist mode of action. This inhibitory role can be understood in terms of evidence that spinosad exposure leads to the removal of $\text{D}\alpha 6$ nAChRs from neuronal membranes and colocalization with lysosomes (**Figure 3.23**). While the

hypothesis suggests that a spinosad-D α 6 complex is translocated to the lysosomes (**Figure 3.20**), the presence of spinosad in the lysosomes is yet to be demonstrated. If the blocked nAChRs are acting as a shuttle for molecules of spinosad, which are brought inside neuronal cells by endocytosis, this insecticide may start to accumulate in the insect brain. The complex lipid nature of spinosad (polyketide macrolactone), could make it less accessible to the attack of metabolic enzymes. To date there are no reports of spinosad metabolites being produced in insects, but it is not clear to what extent this has been investigated. If this lipophilic compound is not digested by the lysosomal acidic hydrolytic enzymes, over time it would accumulate in the lumen of these organelles. It would then impair lysosome function, causing a recycling problem for the neuronal cells, impacting lipid and protein homeostasis and causing neuronal death (Darios and Stevanin 2020). This phenotype would ultimately lead to the severe neurodegeneration observed in adults (**Figure 3.37**) and the death of acutely exposed larvae (**Figure 3.2c**).

LysoTracker staining showed that spinosad exposure causes the enlargement of neuronal lysosomes (**Figure 3.21**), which is characteristic of lysosomal storage diseases (Darios and Stevanin 2020), known to create mitochondrial instability and generate ROS (Kogot-Levin and Saada 2014; Zachman et al. 2010). Most importantly, the LysoTracker staining showed the progressive nature of the lysosomal phenotype in the brains of exposed larvae. After a 1 hr exposure to 2.5 ppm spinosad no enlarged lysosomes are detected in neurons (**Figure 3.22**), whereas the area occupied by lysosomes increases by 8-fold immediately after the 2 hr exposure (**Figure 3.21**), and by 24-fold 6 hr after the 2 hr exposure (**Figure 3.22**). The continued increase in the area occupied by lysosomes even after exposure ceases supports the idea that spinosad is being internalized by lysosomes and not being digested. These data also may explain the delayed impact on larval movement caused by spinosad (**Figure 3.2b**) when compared to imidacloprid (**Figure 2.2b**). In mammalian models, nAChRs known to be involved in key metabolic pathways regulate, for instance, body mass. Interfering with nAChR activity using the agonist nicotine impacts energy metabolism, decreasing lipolysis and triglyceride uptake and storage in adipose tissue (Fornari et al. 2007; Jo et al. 2002). Initially, the blocking of nAChRs by spinosad might result in a neuronal stress that increases the energy demand, and thus induces mitogenesis. This mechanism could explain the initial increase of ATP levels immediately after a 2 hr exposure (**Figure 3.6a**).

Even though the enlarged lysosomes, characteristic of lysosomal storage disease, were not detected after a 1 hr exposure, this does not rule out the possibility that the recycling of blocked receptors had not already started. It could mean that the levels of spinosad and blocked nAChRs in the lysosomes, had not yet reached a threshold level where it would impair lysosomal activity and morphology. The quantification of O_2^- with DHE staining showed the accumulation of ROS in the brain after a 1 hr exposure (**Figure 3.4**), meaning that spinosad was already exerting its neurotoxicity. Hours after the establishment of the lysosomal disorder, when a worsening of this phenotype is observed, the impacts on neuronal function and metabolic tissues could then be translated into the observed reduction in energy (ATP) levels (**Figure 3.6b**).

Stebbins et al. (2002), investigating the toxicity of spinosad in mice found cytoplasmic vacuolation and presence of numerous lysosomal inclusion bodies in liver, lymphoid, renal, and reproductive tissue, and muscles and pancreatic cells. Here a 2 hr spinosad exposure induced a mean 2.2-fold increase in the area occupied by enlarged lysosomes in the larval fat body (**Figure 4.1**). Exposure to imidacloprid under the same conditions did not affect lysosome size or numbers. The similarity between the results presented here (**Figure 4.1**) and the ones obtained by Stebbins et al. (2002) raise the possibility that spinosad could also be internalized by other cell types through mechanisms that are independent of binding to nAChRs.

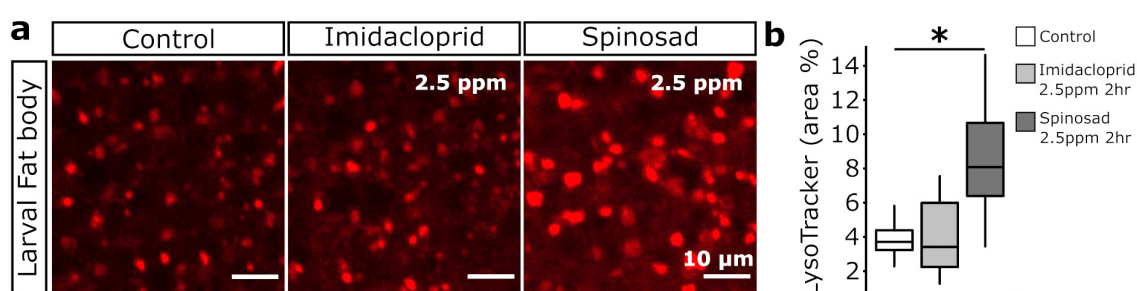


Figure 4.1. Spinosad leads to accumulation of larger lysosomes in the fat body. Larvae exposed to 2.5 ppm of either imidacloprid or spinosad for 2 hr. **a**, The fat body of larvae exposed to spinosad show an increase in the number and size of lysosomes. No differences are observed between controls and imidacloprid exposed groups. LysoTracker staining. Images obtained in Leica SP8 Laser Scanning Confocal Microscope, 400 x magnification. **b**, Quantification of the area occupied by lysosomes in fat body sections of 50 μm x 50 μm (n = 6 larvae / treatment, 3 fat body sections/ larva) (t-test; *P < 0.01).

4.4 Both insecticides trigger a cascade of damage by generating ROS in the brain

The central difference in the mode of action of imidacloprid and spinosad, i.e. the agonist and antagonist action in nAChRs, may explain their contrasting effects on survival. The impacts of imidacloprid were overcome once the acute exposure ceased, as no impact on survival was observed (**Figure 2.2c**). This result reflects the fact that imidacloprid is readily excreted, as are its metabolites, even when produced in the central nervous system (Fusetto et al. 2017). Survival also suggests that at this concentration, mitochondrial damage and deficits in energy metabolism could still be reversed. Once the larvae were removed from the imidacloprid it is likely that the mitogenesis observed with Mitotimer may have restored the number of healthy mitochondria to normal levels, but this was not tested. The acute 2 hr exposure to spinosad, on the other hand, was lethal even when larvae were placed back onto insecticide free media (**Figure 3.2c**), which could be a consequence of irreversible lysosomal storage disease (**Figure 3.22**).

Despite the idiosyncrasies in the mode of action of these insecticides, both imidacloprid and spinosad acute exposure caused a reduction in mitochondrial aconitase activity (**Figure 2.7a** and **Figure 3.6c**), an indicator of the rising oxidative stress in mitochondria (Yan et al. 1997). Both chemicals led to the accumulation of superoxide in the brain: a 45% increase for 2.5 ppm imidacloprid after 1 hr exposure (**Figure 2.5**) and an 89% increase for 2.5 ppm spinosad after 1 hr exposure (**Figure 3.4**). Subsequently there was an increase in the anterior midgut: 44% increase for 2.5 ppm imidacloprid after 2 hr exposure (**Figure 2.5**) and a 28% increase for 2.5 ppm spinosad after 2 hr exposure (**Figure 3.4**). The generation of oxidative stress by these insecticides in the brain almost certainly precipitates the cascade of impacts in the metabolic tissues. The capacity of these insecticides to generate oxidative stress-driven damage was such that 48 ppm imidacloprid (**Figure 2.12**) and 0.5 ppm spinosad (**Figure 3.12**) produced the same level of lipid droplet accumulation in the fat body as 1000 ppm H_2O_2 (**Figure 2.15**), which is an oxidizer, a ROS. Converting these ppm values to molarity values for imidacloprid (1.8×10^{-4} molar), spinosad (6.8×10^{-7} molar) and H_2O_2 (2.9×10^{-2} molar) provides a clearer indication of the relative capacities of these molecules to generate oxidative stress in *Drosophila*. Larvae and adults exposed to much lower numbers of spinosad molecules had far higher levels of downstream metabolic perturbation and damage than was observed with imidacloprid.

The significance of generating oxidative stress in the brain was highlighted when a knockdown of two mitochondrial genes, *ND42* and *Marf*, in neurons was used to investigate impacts outside the central nervous system (**Figure 2.8**). The generation of oxidative stress exclusively in the brain, using genetic tools, disturbed the lipid environment of the fat body and hemolymph, recapitulating the phenotypes created by insecticide exposure. This particular finding may have significance for human disease as the generation of oxidative stress in the brain is common to several pathologies such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (Elfawy and Das 2019; Liu et al. 2015, 2017).

The significance of ROS in the mode of action of insecticides at the doses tested here is also demonstrated by the significant amelioration of the impacts of these insecticides on larval movement (**Figure 2.10** and **Figure 3.9**), accumulation of LD in fat bodies (**Figure 2.9** and **Figure 3.8**), climbing ability (**Figure 2.23**) and survival (**Figure 2.24** and **Figure 3.10**) in flies pre-treated with the antioxidant, AD4. Other studies have associated oxidative stress with insecticide exposure (Balieira et al. 2018; Brinzer et al. 2015; Lukaszewicz-Hussain 2010; Wang et al. 2016), but the experiments reported here are the first to demonstrate a significant contribution of ROS production to insecticide toxicity. That the use of an antioxidant can significantly reduce the impacts of insecticide exposure suggests a promising solution to mitigate impacts in similarly exposed non-pest insects in the field. For example, the use of antioxidants in commercial beehives may provide protection against exposure to these and other insecticides.

So much emphasis is being given to oxidative stress as centre piece in the biological activity of spinosad and imidacloprid, but there is evidence that insecticides from a wide range of chemical classes increase levels of oxidative stress. Organochlorines, organophosphates, carbamates and the pyrethroid, permethrin have all been shown to produce markers of oxidative stress (Balieira et al. 2018; Karami-Mohajeri and Abdollahi 2011; Lukaszewicz-Hussain 2010; Terhzaz et al. 2015; Wang et al. 2016). Including these insecticides/classes with imidacloprid and spinosad, there is evidence that 50% of insecticides sold worldwide (Sparks and Nauen 2015) generate oxidative stress. This percentage is likely to increase if more chemicals are tested for this characteristic.

4.5 The effects of acute insecticide exposures on metabolic tissues

ROS molecules are unstable and have a very short life-span (Magder 2006; Valko et al. 2007), which raises the question of how oxidative stress generated in brain spreads to other tissues. ROS can lead to the generation of more stable oxidized products such as peroxidized lipids, which are highly efficient in perpetuating the oxidative damage to other macromolecules (Magder 2006; Valko et al. 2007). Peroxidized lipids may also have the capacity to pass through lipophilic cell membranes. Considering the small size of the insect body and the close proximity of metabolic tissues to the brain (Padmanabha and Baker 2014) it is possible that relatively stable oxidizing agents, such as peroxidized lipids, could be transported through hemolymph, triggering oxidative stress outside the central nervous system. Downstream phenotypes produced following imidacloprid or spinosad binding to nAChRs were, therefore, similar due to the generation and propagation of oxidative damage.

Spinosad caused a greater accumulation of LDs in fat bodies than the same dose of imidacloprid (2.5 ppm for 2 hr) (**Figure 2.9** and **Figure 3.8**). However, when larvae were exposed to the highest acute doses of insecticide that does not interfere with larval survival, both chemicals caused a similar increase in the area occupied by LDs (**Figure 2.12** and **Figure 3.12**). Even though the spinosad doses were 96 to 240-fold lower than the imidacloprid doses, both insecticides probably induce similar levels of oxidative stress and thus similar phenotypes. The various doses of spinosad and imidacloprid tested, changed the area occupied by LDs in the fat body, but had the same impact on LD dynamics - an increase in the number of small LDs and reduction in the number of the large ones. The overall increase in the area occupied by LDs suggests that not only new small LDs are being generated, but also that large LDs are being split. Under several stress situations, such as the unfolded protein response (UPR), heat shock response, oxidative stress and cancer, cells show an increase in the number of LDs (Shyu et al. 2018), a mechanism which seems to involve the overexpression of PLIN2 (Lsd-2 in *Drosophila*) (Jin et al. 2018). From a biophysical perspective, splitting large LDs into smaller ones increases the total surface area, thus making LDs more accessible to supply fatty acids when the cell is under energetic stress (Suzuki et al. 2011).

In the Malpighian tubules and midgut, however, insecticide exposure led to a decrease in the total number of LDs (**Figure 2.13** and **Figure 3.13**). The levels of lipids in hemolymph were then measured in response to the acute exposure. Here, once again the insecticides showed contrasting effects. No change was observed after a 1 hr spinosad exposure, but after a 2 hr exposure a significant 10% average increase in lipid levels was measured (**Figure 3.14**). This response was in the same direction as the one obtained with H₂O₂ exposure (**Figure 2.15e**) and the opposite of the one obtained with imidacloprid (**Figure 2.14**). The organic insecticide also took a longer time to impact lipid levels, since imidacloprid showed effects on this phenotype after 15 minutes of exposure (**Figure 2.14**). This divergence could emerge from the difference between the quantity of lipids being exported into and imported from the hemolymph by the different metabolic tissues. This was not quantified. The protein levels of lipid transport particles were not investigated in this study. They are likely to participate in the lipid mobilization between tissues and could even serve as a shuttle for oxidized lipid species; ApoE/D is known to be sensitive to ROS (Ioannou et al. 2019; Liu et al. 2017). The investigation of transcriptomic profiles for both insecticides revealed no difference in expression levels of key genes involved in lipid synthesis in the fat body (such as: *mdy*, *Dgat2*, *FASN1*, *FASN2* and *FASN3*). The exceptions were the upregulation of phosphatase *Lipin* (TAG synthesis) and the downregulation of lipase CG6295 by spinosad. Overall, it can be concluded that lipid synthesis is not the major factor in changing the lipid environment. Both insecticides led to the upregulation of the lipoprotein receptor *LRP1* in the fat body, whereas spinosad also downregulated the lipid binding protein *fabp* (**Table 2.2** and **Table 3.2**), suggesting that at least part of the impacts on the lipid environment of metabolic tissues results from changes in lipid mobilization.

One other possibility needs to be considered. Insecticides exposure could be forcing the excretion of lipids via the Malpighian tubules. Li et al. (2020) observed in *Drosophila* that under situations of stress or injury, lipids present in the hemolymph are excreted by Malpighian tubules. This mechanism is dependent on Materazzi, a lipid binding protein. Excretion prevents lipid peroxidation, reducing the deleterious effects of oxidative stress. Whether the same mechanism could be induced under insecticide exposures is a possibility that requires further investigation.

4.6 The effects of acute insecticide exposures on lipidomics profiles

The same lipid classes were impacted by both insecticides: reduction in levels of PE, PC, TAG, and CL (**Figure 2.19** and **Figure 3.18**). Spinosad exposure affected more than twice the number of lipid species; 88 versus 34 out of the 378 lipid species identified. The significant difference in the two lipidomic profiles lies in the number and identity of the lipid species affected and in the magnitude of the effect (**Figure 4.2**). In *Drosophila* PEs and PCs together correspond to more than 70% of cell membrane lipids (Guan et al. 2013). PCs are also key regulators of LD dynamics (Krahmer et al. 2011). TAGs are main lipids stored inside LDs (Azeez et al. 2014) and CLs essential for mitochondria activity. A reduction in the levels of CLs results in ROS (Kagan et al. 2014; Ren et al. 2014). Based on the known functions of these lipid classes, the lipidomic profiles support the observed changes in LD numbers in metabolic tissues, and the mitochondrial impacts (increased MitoTimer red signal and reduced mitochondrial aconitase activity).

Despite the robust evidence of oxidative stress, few peroxidized lipid species were significantly affected by insecticide exposures (**Figure 2.19** and **Figure 3.18**). Brinzer et al. (2015) studying impacts of permethrin on *Drosophila* metabolites found evidence of oxidative stress, but not the peroxidized lipid themselves. The limited evidence of peroxidized lipid species found here is probably due to a methodological difficulty in identifying peroxidized lipid species by LC-MS and is, perhaps, a consequence of performing lipidomic analysis on whole larvae. If increased levels of lipid peroxidation occurred in one tissue, they may have been diluted out and rendered undetectable in analysing the whole larva.

The lipidomic analysis measured the immediate impact of insecticide exposure on the lipid environment, but the severity of these impacts also seems to correlate with the later effects of exposure. The natural biological variation found on control groups was reduced by spinosad exposure and increased by the same dose of imidacloprid. The closer proximity of imidacloprid profiles to control profiles might reflect the ease of recovery under the tested exposure conditions (**Figure 4.2a**). On the other hand, the further distancing of spinosad profiles from control profiles (**Figure 4.2a**), may represent that the dose used was enough to push all the larvae to the same point of non-return where it is no longer possible to recover, a commitment to death. These results not only reflect the

specific lipidome signature of each insecticide but also their distinct modes of action, since a very weak correlation was found between the impacts caused by spinosad and imidacloprid on the lipidomics profiles (**Figure 4.2b**). The identification of lipidome fingerprints for each insecticide, such as the ones found here, may be a useful approach to determine the main culprit for the demise of insect population in a certain region. The identification of lipidome profiles of affected beehives could, for instance, contribute to a better understanding of insecticides role in the colony collapse disorder.

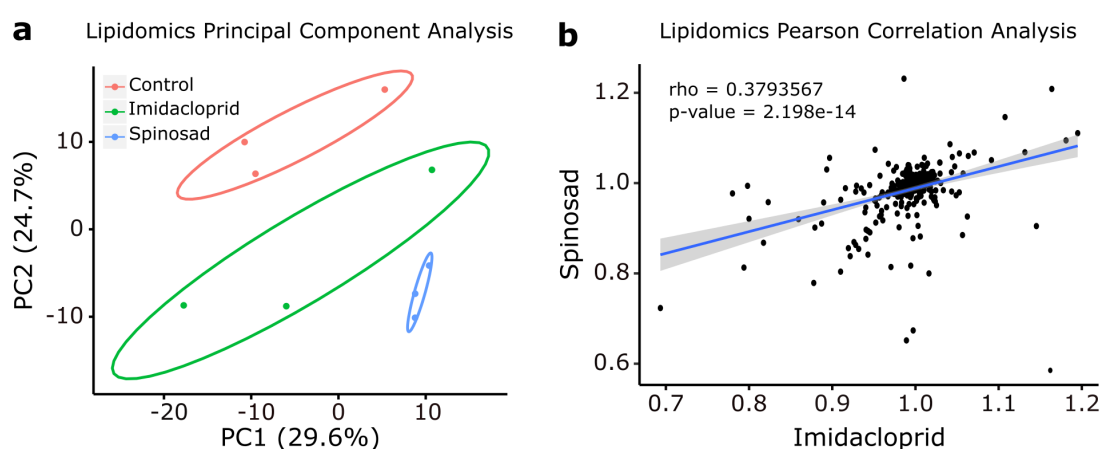


Figure 4.2. Comparison of lipidomic profiles of larvae exposed to imidacloprid or spinosad. Lipidomic analysis of larvae exposed to 2.5 ppm of either imidacloprid or spinosad for 2 hr ($n = 10$ larvae/ sample; 3 samples/ treatment). **a**, Principal Component Analysis of lipid profiles. Each dot represents the lipidome data sum of each sample. First component explains 29.6% of variance and second component explains 24.7% of variance. **b**, Correlation between disturbances caused by Imidacloprid and Spinosad on the lipid compounds. Pearson correlation analysis, $\text{Rho} = 0.3793567$, $\text{p-value} = 2.198 \times 10^{-14}$.

4.7 The effects of acute insecticide exposures on transcriptomics profiles

Unique insecticide profiles were also found when comparing the transcriptomic analysis (**Figure 4.3**). This was particularly true for the brain. Here spinosad impacted more genes involved in P450 metabolism, the oxidation-reduction process and immune response. One particularly noteworthy process that was enhanced in the brains of larvae exposed to spinosad was the activation of the proteasome pathway (**Figure 4.4**). While the proteasome system is commonly known for degrading individual cellular proteins through the ubiquitin-proteasome system (UPS), lysosomes degrade cytoplasmic

components through autophagy (Wang and Robbins 2014). However, proteins can be substrate for both degradative systems, which engage in crosstalk, allowing the cell to reduce the levels of UPS substrates (Korolchuk et al. 2010). A total of 35 genes involved in proteasome pathway were activated in brains of spinosad exposed larvae, including all the β proteasome subunits (β 1-7) and six out of 7 α proteasome subunits (α 2-7). This activation may be a response to the increased endocytic vesicle trafficking and the need to recycle the nAChRs blocked by spinosad.

In comparing the brain and fat body for either insecticide, it is abundantly clear that these tissues are not responding to the demand for energy in the same way. The brain shows a perturbed expression of genes related to pyruvate and citrate metabolism. Regardless of which insecticides was used, genes encoding for lactate dehydrogenase (*ldh* – converts lactate into pyruvate) pyruvate dehydrogenases (*pdha*, *pdhb* – convert pyruvate into acetyl-CoA) and citrate synthase (*kdn* – converts acetyl-CoA into citrate, a substrate for lipogenesis) were upregulated. Citrate connects carbohydrate and lipid metabolism, since it is the first molecule produced in Krebs cycle and, it can be broken down into acetyl-CoA for fatty acid biosynthesis (Williams and O'Neill 2018). In this study, increased levels of acetyl-CoA combined with elevated levels of SREBP could provide the neurons with the ability to increase lipid synthesis (Liu et al. 2015). These lipids are typically peroxidized and exported from neurons via ABCA transporters where they are captured by *Glial Lazarillo* (Liu et al. 2017). In the fat body, no impact on the expression of these genes, or other related genes was observed, suggesting that insecticides did not cause major perturbations in lipid and carbohydrates metabolism in this organ. This difference may be explained by the fact that the brain is under a higher energy demand. From a metabolic perspective, the fat body acts as a lipid reservoir. The triggering of an increase in the number of LDs in fat body is likely to be a protective response to oxidative stress (Bailey et al. 2015). If the accumulation of LDs is preventing or reducing the oxidative damage in the fat body it may explain the minor impacts in expression of stress related genes (GSTs, P450s and genes from JNK and Cnc pathway).

It needs to be acknowledged that these tissues were studied at only one time point, chosen to match the one used for many other experiments reported here. Further, other relevant tissues such as the midgut, Malpighian tubules and hemolymph were not studied at all. Increasing the breadth and depth of transcriptomic analysis would enhance the understanding of insecticide impact at the tissue and organismal level.

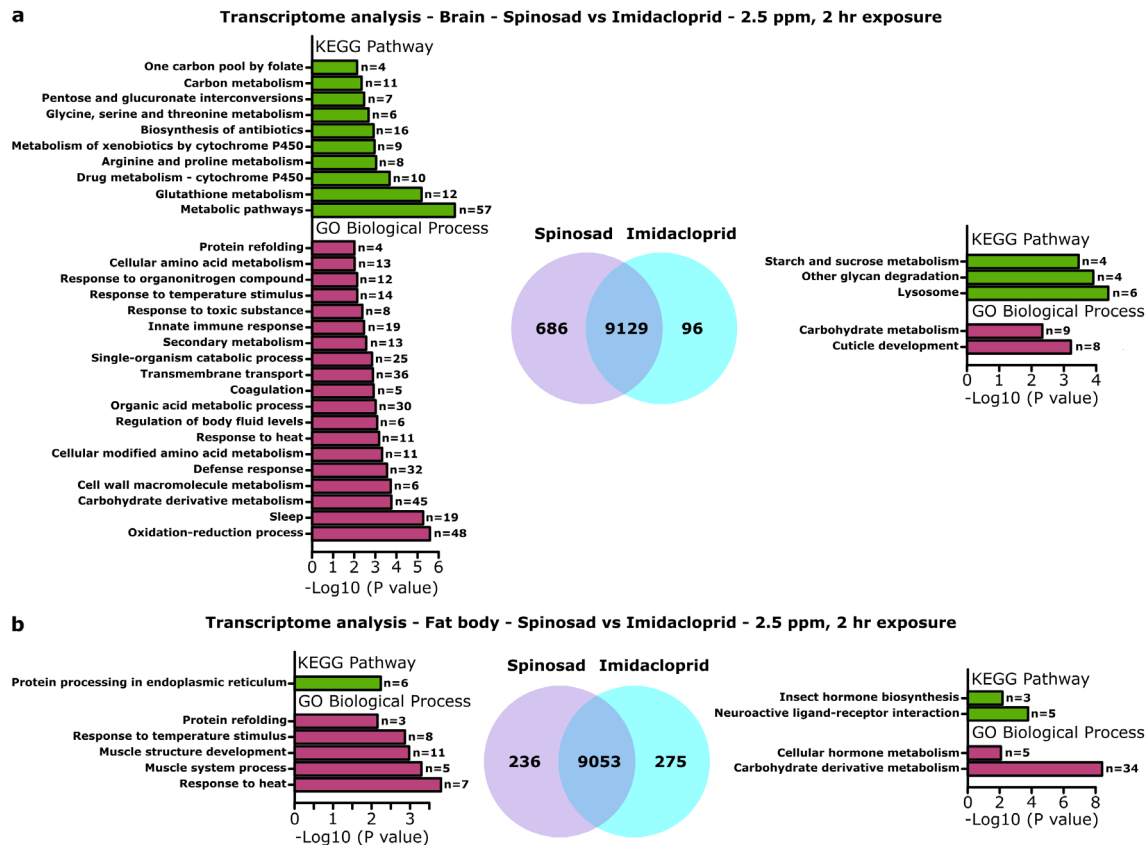


Figure 4.3. Comparison of transcriptomic profiles of larvae exposed to imidacloprid or spinosad. Third instar larvae exposed to either 2.5 ppm imidacloprid or spinosad for 2 hr ($n = 40$ larvae/ sample; 3 samples/ treatment). **a**, Transcriptomic analysis of brains, 686 genes were upregulated in spinosad samples and 96 genes were upregulated in imidacloprid samples (foldchange $>|2|$, $P_{adj} < 0.05$). **b**, Transcriptomic analysis of fat bodies, 236 genes upregulated in spinosad samples and 275 genes upregulated in imidacloprid samples (foldchange $>|2|$, $P_{adj} < 0.05$). KEGG and Gene Ontology analysis were performed in DAVID (Bioinformatics Resources 6.8), the Modified Fisher Exact P-value was transformed using $-\log(10)$ scale. The “n” values in front of the bars indicate the number of genes associated to each KEGG Pathway or GO Biological Process in the dataset.

impacts on the ON transient later (day 5) (**Figure 2.31**), when compared to spinosad (**Figure 3.32**). The impacts of imidacloprid on the lamina were mild (**Figure 2.31**) and more severe on the retina where the vacuolation of glial cells was conspicuous (**Figure 2.28**). Spinosad had almost no impact on ERG amplitude, except for the reduction observed on day 20, but reductions in ON transients were more severe and observed after only 1 day of exposure (**Figure 3.32**). The impact of spinosad was mild on the retina (**Figure 3.30**) but severe on the lamina with high proportion of vacuolated cells (**Figure 3.31**). The L1 and L2 laminar neurons (**Figure 2.29**) are believed to be the main neurons responsible for generating the ON transients observed in the ERGs (Coombe 1986). The vacuolation of the cartridges in the lamina, and thus of L1 and L2, in flies exposed to spinosad, therefore explains the large reduction in the size of the ON transient.

Why spinosad largely affects the lamina and imidacloprid the retina is something that remains to be answered. One factor could be the location of the cells expressing the specific nAChR subunits targeted by these chemicals. $D\alpha 6$ is known to be expressed in antennal mechanosensory and motor centres in adult brain (Nakayama et al. 2016); while $D\beta 1$ and $D\beta 2$ are known to be expressed respectively in the transmedullary neuron Tm2 (Takemura et al. 2011) and in medulla, lobula and lobula plate (Chamaon et al. 2002), involved in the visual system (Namiki and Kanzaki 2016). However, experiments here showed that $D\alpha 6$ is expressed not only in motor centres but in several areas in the adult brain, including the medulla (**Figure 3.29**). The same could be true for the expression of $D\beta 1$ and $D\beta 2$, i.e. the expression pattern of these subunits is not fully known, and a better understanding of it could provide a clue to the results obtained here. Another factor related to the specific impacts of imidacloprid in retina and spinosad in lamina could be the extent to which the insecticides, or chemical signals that they generate (e.g. ROS), penetrate the visual system. Experiments with antibodies specific to the two insecticides and DHE staining may address the penetration issue.

One thing that is clear is that, at the concentrations used here, both insecticides cause severe impact on visual acuity in *Drosophila*.

Both insecticides caused an increase in the number of mitochondria in the visual system - in the retina for imidacloprid (**Figure 2.28**) and in the lamina for spinosad (**Figure 3.31**). The number of mitochondria in a cell emerge from the dynamic balance

between mitochondrial biogenesis and degradation (Melser et al. 2015). Such turnover ensures quality control, eliminating dysfunctional mitochondria through mitophagy and adding new ones through mitochondria biogenesis (Stotland and Gottlieb 2015). Mitochondrial biogenesis is regulated by different factors such as hormones, inflammatory signalling and Ca^{2+} signalling (Wenz 2013). ROS are inducers of mitochondrial biogenesis through the activation of the transcription factor PGC-1 α (Wenz 2013; Wright et al. 2007). In this study, the results obtained by electron microscopy of the visual system match perfectly with the results obtained with MitoTimer in larvae (**Figure 2.6** and **Figure 3.5** – an increase in the signal for healthy mitochondria, but also in the signal of stressed mitochondria) and the lipidomic analysis in larvae (**Figure 2.19** and **Figure 3.17**). CLs are involved in mitochondrial turnover, being externalized to the mitochondrial outer membrane in neuronal cells, where they serve as a mitophagic receptor (Chu et al. 2013; Melser et al. 2015). Compromising CL synthesis and translocation can decrease mitochondrial recycling in neuronal cells (Chu et al. 2013; Melser et al. 2015). This could be the way by which the oxidative stress induced by insecticides led to the accumulation of defective mitochondria.

The nAChR *D α 6* knockout mutants tested also had defective ERGs (**Figure 3.33** - reduced ON transients) and photoreceptor degeneration (**Figure 3.34**). None of these phenotypes showed signs of worsening over time, meaning that these defects are probably congenital. A Genome-Wide Association Analysis showed that the subunit *D α 6* is involved with oxidative stress resistance in *Drosophila melanogaster*, a result that was validated by mutant analysis (Weber et al. 2012). It can be hypothesised that the lack of oxidative stress protection conferred by *D α 6* subunits is causing the visual system phenotypes and even the natural increase of LDs in fat body and lipid levels in hemolymph observed in mutants. Alternatively, it could be due to yet to be discovered *D α 6* functions.

4.9 The onset of neurodegeneration

Chronic exposures to imidacloprid and spinosad caused a progressive loss of climbing ability (**Figure 2.23** and **Figure 3.36b**), a phenotype largely associated with neurodegeneration (McGurk et al. 2015). Moreover, both insecticides increased the bang sensitivity in a progressive manner (**Figure 2.22** and **Figure 3.36a**). Bang-sensitivity

phenotype is associated with defects in the oxidative phosphorylation enzymes and thus mitochondrial dysfunction (Toivonen et al. 2001).

While the evidence for neurodegeneration in imidacloprid exposed flies comes from the behavioural assays, electroretinograms and the vacuolation of glial cells in retina, H&E staining of brains revealed a higher degree of neurodegeneration caused by spinosad (**Figure 2.33** and **Figure 3.38**). It is important here to note that the dose used for chronic exposures to spinosad (0.2 ppm) was 20-fold lower than the dose used for imidacloprid (4 ppm). These data, along with the other data for spinosad strongly underline the message that an organic label cannot be to mean that the product is safer or less toxic than a synthetic alternative. Most chemical insecticides are used because of their high efficiency in killing insects. They are not pest specific; they could have detrimental impacts on non-pest insect species. In a literature review in 2010, Kirst concluded that spinosad has a lesser impact on non-pest insects compared with many other insecticides (Kirst 2010). However, this claim was largely based on studies that measured lethal concentrations, not the impacts of low dose exposures.

4.10 Insecticide exposures and the decline of insect populations

Low doses of imidacloprid and spinosad generate oxidative stress, triggering downstream perturbations in the lipid environment and gene expression and cause neurodegeneration in *D. melanogaster*. In investigating the impacts of low doses of imidacloprid or spinosad in other insect species it is, thus not expected that the responses obtained here will be exactly the same as clearly there are a range of significant biological differences between species. However, it is extremely likely that the impacts of these insecticides in a wide range of insect species will be similar, because the major proteins and pathways are evolutionarily conserved including the nAChR targets (Jones et al. 2007; Zhang et al. 2000), the JNK and Cnc/Keap1 xenobiotic responses (Fuse and Kobayashi 2017; Ríos-Barrera and Riesgo-Escovar 2013) and lipid metabolism and transport (Liu and Huang 2013). The mechanisms whereby ROS causes neurodegeneration is conserved even between insects and mammals (Liu et al. 2017). But while the impacts of these insecticides may be similar in the different insect species, the concentrations at which they occur will almost certainly vary. This is to be expected because there are marked differences in sensitivity to any

given insecticide that are observed between, and even within, species. This can be due to a wide range of factors that influence the amount of insecticide in the brain and the level of ROS that might be generated via the insecticide:target interaction. Variation in the capacity to reduce concentrations present in the brain can exist as a consequence of difference in processes such as insecticide metabolism, uptake or efflux (Scott and Buchon 2019). The level of affinity of a given insecticide for its target could also vary within and between species (Denecke et al. 2017; Manjon et al. 2018).

The mechanism by which imidacloprid and spinosad exert their low dose impacts have not been systematically addressed in any other insect species. There have been many studies on the low dose impacts of one of these insecticides - imidacloprid. Having been associated with the Colony Collapse Disorder (Lu et al. 2014), the impact of imidacloprid on various aspects of behaviour has been analysed in some detail. Honeybees fed an acute dose of 0.5 ppm of imidacloprid showed an impairment in the navigation system which lasted for 24 hr. Doses as low as 0.05 ppm were sufficient to affect the interval between two successive visits to the same feeding site (Thany 2010). Doses as low as 4 or 8 ppb were sufficient to kill 50% of worker bees in the course of a 30 day of chronic exposure (Rondeau et al. 2014). This ultra-sensitivity could be due to reduced number of genes encoding detoxifying enzymes (Arena and Sgolastra 2014; Ugelvig and Cremer 2012).

These studies on honeybees highlight a gap between what has been done in this study and that which is published in the literature. With a focus on Colony Collapse Disorder, the literature has been devoted to aspects of behaviour that, if compromised, would explain this phenomenon. In considering the demise of other non-pest insect species, potential impacts on behaviour and fitness traits will be of real interest. While this study has focused on mechanism more than behaviour or fitness, it does have implications which can be identified:

- (1) Acute exposures to either imidacloprid or spinosad damage mitochondria (**Figure 2.7a** and **Figure 3.6c**) and reduce energy (ATP) levels (**Figure 2.7b** and **Figure 3.6b**). It remains to be tested, but this could impact mating behaviour and reproductive output.

- (2) Chronic exposures to these insecticides shorten lifespan (**Figure 2.21** and **Figure 3.26**), cause neurodegeneration (**Figure 2.28** and **Figures 3.31, 3.37**) and loss of visual acuity (**Figure 2.31** and **Figure 3.32**). Lifespan is critical in social insects such as honeybees, where workers take on different roles in the hive as they age. If impacts such as the ones observed here in the model *Drosophila* are also observed in other insect species such as bees it would affect the viability of the colony. Neurodegeneration could impact a range of behaviours, depending on the regions of the brain that are affected. For spinosad the brain damage is very extensive. The loss of vision detected here would be a threat to the survival of any insect, such as forager worker bees, that rely on visual clues to identify the location of nutritional resources (Srinivasan et al. 2000). Defects in foraging behaviour and visual learning have already been associated with imidacloprid exposure in honeybees (Belzunces et al. 2012). A reduction in energy levels would reduce the capacity to sustain flight.
- (3) The transcriptomic analysis revealed a severe disruption in the expression of genes involved in immune response (**Table 2.3** and **Table 3.3**). Several antimicrobial peptides (such as Dipterecin and Drosomycin) were among the most downregulated genes in the transcriptomic analysis. It has already been suggested that the humoral immune response of honeybees is compromised by insecticide exposure (Perveen and Ahmad 2017). The level of disruption of gene expression detected here demonstrates a great potential for low dose insecticide exposures to synergise with pathogens and parasites, contributing to the decline of insect populations.
- (4) Adaptation to any particular environment involves the regulation of gene expression to optimize phenotypic outcomes. The significant perturbations of gene expression observed here under conditions of acute exposure are likely to disrupt this optimal pattern of gene expression. This may impact fitness and synergize with the effects of other stressors, such as climate change, that have been implicated in the demise of insect populations. For honeybees it may be a factor in determining the extent damage inflicted by the parasitic Varroa mite (Nazzi and Le Conte 2016).

At the end of this study so much remains to be done in *Drosophila* and non-pest insects. In *Drosophila* a detailed analysis of different aspects of behaviour and

components of fitness during or following exposure to imidacloprid and spinosad would be an obvious sequel to the research conducted here. Recent research indicates that impacts that span life stages should also be considered. Imidacloprid concentrations in the parts per billion range administered in larval development, impact a suite of adult behaviours in *D. melanogaster* indicating the potential for carryover affects (Young et al. 2020). Non-pest insects could be examined to determine whether exposure to low doses of these insecticides triggers oxidative stress and the downstream impacts on the lipid environment and gene expression observed here. This study has thrown a spotlight on the deleterious impacts of an organic insecticide, spinosad. Imidacloprid research has dominated the research literature because of its links to Colony Collapse Disorder. Many other insecticides have therefore not had the same level of scrutiny. That the key to the low dose impacts of both imidacloprid and spinosad is the oxidative stress they generate is of real concern because insecticides from several other chemical classes have been associated with increases in oxidative stress. These insecticides need to be systematically investigated using a pattern of examination of the type that has been used here. If a wide range of insecticides do indeed cause oxidative stress, then the cumulative dose of insecticides that non-pest insects are exposed to will be of real interest.

4.11 Conclusions

Unfortunately, the widespread use of insecticides creates the potential for a wide range of organisms, including insects and humans to be exposed (Khorram et al. 2016; Yadav et al. 2015). Around 600,000 tonnes of insecticides are used annually around the world and sublethal concentrations of these chemicals can affect behaviour, fitness and development of target and non-target insects (Müller 2018).

This study has proposed a mechanism to explain how exposures to low doses of insecticide could be contributing to the worldwide decline of insect populations. Low doses of the insecticides tested created oxidative stress in the *Drosophila* central nervous system, precipitating a cascade of impacts in *Drosophila* biology (**Table 4.1**). Under conditions of acute exposure the accumulation of ROS in the brain impacted the lipid environment of metabolic tissues, disrupted the expression of many genes involved in stress and xenobiotic response, severely impaired the immune response and launched patterns of gene expression that would lead to neurodegeneration. The overall response:

energetic failure demonstrated by the mitochondrial impacts and systemic depletion of ATP levels. Under chronic doses the oxidative stress and mitochondrial impairment caused the progressive loss of vision and neurodegeneration. That is the first study to demonstrate that excessive ROS production is possibly the centrepiece in the impact of low dose insecticide exposures.

Table 4.1. The cascade of impacts precipitated by small doses of imidacloprid and spinosad on *Drosophila melanogaster*.

	Imidacloprid	Spinosad
<i>Effect on nAChR</i>	Agonist	Antagonist
<i>nAChR response</i>	Low, enduring Ca ²⁺ influx	nAChR endocytose and digestion by lysosome
<i>Initial effect</i>	ROS production in the brain	Lysosomal disorder and ROS production in the brain
<i>Acute effect on larval movement</i>	Rapid decrease in larval movement	Slow decrease in larval movement
<i>Acute effect on energy metabolism</i>	Increased mitochondrial turnover, immediate reduction in ATP levels	Increased mitochondrial turnover, late reduction in ATP levels
<i>Acute effect on fat body</i>	Increased lipid stores, with reduction of large LD numbers and increase of small LD numbers	Increased lipid stores, with reduction of large LD numbers and increase of small LD numbers
<i>Acute effect on Malpighian tubules and midguts</i>	Decreased lipid stores	Decreased lipid stores
<i>Acute effect on hemolymph</i>	Decrease lipid levels	Increased lipid levels
<i>Acute effect on lipidomic profiles</i>	Overall reduction of TAGs, PCs, PEs, and CLs	Overall reduction of TAGs, PCs, PEs, and CLs
<i>Acute effect on brain's transcriptomics profiles</i>	Affected genes related to xenobiotic metabolism, antioxidant response, neurodegeneration, and immunity	Affected genes related to xenobiotic metabolism, antioxidant response, lysosome and proteasome activity, neurodegeneration, and immunity
<i>Acute effect on fat body's transcriptomics profiles</i>	Affected genes related to xenobiotic metabolism, antioxidant response and immunity	Affected genes related to xenobiotic metabolism, antioxidant response and immunity
<i>Response of resistant mutants to exposure</i>	Showed impacts on fat body and hemolymph	Reduced impacts on fat body and hemolymph
<i>Chronic effects on vision</i>	Early reduction in amplitude, late reduction in ON transient.	Late reduction in amplitude, early reduction in ON transient
<i>Chronic effects on neurodegeneration</i>	Neurodegeneration in retinas	Neurodegeneration in lamina and vacuolation of midbrain
<i>Chronic effects on behaviour</i>	Decreased climbing ability and increased bang-sensitivity	Decreased climbing ability and increased bang-sensitivity
<i>Response to antioxidant treatment</i>	Reduced LD accumulation in fat body; improved larvae movement, climbing ability and chronic exposure survival	Reduced LD accumulation in fat body; improved larvae movement and acute exposure survival

A number of biomarkers identified here, such as impacts on ERGs, anatomy of the retina, aconitase activity, ATP and superoxide levels and lipid droplet dynamics, can be used to investigate the systemic impact of low-dose insecticide exposures on other insect species. Several phenotypes were ameliorated with antioxidant treatment. Given the conservation of nAChRs in insects and the ability of other insecticides to generate oxidative stress, the downstream impacts of these other insecticide:receptor interactions merit similar investigation, since they could potentially trigger similar impacts. The impacts of imidacloprid and spinosad should be investigated in other non-pest species. Studies on other insecticides should be initiated in *Drosophila* following the template established here before other non-pest species are investigated. In cases where oxidative stress is shown to play a central role in insecticide toxicity, the use of antioxidants could provide a possible mitigating solution. This may be particularly useful in reducing the impact of insecticides on commercial beehives. This should be tested.

Finally, the results obtained here, suggest that the impacts of low insecticide doses on *Drosophila* biology could synergistically interact with the other threats. If these impacts are verified in other species the neurodegeneration, reduction of visual acuity, and impairments in energy metabolism and immune response could turn insects more susceptible to climate change, habitat loss, pathogens, and parasites, contributing to the “Insect Apocalypse”. This work demonstrated how detrimental low dose of neurotoxic insecticide exposures can be. It urges action. We must rethink the way we use insecticides.

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