School of Biosciences, Cardiff University, United Kingdom

Defining species sensitivity and synergism potential for pesticides and pesticide mixtures through physiological traits analysis

A thesis submitted to Cardiff University in candidature for the degree of Dr of Philosophy

Claire E. Badder

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Supervisory Group –

Prof. D. J. Spurgeon [2], P. Kille[1], S. Short [2]

[1] Cardiff University, School of Biosciences, Sir Martin Evans Building, Museum Avenue, Cardiff, CF10 3AX,

[2] UK 2 UK Centre for Ecology and Hydrology, Maclean Building, Benson lane, Wallingford, OX10 8BB, UK

SUMMARY

This thesis investigates mechanisms and effects of chemical interactions on lepidopterans by analysing how toxicity is underpinned by toxicokinetic and toxicodynamic traits. *Mamestra brassicae* were exposed to a range of insecticides and fungicides representing several modes of actions, to identify synergistic or antagonistic effects. A novel high-throughput lepidopteran toxicity test was designed and trialled with a known toxicant, the organophosphate chlorpyrifos. This assay was suitable for assessing acute toxicity and toxicant effects on growth rates.

To investigate chemical interactions, the effects of single compounds must be known. Using the novel bioassay, a range of five insecticides and four fungicides were screened for toxicity. Insecticides were toxic to larvae, except those targeting nicotinic acetylcholine receptors (nAChRs) such as imidacloprid and sulfoxaflor. Fungicide exposures caused no mortality. Of 13 binary mixture combinations tested, six showed a synergistic interaction, one an antagonistic and six no interaction (additive). The synergistic mixture of cypermethrin and prochloraz was taken forward for further testing. Sublethal and acute effects of this mixture were analysed in 2nd to 6th instar of *M. brassicae* larvae using the TKTD DEBTox model, highlighting synergistic effects on growth and survival.

Finally, adult and larval sensitivity was compared to investigate effects of transcriptomic differences between the morphologically distinct life stages. Larvae and adults showed differential sensitivity to imidacloprid. A *M. brassicae* genome was sequenced and a larval and adult transcriptome generated to investigate this. Three detoxification enzyme families representing phase I, II and III metabolism were catalogued and transcriptomic differences analysed. Several of these enzymes were over 1000x upregulated in larvae, so represent potential drivers of this differential sensitivity. This work explores the mechanisms of synergistic chemical interactions via a multi-faceted approach including toxicity testing, TKTD modelling and metabolomics. This work will provide evidence to improve control of crop pests by implementing targeted pest management strategies based on life-stage sensitivities and also investigates the synergistic potential of pesticide mixtures, informing future pest management strategies and minimising risk of adverse effects to beneficial species.

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CHAPTER 1 – INTRODUCTION

1.1. Pesticide Use

Anthropogenic activity has exposed the natural environment to a wide range of chemical pollutants that can threaten both the structure and function of ecosystems (IPBES, 2019). An example of this is the routine use of pesticides in agricultural practices. The term pesticide can encompass a wide range of compounds including fungicides, herbicides, insecticides, rodenticides and molluscicides (Aktar et al. 2009). In many cases, these chemicals are designed to disrupt or inhibit the physiological functions of their target species leading to effects on key vital rates (e.g., survival, growth, sexual development, reproduction) that underpin population growth and stability. A study by Pimentel (1995) indicated that only 0.1% of applied pesticides reach their target pest while 99.9% entered into the wider environment, where they may interact with non-target organisms. These wider effects are a concern, as many of the unintended consequences of pesticide use arise from their impacts on wider species present in ecosystems.

It is widely acknowledged that many insects and annelids do not threaten the agricultural environment, but rather offer beneficial services to the ecosystem. For example, insects such as hymenopterans and lepidopterans may act as pollinators (Sakagami and Sugiura 2019; Brock et al. 2021), provide services as predators of pests, and parasites or species such as collembolans have a role in nutrient cycling (Ndakidemi et al. 2016; Fedorov 2021). Yet, despite the environmental concerns about the conservation of populations of these beneficial species, pesticide usage has increased considerably in recent decades. This is most notable in the agricultural sector, where the economic benefit of pesticides have encouraged the widespread prophylactic use of a range of different active ingredients in all stages of production. A report by FERA (2019) showed that 30 million more hectares were treated with pesticides in 2016 compared to 1990 indicating that the total area treated by pesticides is still increasing. Agriculture has a substantial benefit to the UK economy with estimates suggesting that the farming sector produces 60% of all food consumed in the UK at a worth of around £18 billion per year in exports (Development Economics, 2017). Therefore, modern agricultural practices are reliant on the use of combined treatments of agrochemicals for pest management, either used in combination or sequentially throughout the growing season, are and will in all likelihood remain a key part of environmental management practices for farmed land.

Because of their potential to impact non-target species, pesticides have been studied for their ecotoxicological effects perhaps more than any class of contaminant (with metal being perhaps a close second). As a result of this work, there is a large body of research documenting the effects of single pesticide active ingredients or formulated products on both non-target and pest species. However, this focus on the effects of a single chemical may not realistically reflect the environment, as it is much more likely that organisms will be exposed to mixtures of historic and recently applied agrochemicals (Hutchinson 2011; Cedergreen 2014). Although it is crucial to understand the impact of single chemicals, it is also essential to understand the interactions between pesticides in combinations. Improving our understanding of these mixture effects on non-target species has the potential to reduce the harmful effects to beneficial invertebrates arising from applying mixtures of chemicals, e.g., fungicides and insecticides to beneficial invertebrates in arable landscapes.

1.1.2 Insecticides

Insecticides are one of the major classes of pesticides that are in widespread use for agricultural management. To exert their toxic effects, insecticides target one of four key aspects of insect physiology: growth and development, nerve and muscle, respiration or midgut function (IRAC, 2018).

Insecticides that target nerve and muscle functions can include those that inhibit Acetylcholinesterase (AChE), such as organophosphates or carbamates. Acetylcholine (ACh) is a neurotransmitting agent, which plays a role in the transmission of impulses to cells at synaptic, cholinergic and neuromuscular junctions (Westfall, 2009). AChE is an enzyme that acts as a regulator of nerve transmission by controlling levels of ACh in the synaptic junction by hydrolysing ACh into Acetic acid (A) and Choline (Ch); metabolic products that do not stimulate synaptic membranes. AChE inhibitors disrupt ACh by inhibiting ACh hydrolysis, therefore, blocking the transmission of impulses to target cells, leading to sustained excitation of the nervous system causing paralysis and death (Fukuto, 1990). ACh is not solely found in insect species. Indeed, their presence is widely conserved in species across a range of phyla, including all mammals. As a result of the widespread occurrence of the Ach target across the tree of life, organophosphates are potentially harmful to a wide range of species, including plants (Zobiole et al. 2012; Mishra et al. 2015), fish, (Benli and Özkul 2010), amphibians (Wijesinghe et al. 2011) and mammals, including humans (Badr 2020).

A further group of nerve-acting insecticides that have passed into widespread use in agriculture are the neonicotinoids, examples of which include clothianidin, thiamethoxam, thiacloprid and imidacloprid. Although relatively recently banned for all except emergency and veterinary uses in Europe and the UK, neonicotinoids remain widely used worldwide. Neonicotinoid insecticides are similar in chemical structure to nicotine (Goulson, 2013). The class can include compounds that have systemic properties, which enable them to enter plants through the roots or shoots and then transport them through the plant tissues regardless of the method of application (Simon-Delso et al., 2015). These systemic properties mean that all plant tissues contain the pesticide, making the plant toxic to insects that feed upon sap or leaf tissues (Goulson, 2013). Consistent with their systemic use, neonicotinoids are relatively soluble in water and have relatively long-half lives in the environment, meaning that they remain present to allow exposure to occur through the growth period of the crop plant.

The mode of action (MoA) of neonicotinoids is to act as an antagonist of nicotinic acetylcholine receptors (nAChRs). Neonicotinoids bind to the active site of this complex multi-sub-unit receptor, thereby, opening cation channels and stimulating nerve impulse transmission (Casida and Durkin, 2013). Similarly to AChE-inhibiting insecticides, this binding leads to continuous stimulation of the neuronal membranes which can then lead to cell energy depletion and paralysis (Simon-Delso et al., 2015). Neonicotinoids interact with nAChRs of insects in a specific manner, therefore unlike AChE inhibitors, this class of insecticide is more selective in their toxicity to insects than to mammalian species (Tomizawa and Casida, 2005). Furthermore, the receptor target is expressed in much higher numbers in insects than in mammals. Therefore, the toxicity of neonicotinoids to mammalian species is reduced in comparison to broad-range insecticides such as organophosphates. Due to the taxon-targeted nature of this insecticide group, 24% of the global insecticide market has consisted of neonicotinoids, making the nAchR the most targeted receptor site for pesticides sold globally (Sparks et al. 2020).

Although active against AChRs in insects, this specificity for the insect receptors is not complete. Consequently, non-target species with similar nAChR receptor structures can also be impacted, for example, annelids such as earthworms (Short et al. 2021). For example, Wang *et al.* (2015) found that exposure to clothianidin inhibited cellulase activity, an important enzyme in earthworms that aids in the decomposition of plant litter. Further,

exposure to neonicotinoid insecticides caused disfigurement of epidermal tissue in *Eisenia fetida* (Wang *et al.* 2015). This study also found that cells within midgut tissue had irregular-shaped nuclei after neonicotinoid exposure. Furthermore, Ge *et al.* (2018) found that *E. fetida* reproductive rates deteriorated and that any cocoons produced were smaller after exposure to six different neonicotinoids even at environmentally relevant concentrations.

A further example of the potency of neonicotinoids on beneficial species can be seen for nematodes. Bradford *et al.* (2020) found that exposure to imidacloprid impacted both the growth and fecundity of the nematode *Caenorhabditis elegans.* Further studies have also reported the impacts of neonicotinoid exposure on the movement of nematodes, such as reduction of locomotion and head-thrashing (Ruan et al. 2009; Kudelska et al. 2017; Sakaguchi et al. 2022). However, acute exposure suggests that *C. elegans* is not a susceptible species to field-relevant concentrations of neonicotinoids (Neury-Ormanni et al. 2019); perhaps due to low cuticle permeability affecting the extent of exposure (Kudelska et al. 2017).

A third major class of insecticides currently in use worldwide are the pyrethroids. Chemicals in this class were first synthesised from natural pyrethrins and are often used as broad-spectrum insecticides (Vijverberg and van den Bercken, 1990). Pyrethroids have been shown to have low oral toxicity to mammalian species but have been seen to exhibit clear toxic responses to insect pests (Mueller-Beilschmidt 1990). Like organophosphates and neonicotinoids; pyrethroids are also neurotoxins. This class of insecticide act on the voltage-gated sodium channels (VGSC) in the nervous system (Singh et al. 2012). However, chloride and calcium channels can also be affected by altering the electrical signalling functions of these channels (Soderlund 2010). Such interactions and their effect on nerve function can lead to paralysis and death (Vijverberg and van den Bercken 1990). It is thought that the decreased sensitivity of mammals to pyrethroids can be, in part, explained by differences in physiological temperature (Chrustek et al. 2018). Pyrethroids form stronger bonds with the sodium channel at lower temperatures, therefore, they are likely to be more effective at the lower physiological temperatures of insect bodies (around 25°c), as compared to the higher physiological temperatures of mammals up to 37°c (Ensley, 2007).

Pyrethroids such as cypermethrin and related active ingredients may also be toxic to other beneficial invertebrate species such as earthworms and collembolans. Even in sublethal

concentrations cypermethrin was found to be genotoxic to the annelid *Pheretima peguana* (Muangphra et al. 2015). Furthermore, reproductive rates were decreased in both earthworms (Zhou et al. 2011), springtails (*Folsomia candida*) and *C. elegans* (Ruan et al. 2009) exposed to levels at recommended application range concentrations (Zortéa et al. 2015).

Diamide insecticides such as cyantraniliprole, are ryanodine receptor modulators. They act on the nervous system by activating muscle ryanodine receptors, causing uncontrollable release and depletion of calcium needed for muscle contraction (Troczka et al. 2012; Grávalos et al. 2015). Calcium channels are a common choice target for pest management, mainly because of their role in fertilisation, alongside the aforementioned role in muscle contraction (Hall et al. 1995). Diamide insecticides are selectively toxic to invertebrates over mammalian species, due to differential selectivity of the ryanodine receptor (Selby et al. 2013); most probably based on differences in receptor type found in mammals and insects (Cordova et al. 2006).

Anthranilic diamides, such as chlorantraniliprole and cyantraniliprole, are often introduced into the environment as a seed treatment, directly into the soil or through plant application (Cordova et al. 2006). Studies have suggested that the efficacy of cyantraniliprole and chlorantraniliprole may be improved by seed coating or soil application rather than when used as a foliar spray. This is due to the translocation of the toxin throughout plant tissue based on the relative water solubility of these actives and their prolonged half-life (Pes et al. 2020; Huynh et al. 2021). In a study of systemic transfer, Huynh et al (2021) found that metabolites of cyantraniliprole were present in all tissues of tomato plants except for the fruit. This compared to the results of Pes et al (2020) who found that if cyantraniliprole was applied via foliar application, the pesticide was not translocated to new leaves indicating reduced systemic transfer via this route.

Although anthranilic diamides demonstrate such strong specificity for insect target taxa, their application can still be detrimental to beneficial species. Liu et al (2018b) found that at concentrations of 5 mg/kg and above of chlorantraniliprole, weight of *E. fetida* was significantly reduced; a result mirrored in a study by Qiao et al (2019). However, anthranilic diamides have been proposed as a bee-friendly alternative to neonicotinoid pesticide application (Larson et al. 2013; Larson et al. 2014). However, despite such claims, there is

growing evidence of sublethal effects on pollinator species. For example, exposure to chlorantraniliprole has been found to impair the locomotion of honeybees for periods of greater than one-week post-exposure (Selby et al. 2013; Kadala et al. 2019); indicating the potential for adverse effects.

1.1.3 Fungicides

Fungicides are biocidal chemicals that are applied to reduce crop damage by eradicating fungal crop diseases. Fungicides are designed to disrupt a range of processes associated with specific aspects of fungal metabolism, to provide targeted impacts on this group, with the anticipation of minimal effects on non-target taxa. Despite the intended targeting of fungi, there are many reports of fungicide use resulting in acute or chronic effects on aquatic and terrestrial invertebrate species. Like insecticides, different fungicide classes are in use that have several different effect mechanisms. Modes of action most widely linked to fungicide action include effects on lipids or other membrane components, effects on amino acids and protein synthesis, effects on signal transduction, effects on respiration and effects on cell divisions by affecting mitosis (FRAC, 2019).

Fungicides are often considered of comparatively low risk for terrestrial invertebrate species, as they are not designed to target receptors and functions that are associated with key aspects of relevent species physiology. However, there is an increasing body of research that indicates their usage has had detrimental effects on invertebrate populations. Notable examples of this are the association between fungicide application and declines in pollinator populations and impairment of larval development (Mengoni Goñalons and Farina 2018; Pettis et al. 2013; Simon-Delso et al. 2018). Sublethal effects of fungicide application have also been documented in soil species such as *C. elegans*. For example, fungicide exposure has been reported to reduce nematode growth and impair neuronal function (Easton et al. 2001; Negga et al. 2012; Harrison Brody et al. 2013).

Azole and aromatic fungicides are the class of fungicides that have been in widest use in the UK over the last two decades. The mode of action of this class of fungicide has been linked to the disruption of lipid and membrane function. Aromatic fungicides such as chlorothalonil can be phototoxic. This means that the membrane of fungal cells becomes sensitive to solar

radiation, which can then destroy membrane lipids such as linoleic acid (Boscá et al., 1998). Effects on membrane integrity is also associated with exposure to the azole sub-group of triazole fungicides, for example, propiconazole. This class of fungicide also causes membrane dysfunction by altering the chemical structure of sterols; which can eventually lead to cell apoptosis. Despite this potential, propiconazole is generally considered low risk for non-target vertebrate and invertebrate species. A study of earthworm mortality by Rico et al (2016) indicated that mortality rates of *E. fetida* exposed to the triazole tebuconazole were low and that *E. fetida* even had a slight attraction to tebuconazole in soil based on the results of an avoidance bioassay. However, this study also found that the worms suffered weight loss and exhibited unusual body constrictions such as coiling which was taken to be the result of physiological damage when exposed to high concentrations. This apparent sublethal effect could affect wider physiology, especially as tebuconazole has a relatively long half-life (32 to 216 days depending on soil type) (Cui *et al.* 2018) meaning that prolonged exposure of earthworms to this substance in soil may be anticipated. The triazole, Myclobutanil, displays further adverse effects on non-target species and is moderately toxic to avian and bee species (Bishop et al. 2000; Sanchez-Bayo and Goka 2016). Hence, direct toxicity can result for some species following azole fungicide exposure.

Another known mechanism of action of azole fungicides is through the inhibition of the functioning of enzymes from the cytochrome P450 superfamily. Cytochrome P450s (*CYP450*s) are a family of monooxygenase enzymes used in various biosynthetic functions in both plants and animals (Yang et al., 2011). For example, CYP51 takes part in the oxidative removal of methyl groups from steroid precursors such as lanosterol. This transforms the precursors into ergosterol, which is vital for maintaining all membrane fluidity and permeability (Zhang et al., 2019). It is also thought that CYP51 plays a role in immune function, as cells without CYP51 are more susceptible to phagocytosis by macrophages (Wu et al., 2018). Azoles are known to inhibit the activity of CYP51 and as a result ergosterol biosynthesis (a key fungal sterol that is involved in cell wall formation). While azoles are designed to target the specific *CYP450* enzyme that is involved in sterol metabolism, they are also known to inhibit other enzymes from this class. Given the role of *CYP450s* in a range of metabolic reactions, including those involved in hormone cycling and xenobiotic breakdown, such inhibition could potentially have effects on hormone status of the toxicity of co-exposed substances.

The mode of action for strobilurin fungicides such as azoxystrobin is the inhibition of mitochondrial respiration, spore germination and mycelial growth (Feng et al. 2020). Given the ubiquity of mitochondrial functioning in energy metabolisms across all eukaryote species, strobulins might be anticipated to have a general impact on metabolic traits in a range of different taxa. Despite this potential, azoxystrobin is currently identified as having low toxicity to birds, mammals, bees and several other non-target terrestrial invertebrates. Despite its relatively low toxicity to some groups, Wang *et al.* (2011) found that azoxystrobin was extremely toxic to the earthworm *E. fetida*. However, this study appears to contradict the findings of Leitão et al (2013) who found low mortality of this fungicide in a range of invertebrate species including *Folsomia candida*, *Enchytraeus crypticus* and *Eisenia fetida*. This study, did however, find chronic effects of azoxystrobin on reproduction in all three species. Further, an exposure study of pyraclostrobin to silkworm (*Bombyx mori*) found that oxygen consumption was reduced in mitochondria of the head and gut, leading to reduced mortality and weight of cocoons (Nicodemo *et al.* 2018).

Studies in aquatic species have also indicated the potential for sublethal effects as a result of azoxystrobin exposure. In fish, it has been reported that exposure to strobilurin fungicides can disrupt mitochondrial function (Li *et al.* 2021). In the zebrafish *Danio rerio*, this effect on mitochondria induced oxidative stress, in turn reducing both body length and movement (Kumar et al. 2020). This suggests strobilurin fungicides share a similar mode of action in animals as fungi by interfering with electron transfer and therefore, affecting mitochondrial functioning.

Chlorothalonil is a broad-spectrum organochlorine fungicide. Unlike azoxystrobin, which has a specific target; chlorothalonil is able to impact multiple biochemical pathways and processes. Chlorothalonil is known to bind to and deplete glutathione, thereby affecting cellular respiration and further preventing the germination of spores and motility of zoospores (Van Scoy and Tjeerdema 2014). This broad MoA and persistence of metabolites makes chlorothalonil a chemical of concern to non-target species. The persistence of this chemical, with a half-life that can be up to 150 hours and its metabolites means that there is significant potential for non-target species exposure to this fungicide. Further, as metabolites may be more persistent and toxic than the parent compound, these effects may persist even after the parent compound breaks down (Caux et al. 1996).

Although it is not always fully clear how chlorothalonil interacts with invertebrate species, sublethal effects have been reported in exposed pollinators. For example, Pettis *et al.* (2013) found that infection rates of the fungal parasite *N. apis* were twice as high in bees exposed to chlorothalonil as in unexposed individuals. Extreme effects as a result of exposure to the fungal parasite could include complete colony collapse giving the potential for chlorothalonil to have effects on colony dynamics. In addition to effects on pathogens, chlorothalonil can alter bee nutrition, affecting both protein and carbohydrate levels. In honey bees, this effect can decrease body weight and wing length (O'Neal et al. 2019). The potential for such sublethal effects of chlorothalonil on other pollinator species are unknown and should give cause for further study. However, despite this potential for interactions based on some shared mechanistic aspects, the US-EPA (EPA 1999) in a review of the agricultural use of this fungicide categorized chlorothalonil as 'relatively non-toxic' to honey bees.

As fungicides are widely used in agriculture, this has led to concern about the potential for the development of fungicide resistance FRAC (2019). As a common management strategy used to combat resistance, it is recommended that fungicides with different modes of action are applied either in mixtures or alternately in sequential exposures (van den Bosch et al. 2014). This approach is recommended to maintain the efficacy of fungal disease control in arable land, as it means that fungi are not subject to sustained directional selection as a result of the application of a single class of active ingredient (Massi et al. 2021). Indeed, this practical management approach is common among all types of pest control chemical biocides. For this reason, it is likely that non-target species are more likely to encounter exposure to a mixture of fungicides, rather than any single application. As fungicides are used in management regimes that can also include insecticides (and herbicides), this co-exposure will involve simultaneous exposures to multiple active ingredients.

1.2. Chemical Interactions and Metabolism

1.2.1 Concentration Addition and Independent Action

Managing chemical risk often relies on the results of single chemical data. However, this may not apply to realistic situations where organisms are exposed to several mixtures of chemicals simultaneously. Such mixture scenarios are of high relevance to agricultural management systems, where multiple pesticides may be used either simultaneously or across a growing season. Due to the variability and scale of mixtures in the environment; it is impractical to directly assess the effect of all chemical mixtures through direct experimentation (Altenburger et al. 2000). Therefore, several theoretical approaches have been developed that can be used to provide a robust basis through which to model the potential joint effects of exposures to mixtures.

Historically, two models have become established as the dominant paradigms used to assess the additive toxicity of chemical mixtures. The Concentration Addition (CA) model (Loewe and Muischnek 1926) is based on the assumption that the chemicals present in a mixture share the same biological target and,- therefore, have the same MoA. In this model, it is perhaps simpler to consider each chemical as a dilution of each other, differing only in terms of their potency (Cedergreen et al. 2008). According to the concentration addition model, the joint effects of chemicals present in a mixture can be calculated by the sum of their concentrations divided by a measure of their potency (e.g. an LC_{50} value).

The second model that has been widely used for the prediction of mixture effects is the Independent Action (IA) model (Bliss 1939). IA assumes that the two chemicals affect the same endpoint but do so through differing MoAs. Berenbaum (1981) illustrated this by likening it to the example of throwing nails and pebbles at eggs. Neither nails nor pebbles will cooperate in cracking the eggs, but each nail or pebble has a probability to hit the egg (this probability will likely differ between the two causes) and any damage done to the eggs is, therefore, the result of the combining of these probabilities of damage. The IA model acts under the assumption that chemicals behave differently in a statistical sense when part of a mixture. When used in a chemical mixture context, the joint effects of two chemicals

calculated according to IA can be calculated as the product of the unaffected fractions of the effects resulting for each chemical.

The development of CA and IA and their implementation for mixture toxicity assessment provides the structure behind much of the literature on chemical mixture theory and its application. Studies to assess the predictive potential of CA have demonstrated the capacity of this model to provide an accurate prediction of mixture toxicity in studies designed to test the joint effects of a large number of similarly acting chemicals (Altenburger et al. 2000; Arrhenius et al. 2004). The CA model is also efficient to use, as it provides a relatively simple framework for predicting the effects of a large number of chemicals present in combination. In particular, the model requires only a calculated single toxicity value (e.g. an LC_{50}) for its implementation. Thus, these models have relatively simple formulations. In addition, they can be applied to studies with field-relevant concentrations and those with mixtures of an infinite number of chemicals (Backhaus et al. 2004; Cedergreen 2014).

Despite their established history with mixture effect prediction, both CA and IA have faced criticisms in their application as default mixture effect prediction models. One major concern is their strict mechanistic focus. While this may be relevant to designed laboratory experiments, organisms in polluted natural ecosystems are likely to encounter chemicals with both similar and different MoAs at the same time (Mwense et al. 2004). Further, to predict the joint effects of mixtures , the models require the MoA of any mixture compound to be known. Such understanding is still not the case for many chemicals likely to be encountered by organisms, especially when exposure is to non-target species for pesticides (e.g. the mode of action of a fungicide exposure in an invertebrate species). A further limitation of the CA and IA models is the assumption of independence for responses to mixtures of chemicals in which chemicals interact. When such interactions occur this can lead to antagonistic or synergistic effects that are not captured by either model.

Although synergy has no universal definition, it can describe a joint action of mixture chemicals in which the total effect is greater than the sum of the effects calculated by the CA or IA model (Howard and Webster 2009; Cedergreen 2014). Conversely, antagonism is a smaller effect than predicted (Greco et al. 1995). Interactions between chemicals leading to synergism or antagonism may occur for a number of mechanistic reasons. Such effects could be due to toxicokinetics, such as when one chemical affects the assimilation, metabolism,

distribution or elimination of others leading to changes in internal exposure; or to toxicodynamics, such as when one chemical affects the molecular interactions, damage accrual or damage recovery of another. In both cases, the greater internal exposure or increased molecular interaction and cellular damage can result in greater effects on apical endpoints (e.g. mortality, growth, reproduction). In the context of risk assessment, CA generally results in the more conservative prediction of effects (i.e. high overall effect prediction) of the two models and therefore, may over-predict the effect of a mixture where chemicals do not have the same mode of action. Hence, assessment according to CA provides some marginal protection against synergistic effects (Cedergreen et al. 2008; Bjergager et al. 2017), although only in such cases where the scale of interaction is small.

A further significant limitation of the CA and IA models is that they completely ignore the time dimension of toxicity (Baas et al. 2010). The CA and IA are based on the use of dose-response models that draw on concentration-response data that is collected for a single time point to calculate the LC_x/EC_x. This value then being used for the mixture effect prediction. The problems inherent with LC_x/EC_x values have been widely discussed in the literature. It is recognised that these values are limited in the nature of the information that they provide on true toxicity, as both provide a measure of toxicity at a single snapshot in time (Jager 2011). Thus, if dose-response data from a different time point were used, a different LC_x/EC_x would be estimated. As a result of this time dependence, results obtained can be uninformative as EC_x values can decrease in time. For these reasons, it is suggested that toxicity should not be studied as a snapshot in time but rather as a dynamic process that takes into account temporal aspects of exposure and how over time this leads to the development of apical toxic effects.

1.2.2 Toxicokinetic/ Toxicodynamic models

A promising alternative to the dose response CA and IA mixture models can be seen in the development of various toxicokinetic and toxicodynamic (TKTD) approaches such as Dynamic Energy Budget Theory (DEB), and the General Unified Threshold model of Survival (GUTS)

(Jager et al. 2011; Jager et al. 2006). TKTD models are specifically designed to track the development of toxic effects over time. As such, these models use knowledge of toxicokinetic and toxicodynamic trait structure to mechanistically model the effects of a toxicant on organisms. Therefore, processes such as uptake, distribution, metabolism and elimination are accounted for which, unlike the dose response based CA and IA models allow a link between exposure concentrations and effects over time. This, in turn, allows for meaningful comparison between distinct chemicals and species, whilst considering the effects of time within the model, rather than it acting as a potentially confounding factor in any comparison (Jager et al. 2006). The temporal consideration of TKTD modelling allows analyses of different exposure scenarios that are not well accounted for by conventional concentration-response models, such as pulsed or fluctuating exposure (Ashauer and Jager 2018). The mechanistic basis of these models importantly also allows predictions of untested scenarios (i.e., longer exposure, new exposure concentrations etc.) once models are calibrated, something not possible with convention single time point concentration-response models.

1.2.2.1 The GUTS Model Framework

The GUTS model is a TKTD-based model that is specifically designed for describing effects on survival over time (Jager et al. 2011). GUTS is designed to simulate the temporal process of toxicity in time that leads up to the death of an organism (Jager 2011; Jager and Ashauer 2018). The GUTS framework is divided into three stages, a toxicokinetics module that transforms external concentration into internal concentration, a module where this internal exposure is translated into damage dynamics and finally the death mechanism that is calculated from the damage level to provide analysis of survival over time. Together, the damage dynamic and death mechanism form the toxicodynamic part of the model (Jager and Ashauer 2018). In the absence of internal concentration data, a reduced version of the model (GUTS-RED) can be used to model responses. This version combines both the TK and TD parameters into a one-compartment model with a dominant constant rate (k_d). The GUTS framework contains two assumptions regarding the death mechanism following the modelling of the TK and TD processes: these are that mortality results due to stochastic death, or that it is driven by individual tolerance. Stochastic death assumes that all individuals are identical so that when death occurs, it does so as a random event with a certain probability once a concentration threshold is exceeded, within this approach such individuals that survive exposure to a toxicant are not less sensitive than their counterparts but are

simply more fortunate (Newman and McCloskey 2000). Individual Tolerance (IT), in contrast, implies a concentration threshold at which an individual is killed immediately by a toxicant. In this case individuals do have different inherent sensitivities. Both approaches can be unified but using only SD or IT separately provides in most cases, a satisfactory prediction of survival (Jager and Ashauer 2018).

The GUTS framework has also been extended to simulate survival of organisms exposed to mixtures of toxicants (Bart et al. 2021). These developed models can use either individual tolerance or stochastic death in a model formulation that is similar to CA or IA analyses depending on whether the MoAs for the toxicants in the mixture are similar or dissimilar. In the CA like model, chemicals share the calculated mortality parameter, however, in the IA like model, survival probability is calculated by multiplying the survival probability of each chemical in the mixture. In addition to providing a simulation of survival, the GUTS mixture model can also highlight interactions between chemicals and classify mixtures as additive, synergistic or antagonistic (Bart et al. 2022). This is done by accurately estimating, overestimating or under-estimating mortality. For example, in a situation where chemicals are found to interact, mortality will not be accurately calculated by the model, shown either with under (synergistic) or over (antagonistic) predictions (Figure 1.1). Thus, the use of this mixture model can allow for the modelling of organism response mixtures of chemicals with differing MoAs, including in cases where synergistic or antagonistic effects are known to occur. This capacity to identify and mechanistically incorporate interaction in mixture means that TKTD models based on the GUTS framework can form an important part of any assessment to develop pest management strategies or to identify the risk of population losses of beneficial species as a result of exposure to mixtures.

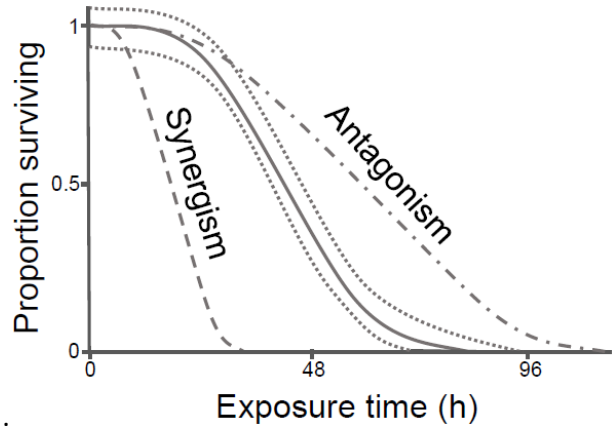


Figure 1.1. Schematic of TKTD interactions between chemicals. The prediction of concentration addition is shown as the solid line and 95% CIs are shown as close dashed lines. Synergy is simulated as a more rapid mortality effect and antagonism as a more delayed response.

1.2.2.2. DEB TK/TD Models

The sublethal effects of pesticides on apical endpoints relevant to population growth and stability, such as reproductive outputs, maturation and growth, are also often seen as a consequence of pesticide application. These sublethal effects have direct consequences on population stability by reducing broods per year, increasing predation rates and changing disease susceptibility (Pestana et al. 2009; Wang et al. 2009; Pettis et al. 2013; Krishnan et al. 2021).

Growth effects were originally analysed by energy apportionment models such as the 'Scope for Growth' model (Warren and Davis 1967) in which surplus power is converted into growth. In more recent years, this model has been refined and currently the most established model used to assess the sublethal effects of chronic pesticide exposure is the Dynamic Energy Budget (DEB) model (Baas et al. 2010; Jusup et al. 2017). DEB theory is designed to simulate the physiological process that describe the acquisition and use of mass and energy throughout the life of an individual to ensure survival, growth and reproduction (Kooijman 2010). DEB models can range from a complex system comprising of different levels such as

cells, organisms and even whole populations that require extensive parameterisation or can be largely simplified to simulate effects of a stressor on an individual in models that require much more limited data. The basic principle of the DEB framework is in metabolic theory (Kooijman 2010). DEB simulates the uptake of energy and its utilisation in biological processes. The theory suggests that organisms are made up of two compartments, energy reserve and structure. Structures require energy for maintenance, while the reserve compartment does not have a maintenance energy cost. Food is taken up by live organisms and assimilated proportionally to body surface area. The assimilated energy is stored in reserves, which are then mobilised for use by the organism. A fraction (κ) of the energy is used for the soma (growth and somatic maintenance), while the remainder (1 − κ) is used for maturation (of juveniles), maturity maintenance, and reproduction (in adults).

In DEB models, the maintenance requirements are supposed to take precedence over growth or structure (Kooijman 2001). Assimilation is governed by surface area, whereas maintenance is proportional to volume. Therefore, with a faster increase of the volume than surface area with growth, a growth curve with a maximal asymptotic length is expected. This pattern is captured by the Von Bertalanffy growth curve, under constant thermal and feeding conditions. For an easier application, DEB models can be simplified to DEBkiss models in which the reserve compartment is removed and the organism is viewed as a single compartment system where energy is assimilated directly from food and used to fuel metabolism (Jager 2020). Therefore, DEBkiss builds upon an explicit mass balance and excludes the distinction of biomass in a structure and reserve compartment: all biomass is treated as “structure”. This restricted design has been shown as a reliable simplification for invertebrate species (Figure 1.2).

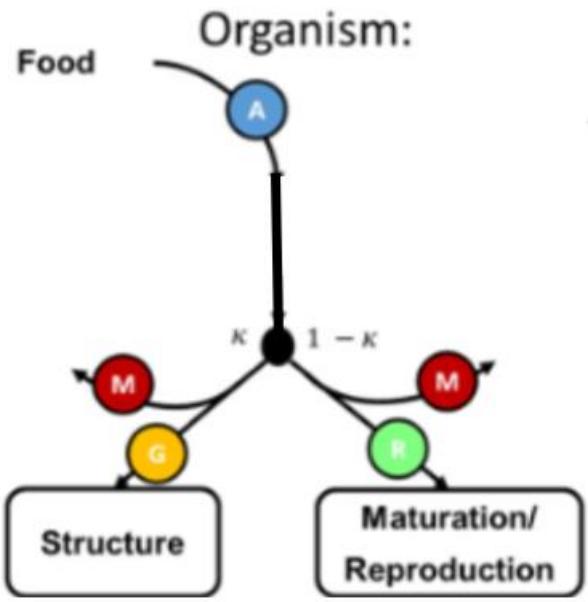


Figure 1.2. Schematic of the DEBKiss model, adapted from Sherborne et al (2020). Here food is taken in by the organism and assimilated into energy. Maintenance and biomass costs (labelled here as structure) are satisfied first and remaining energy (1-κ) is used to fuel maturation and reproduction costs.

In DEB-TKTD models (i.e. DEBtox and DEBKiss) the toxicant acts as a stressor that targets energy processes, by, for example, increasing the energy maintenance cost. To account for this, the DEB (or DEBkiss) model is linked to a TKTD model, which accounts for the accrual of and recovery from damage (toxicodynamics, TD) related to the bioaccumulation, distribution, biotransformation, and elimination of the chemicals in the organism (toxicokinetics, TK). Like in the GUTS framework, if there is no information on body residues (measurements or predictions); the TK and TD parts are combined into a one-compartment model linking the external concentration to the damage, over time. From the scaled damage level, a dimensionless stress level is calculated and can modify the value of one or more of the DEB physiological parameters (Jager 2020). Depending on the impacted physiological parameter(s), the DEB-TKTD model offers access to four metabolic processes to be affected by the toxicant: 1) assimilation, 2) maintenance, 3) growth and 4) reproduction. The most affected metabolic process is generally referred to as a physiological mode of action (pMoA). Physically, this would appear as a reduction in growth or reproductive rate of the organism.

The DEBTox framework can also be used to assess the sublethal effects of chemical mixtures on organisms (Vlaeminck et al. 2021). Using this model to assess mixtures can identify synergistic interactions between chemicals and also provide information on the pMoA of chemicals in the mixture. This mechanistic information may be able to provide better targeted pest management strategies by simulating not only which life stage of an organism is more at risk from agrochemicals, but also when to apply agrochemicals to avoid unwanted interactions. To include additional stressor effects, further parameters can be added to the model to simulate the effects of starvation or, pulsed exposure (Jager 2020). This wide range of applications has made DEB models and their simplified variations an area of interest in risk assessment. Although the model is not yet deemed ready to use by regulatory bodies, (EFSA Panel on Plant Protection Products and their Residues (PPR) et al. 2018), the methods do provide the user with analyses of toxicity of both time and a range of endpoints.

1.3.0 Pesticide Metabolism

The toxicokinetic compartment of TKTD models parameterises the bioaccumulation, distribution, biotransformation and elimination of toxic compounds in an organism. As TK traits determine the ability of a species to metabolise and excrete xenobiotics, an understanding of metabolic processes is essential to understand single pesticide and mixture effects. For example, xeno-metabolic inhibition can be responsible for the occurrence of chemical interactions, i.e. when the capability of an organism to metabolise a chemical is hindered. In such cases, this may increase the toxicity of other toxicants that the organism is also exposed to. Understanding the metabolic process of xenobiotics is, therefore, essential to investigate the mechanisms underlying synergistic interactions.

1.3.1 Phase I metabolism

Pesticide exposure is routine for many insect species and therefore, many will encounter a chemical stressor at some point in their lifetime (Goulson, 2019). Over evolutionary history,

such exposures occur naturally as a result of the presence of toxic substances produced by plants as a defence against insect herbivory. In the Anthropocene, exposure to toxic organic chemicals can additionally be of anthropogenic origins, such as through pesticide use. From this initial contact, the organism must neutralise the threat of such chemicals to mitigate toxicity relating to the exposure to ensure individual survival and reproduction. Metabolism of xenobiotic compounds is broadly divided into three phases, with each having characteristic protein components, phase I - modification, phase II – conjugation and phase III - excretion. The general aim of phase I and II metabolism is to alter the chemical properties of the compound so it is easier to excrete in phase III.

In phase I metabolism, the structure of the toxicant is altered enzymatically, generally into a polar and, thus, more water-soluble molecule (Phang-Lyn and Llerena 2022). The modification of the toxicant by the phase I enzyme systems may occur as a result of oxidation, reduction or hydrolysis reactions. All have three possible outcomes; the chemical is rendered inactive, the metabolites of the chemical are still active but less so than the original chemical or finally, the original chemical is no longer active but one or more of its metabolites remain active. Where molecules remain active, the structurally altered molecule is often less toxic than its parent compound, however, this decrease in toxicity does not apply to every compound as there are a number of notable exceptions where metabolites have higher toxicity including the production of oxon metabolites from organophosphorus insecticide compounds (Feng et al. 2020; Huang et al. 2021).

Among the range of different phase I reactions, oxidation is the most common (Dibetso 2020). In an oxidation reaction, the chemical substrate gains oxygen or loses hydrogen, resulting in a loss of electrons. Reduction reactions occur concurrently with oxidation reactions as an electron is received by this transfer (Hardin and Jeff 2011). This reaction is often catalysed by a superfamily of *CYP450* enzymes in the smooth endoplasmic reticulum of eukaryotic cells (Claudianos et al. 2006). Enzymes from this superfamily exist in multiple forms in insects, with different *CYP450s* performing functions such as detoxification of xenobiotics, steroid synthesis, metabolic reactions and hormone synthesis. In insects, *CYP450*s have also been attributed to the development of resistance to agrochemicals, e.g. insecticides (Lu et al. 2021). Hence, they have become recognised as a critical family of proteins in the context of agrochemical pest control and the identification of non-target ecotoxicological effects.

In insects, *CYP450*s are divided into four phylogenetic clans, the CYP2, CYP3, CYP4 and mitochondrial *CYP450*s (Feyereisen 2006). It is thought that clan 2 CYPs and CYPs from the mitochondrial clan have roles in essential life functions, such as ecdysteroid, pheromone and allomone processes (Feyereisen 2006; Feyereisen 2012). For example, biosynthesis, activation and catabolism of ecdysteroids and juvenile hormone have all been linked to the activity of *CYP450*s from these clans (Feyereisen 2012; Tu et al. 2022). Clan 3 and 4 *CYP450*s are more commonly linked to xenobiotic detoxification and resistance functions . A high number of xenometabolic *CYP450*s are found in families 4 and 6 (clan 4 and 3 members respectively) (Feyereisen 2012; Liu et al. 2018). For example, *CYP450*s from these families have been widely attributed to the detoxification of organophosphate, pyrethroid and neonicotinoid insecticides (Komagata et al. 2010; Puinean et al. 2010; Wang et al. 2018).

Another group of detoxification enzymes with a role in phase I metabolism are esterases. Like *CYP450s*, esterases are also thought to be involved in the metabolism of organophosphate, pyrethroid and neonicotinoid pesticides (Whalon et al. 2008; Zhu and Luttrell 2015). Pyrethroids in particular are lipophilic, possessing an ester bond (Ross et al. 2010). Thus, the hydrolytic activity of carboxylesterases renders pyrethroids water-soluble by increasing polarity. Esterase enzymes detoxify insecticide molecules by cleavage or sequestration (Panini et al. 2016). This ensures that toxic molecules can no longer interact with target proteins and may also induce resistance to insecticides such as for carbamates and pyrethroids in aphids (Lan et al. 2005; Wang et al. 2018).

1.3.2 Phase II and III Metabolism

The next stage of metabolism, phase II, is achieved through the conjugation of the parent compound or more often the metabolites of phase I metabolism. The metabolites that are produced in phase I are conjugated with different charged species, for example, glutathione, uridine 5'-diphospho (UDP)-glucosyltransferases (UGTs) and methyltransferases (MTs) (Gupta 2016). The resulting product of a conjugation reaction is often less active than the substrate. Furthermore, these metabolites are more polar and therefore, cannot diffuse across membranes. This allows the metabolites to be actively transported from the cell and into the excretory systems where they can lost from the body (Enayati et al. 2005).

Phase II metabolic enzyme families are usually transferases and can include UDP-glucuronosyltransferases (UGTs), sulfotransferases, N-acetyltransferases (Jancova et al. 2010). However, arguably the most diverse family of phase II metabolic enzymes are *GST*s. In insects, elevated *GST* activity has been associated with resistance and detoxification of every major insecticide type (Kostaropoulos et al. 2001; Enayati et al. 2005; Hu et al. 2014; Zhu et al. 2015; Khan et al. 2020). This family of enzymes is consequently well represented in insect genomics and knowledge of specific *GST* expression is expanding rapidly (Pavlidi et al. 2018). *GST*s can undertake both phases I and II metabolism, but are most known for glutathione (GSH) conjugation reactions (Pavlidi et al. 2018).

The final stage of metabolism is excretion (Guidotti 2013). Here, protein carriers such as ATP-binding cassette transporters (ABC transporters) actively remove the products of phase II metabolism outside of the cell membrane (Phang-Lyn and Llerena 2022). Once outside the cell and into the excretory systems, metabolites may either metabolise further or can be lost from the body. The most important components of phase III metabolism are the range of different ABC-transporters. In insects, the ABC superfamily can be divided into 8 subgroups; depending on their ATP binding site (ABCA to ABCH) (Merzendorfer 2014). Members of the ABCG subgroup in particular are reported to infer insecticide resistance (Dermauw and Van Leeuwen 2014). The most notable example of this being to cypermethrin (Bariami et al. 2012; Bonizzoni et al. 2012). Studies of Lepidoptera have also shown that upregulation of the ABC C sub-group transporters can convey resistance to *Bacillus thuringiensis* (Bt) by reducing binding affinity to the toxin Cry (Xiao et al. 2014; Chen et al. 2018).

Although the role of the different metabolic enzyme systems have been studied for decades due to their role in mediating toxicity and in the development of phenomena such as insecticide resistance, there is still much to learn about their role in different non-target species (Wu et al. 2019). A further understanding of all three phases of xeno-metabolic processes is essential to predict how chemicals will interact within the organism. Therefore, this knowledge can be applied to mixture toxicity to understand if inhibition of any of these metabolic processes can cause a chemical interaction to occur.

1.4. The Role of Metabolic Enzyme Systems in Synergy

While synergistic (and antagonistic) effects in mixture exposures are often reported in the literature, the mechanisms behind these interactions are often unknown (Altenburger et al. 2013). Although it is recognised that a range of mechanisms can be responsible for synergistic interactions, such as bioavailability, excretion and uptake rates; potentially the best documented mechanism is changed to the organism's xenometabolic capacity (Holmstrup et al. 2010). Such cases of synergy arise when one chemical acts in enzyme systems (e.g. through inhibition) to decrease the metabolic rates of the other chemical(s) in the mixture. As such, detoxification of the toxicants is hindered leading to a greater toxic effect inside the organism as a result of the presence of a higher concentration of the unmetabolised and therefore, generally (although not always), more toxic, compound. Such cases are most likely to arise when one chemical acts to inhibit components of either phase I, phase II or phase III metabolic enzyme systems. The inhibition of metabolic enzymes may not, however, always result in synergy. This is the case where metabolites are more toxic than the parent compound, in which case enzyme inhibition may lead to antagonism. This case has been reported for example, for imidacloprid (de Perre et al. 2017; Huang et al. 2021).

The inhibition of *CYP450*s has been recorded in many incidences of synergy and is recognised as the most likely cause of some of the clearest cases. Azole fungicides are known inhibitors of many *CYP450*s and, therefore, have the potential to impact biotransformation processes, particularly of lipophilic compounds, such as cypermethrin (Cedergreen 2014). For example, many studies of bees have found that if *CYP450* activity was inhibited by exposure to a toxicant before cases of pyrethroid exposure where enhanced toxicity was seen insecticides toxicity of pyrethroids was greatly enhanced (Papaefthimiou and Theophilidis 2001; Johnson et al. 2006; Wang et al. 2020). Further synergistic interactions have been reported in *Daphnia magna* exposed to a mixture of α-cypermethrin and propiconazole or prochloraz. Here, toxicity of α-cypermethrin was increased by a factor of 13 and 61 fold; probably due to the inability of *D. magnia* to biotransform α-cypermethrin to an excretable form (Kretschmann et al. 2015).

Azole fungicides are known to inhibit the activity of a wide range of *CYP450*s, therefore, potentially reducing the ability of the exposed organism to detoxify lipophilic insecticides;

particularly pyrethroids (Papaefthimiou and Theophilidis 2001; Cedergreen 2014; Kretschmann et al. 2015; Dalhoff et al. 2016). However, *CYP450* inhibition has also been reported to increase the toxicity of neonicotinoids, organophosphates and diamide insecticides (Ghaffar et al. 2020; Zhao et al. 2020; Haas et al. 2022), indicating the complexities of understanding how change in xenometabolism rates resulting from exposure can affect the toxicity of mixtures. Such cases may result in incidences where metabolites have a higher toxicity than the parent compound.

In addition to chemical interference of phase I metabolism processes, some pesticides can also modify phase II metabolism reactions. For example, inhibition of UGTs, which metabolise among other xenobiotics, neonicotinoids and pyrethroids (Pan et al. 2018; Cui et al. 2020) . One of the ways that chemicals can affect phase II metabolism is through depletion of the pool of conjugating metabolites. Thus, the activities of phase II *GST*s may be reduced by compounds such as chlorothalonil that are known to affect the sizes of the free glutathione pool (Van Scoy and Tjeerdema 2014). This reduction in available glutathione prevents binding to metabolites activated in phase I metabolism, thereby preventing their excretion (Fernández et al. 2012; Reyna et al. 2021). Hence, in an observation of synergistic effects, the potential for chemical exposure to lead to metabolic change should be considered as a possible mechanistic cause.

1.5. Scope and Objectives of Project

The toxic impacts of pesticides on insect species have been the topic of studies for many years. Recently, some of this focus has shifted from single pesticide application to more environmentally relevant chemical cocktails scenarios. However, to understand the effects on chemical mixtures, the effects of individual chemicals must first be analysed; particularly with regards to uptake, metabolic and excretory (TK) pathways and how these traits impact the sensitivity of an organism to a xenobiotic. Once the impacts of single chemicals are known, this information can be used to explore and predict the effects of chemical mixtures including any interactions.

In this thesis, the following chapters revolve around the central theme of:

‘Defining species sensitivity and synergism potential for pesticides and pesticide mixtures through physiological traits analysis’ in an insect species.

To explore this theme, a detailed analysis of the TK and TD traits of the polyphagous lepidopteran *Mamestra brassicae* will be undertaken in response to a range of pesticides, including insecticides and fungicides. *M. brassicae* is a polyphageous species of the Noctuid family (Popov and Popova). This species inhabits Europe and temperate Asia. The eggs of *M. brassicae* are pale white with an often-brown marking in their centre and hatch within six to ten days (Devetak, 2010). There are usually 6 distinct larval stages, each morphologically different to the naked eye (Figure 1.3). The first instar has a light green body with three pairs of legs along the thorax. The colouration of larvae remains largely consistent until the fourth instar in which the doral region begins to darken. By sixth instar the dorsal region is dark in colour and the ventral region is yellow (Devetek, 2010). Larval development takes approximately six weeks and the final body length is approximately 40-50 mm. Pupation often takes place over winter where the dark brown pupae can be found buried in soil between 2 and 10 mm deep. Adults begin mating on the evening of their emergence from the pupa. A female may deposit around 350 eggs on the underside of a leaf, but can lay around 2500 eggs per copulation (Devetek, 2010).

Here, *M. brassicae* is used not only to inform pest management strategies for this destructive crop pest but also to act as a model species for other members of the Noctuidae family, the second largest family of moths (Weller *et al*. 1994). Members of this family act as important pollinators for many plant species and some have even developed mutualistic relationships with their plant hosts whereby larvae feed on plant leaves while adults act as pollinators for the flowers (Bopp and Gottsberger, 2004).

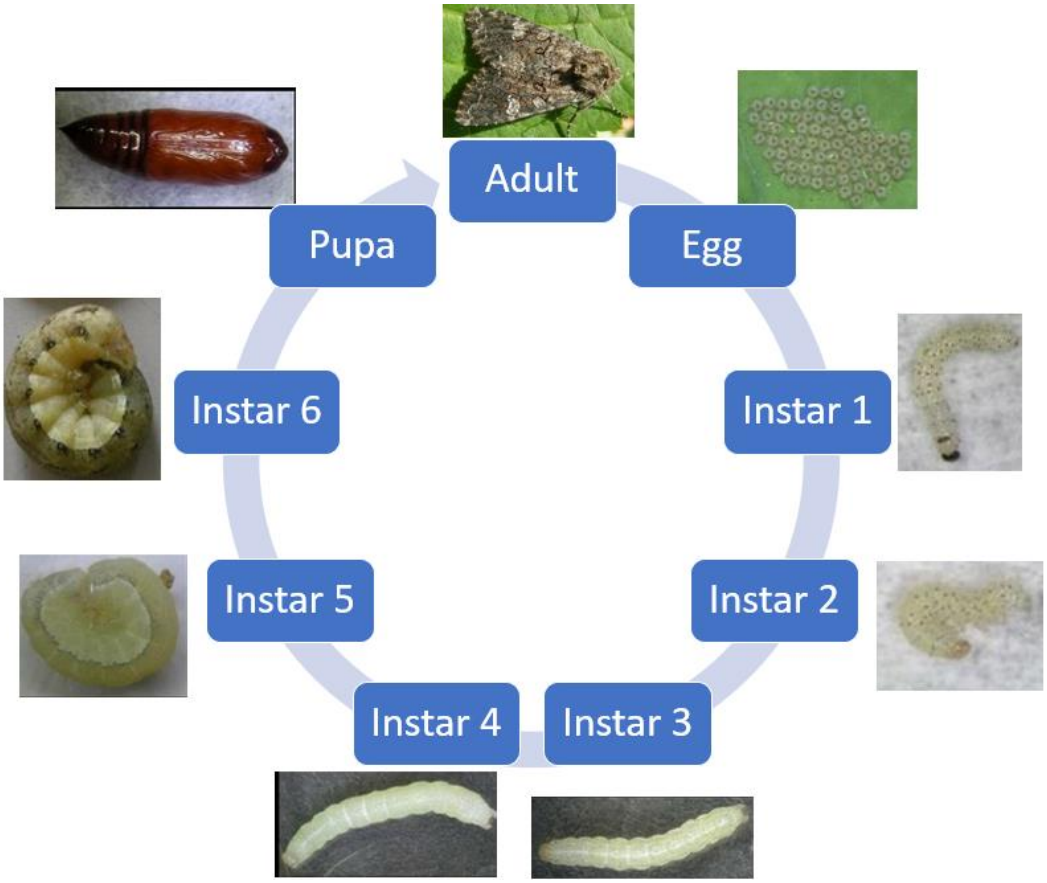


Figure 1.3. Life cycle of *Mamestra brassicae,* detailing from egg through all larval instar stages to pupation and adult.

This thesis will particularly explore how TKTD traits contribute to interactions between chemical pairings and the synergism potential of these binary combinations. Analysis will also compare differential sensitivity in larval and adult stages, with a focus on xenometabolism to predict the synergism potential of insecticide and fungicide pairs. A brief introduction to the hypothesis of each chapter is given below.

In Chapter 2, the design and initial testing of a novel bioassay for the toxicity testing of lepidopteran larvae over a 96-hour exposure period is described. This assay was designed to be high-throughput and capable of testing a large range of insecticides and fungicides with different modes of action. In this initial experimental chapter, the assay is developed using a known toxic substance to Lepidoptera, the organophosphate chlorpyrifos. The bioassay is optimised to produce a minimum of 80% background mortality and survival in time data is verified using a variant of the GUTS model and probit analysis. Sublethal effects on weight

were also analysed at the end of the 96-hour exposure to demonstrate that both chronic and acute data can be obtained from the exposure method.

In Chapter 3, the novel high-throughput bioassay developed and optimised in Chapter 2 is used to test the effects on survival and weight change of a range of 9 pesticides. 5 insecticides and 4 fungicides. Survival in time data for each compound was used to inform the TKTD traits of the single pesticides e.g. excretion and scaled damage parameters. Sublethal effects are monitored using relative weight change. The bioassay is then further developed to allow the testing of binary mixtures of the pesticides. Here, a modification of the GUTS model is used to identify cases of synergism, or antagonism for effects on survival data. Sublethal data is analysed to identify significant changes of weight of larvae exposed to the pesticide mixture. It is hypothesised that due to their targeted mode of action, insecticides will have an impact on *M. brassicae* survival and weight but fungicides will not have either sublethal or acute effects.

In Chapter 4, the joint effects of two pesticides that were found to show a consistent example of synergy in Chapter 3 are explored over the whole larval growth cycle rather than the short 96-hour time frame of the bioassay. A simplified DEB model (DEBKiss) is applied to the survival and growth data for the larvae from instar 2 up until pupation to explore the effects of chronic pesticide exposure to the single chemicals and their binary mixtures. This chapter also explores differential sensitivity between larvae instars and the effects of the pesticide mixtures on pupation. As synergism was seen between the pair of chemicals used in this chapter over the relatively short 96-hour exposure, it is hypothesised that larvae exposed to the binary mixture of pesticides will develop much slower than larvae only exposed to single chemical. This is expected to be displayed as smaller larvae that take much longer to moult and reach sixth instar in comparison to single chemical and control treatments.

Chapter 5 – The final experimental chapter aims to assess the differential sensitivity of adult and larval stages of *M. brassicae* exposed to the pesticides used in earlier chapters. A bioassay for adults was developed and tested against the same pesticides used in the previous chapters for larvae, both singly and in binary mixtures. This aimed to identify any differences in TKTD traits between life stages, which are phenotypically very different. It is hypothesised that due to differences in bioavailability and gene expression, there will be

differential sensitivity between the two species. However, it is expected that this will be small (less than 10-fold).

Chapter 6 - This chapter focuses on the differential expression of xeno-metabolic enzymes in the adult and larval stages of *M. brassicae*. A genome and transcriptome were generated for both life stages and expression of *CYP450*s, *GST*s and ABC transporters, representing phase I, II and III metabolism are compared in an attempt to account for differences in sensitivity seen between the two life stages. This chapter aims to compare the expression of these enzyme superfamilies to that of *D. melanogaster,* as an indicator species to determine if the *M. brassicae* metabolic enzyme complement is a good model species for further ecotoxicological studies of lepidopterans.

CHAPTER 2 – DESIGNING AN ENHANCED NOVEL BIOASSAY FOR LEPIDOPTERAN TOXICITY TESTING

2.1. Introduction

In agricultural landscapes, prophylactic chemical application is widely used as an effective and efficient method of controlling insect pest populations. However, this widespread pesticide use can also damage both the structure and function of ecosystems. Pimentel (1995) estimated that less than 0.1% of applied pesticides reach their target pest while the remaining 99.9% enter into the wider environment where they may interact with non-target organisms and beneficial species. Important non-target species, such as pollinators, can be adversely affected by pesticide exposures. Hence, it is important to understand how pesticides interact with different species from a range of different important pollinator groups.

Worldwide, pollination is responsible for contributing an estimated $577 billion to the global economy (IPBES, 2019). However, due to a range of factors including chemical control practices, many pollinator populations are in decline (Goulson et al. 2015; Kessler et al. 2015). In recent years, the decline of pollinators has provoked an increase in research into the effects of agrochemical exposure on these taxa (Iverson et al. 2019). These studies have largely centred on honeybees. This is due to their noted value in pollination and honey production and also because their eusocial nature makes it possible to easily rear large numbers of individuals for use in testing. However, as well as bee species, lepidopterans (moths and butterflies) are also important pollinators (Hahn and Brühl 2016). This group is, however, currently largely overlooked in studies that consider the effects of pesticide exposure on insects.

Moths are considered to be the most speciose order of lepidopteran flower visitors, aiding in the pollination of almost 300 known plant species (MacGregor et al. 2015; Rader et al. 2020). One indication of the value of lepidopterans as pollinators is the suggestion that butterflies or moths are responsible for half of all floral visits to Macadamia plants in Brazil (Santos et al. 2020). This indicates that pollination by lepidopteran species can make significant economic contributions to crop production as well as for wildflower species. Therefore, understanding the effect of chemicals on lepidopterans is critical to agricultural

economics and conservation biology as well as to the conservation of this widely admired and charismatic group of insects.

Lepidopterans represent a complex of different species with different ecological traits that may bring both disbenefits, as well as positive effects that can arise through their pollination activities. Lepidopteran larvae can also be voracious pests of many crops (Reed and Pawar 1982; Cartea et al. 2010). For example, the Cabbage Moth (*Mamestra brassicae*) is a polyphagous pest species of many vegetable crops and is found in almost all Palearctic regions (Masaki 1968). Despite a wide variance in diet, Cabbage moths have been shown to prefer host plants in the *Brassicaceae* and *Chenopodiaceae* families such as cabbage and broccoli (Popov and Popova 1993). With their rapid feeding rate, Cabbage moths are important pests of Brassica vegetation throughout Europe and Asia (Finch and Thompson 1992). This juxtaposition between larvae and adult, and status as a pest or pollinator, gives many lepidopterans the unusual role of being both beneficial and pest species. For this reason, understanding the effects of agrochemicals on lepidopterans following typical continuous exposure scenarios can help understand the dynamics of pesticide effects on species in this taxon. Such understanding can help to assess impacts on key processes that come through the beneficial or pest actions. The development of a robust and repeatable approach for toxicity testing for lepidopterans would be beneficial to the aims of quantifying the effects of exposures on this group.

In many previous Lepidoptera toxicity tests, the leaf dip method has been utilised as the basis for bioassay (Morse et al. 1986; Hill and Foster 2000; Santos et al. 2011). This protocol involves, dipping a section of leaf into a chemical that is then fed to the larval stage. Survival is assessed usually at a single 48-hour time point. The leaf dip method has become widely adopted for lepidopteran toxicity testing, primarily because of its ease of use. However, this method is restricted in its application as a bioassay by factors such as leaf freshness. These effects mean that this approach may not be practical for longer-term and sublethal monitoring. Further, the current leaf dip methods generate data only suitable for *Ad hoc* statistics at a single time point to calculate a no observed effect concentrations (NOEC), lowest observed effect concentrations (LOEC), or lethal concentrations (LC_x). Such parameters cannot be extrapolated to other (shorter or longer) exposure times than that tested. As such the results are operationally defined by the exposure length and so lack biological meaning for other time scales.

To move toward a more mechanistically based approach for interpreting toxicity test results, the use of toxicokinetic-toxicodynamic (TK-TD) models was proposed more than 20 years ago (Bedaux and Kooijman 1994). Among these TK-TD models, the General Unified Threshold model for Survival (GUTS) provides a framework for TKTD modelling, to predict the effect of exposure on survival over time (Jager et al. 2011). Once calibrated the GUTS model can also be used to calculate the LC_x and EC_x values of a chemical, at any time point, and predict the survival of new exposure scenarios, in both constant and pulsed exposures (Baudrot et al. 2018). Bioassay exposure systems that support GUTS modelling, thus, have far greater values for the mechanistic interpretation of chemical effects than single time point assays.

To date, there is little published work on methods to generate suitable data for TKTD analyses in insect species beyond bee species, and none for lepidopterans. This chapter aimed to develop a new and enhanced technique capable of monitoring acute and sublethal effects of agrochemicals on *M. brassicae* larvae, through repeat measurement over an extended exposure time in comparison to traditional test methods. The bioassay was designed to be i) efficient to allow high through-put of organisms to allow high replication testing, ii) repeatable and capable of supporting normal larval biology and iii) capable of assessing lethal and sublethal effects over time allowing a TKTD modelling approach; more specifically the use of the GUTS model. Furthermore, the bioassay was required to have the capacity to measure effects of relevant endpoints such as a change in body weight.

2.2. Methods

2.2.1 Culture of Test Species

M. brassicae larvae used were collected from a laboratory strain, kept in culture for over 40 years at the UK Centre for Ecology and Hydrology. Over this time, the larvae have been raised on a modification of Hoffman's tobacco hornworm diet (Smith, 1966) - from this point referred to as an 'artificial diet' (Table 2.1). Larvae were kept in culture in a controlled room at 20°c ±2 °c under a 16:8 light: dark pattern.

Table 2.1. Recipe for artificial diet used in insect culture. The two items in italics are added to the diet once it has cooled to 60°C due to degradation.

Ingredient	Measure (g)
Ground Wheatgerm	71.25
Caesin	32.5
Sugar	28.75
Agar	13.75
Yeast	13.75
Wessons	5.625
Water	800
Sorbic acid	1.875
Methyl-4-hydroxybenzoate	1.4
Vitamins and antibiotics	*4.05*
Choline chloride	*0.9375*

During culture and in rearing for experiments, the adult populations were maintained in flight cages with continuous access to 10:90% honey: water solution. Eggs were collected three times within the week to ensure that the selected collection consisted only of egg batches laid within the previous 24-hour period. Eggs from each collection were then added to separate rearing boxes containing the artificial diet and raised until hatchlings were 3 weeks old; at which time they were used either for the bioassays or raised to adults from which the next generation was reared.

To observe normal larval growth in the absence of chemicals, an observation of larvae growth took place to determine the optimum size of larvae used for testing. Individual pots containing 10ml of artificial diet were given an individual 7-day-old 2nd instar larva (n=15). Larvae were weighed every 2 days and the day of each moult was recorded. It was noted that random mortality increases with time, therefore, the ideal instar for bioassays would take a short time to reach but be large enough to notice sublethal weight changes and preferably have a time of at least 96 hours between moults.

Results of this initial observation suggested that the most suitable instar of larvae for bioassay testing was 4th instar (Figure 2.2). Larvae of this size could be collected at only 12 days post-hatching and were unlikely to moult during the 96-hour test duration.

2.2.2. Preparation of Test Chemicals

The bioassay was designed to assess the toxicity of the tested chemical on larval survival and growth over time. To initially assess test suitability and repeatability for time series survival and growth effect measurements, a chemical that is known to exert toxicity on lepidopteran species was selected - the organophosphorus insecticide chlorpyrifos. High purity analytical standard chlorpyrifos >99% (Sigma Aldrich, Poole, UK) was used to make an initial stock for dosing. Chlorpyrifos was diluted in acetone to create a 1% solution. This master stock was then diluted in series in further acetone to generate the required series of exposure concentrations needed for testing.

Three tests were conducted each using different concentration ranges. For the first bioassay, the dietary concentrations of chlorpyrifos assessed were 0, 0.27, 0.82, 2.4, 7.4, 22.2, 66.6 and 200 mg/kg wet weight of artificial diet. This test provided a broad range of concentrations for the initial range finding for the toxic effect. The second test was conducted using a narrower range of tested concentrations of 0, 3.93, 6.29, 10.07, 16.11, 25.78, 41.25, and 66 mg/kg to allow a more refined assessment of survival between concentrations and to allow more robust modelling of the effects of exposure for both GUTS and standard dose-response modelling. This range was then further refined for the final bioassay in which concentrations of 0, 10.35, 13.46, 17.5, 22.75, 29.58, 38.46, and 50 mg/kg were used. The same volume of acetone without adding chlorpyrifos was added to the

control to ensure that any effects that occurred were not related to the presence of the acetone carrier in the test medium.

2.2.3 Bioassay Procedure

Artificial diet was made up following the same adapted Hoffman's tobacco hornworm recipe as used in culture; but with 1000 ml of water used instead of 800 ml. This change was to ensure that the consistency of the diet was suitable for dispensing evenly to allow accurate dosing using a positive pressure pipette. The required amount of diet needed for all replicates for each tested concentration was initially weighed out and the required aliquots of the appropriately diluted test chemical were added to give the desired concentration in the dietary medium. After preparation, the artificial diet was kept in a water bath at a constant temperature of 50˚C, to prevent setting and chemical was added while the diet was still in its liquid form. Although the pesticide, in this case, chlorpyrifos, is only maintained at 50˚C for a very short time, care should be taken to ensure that the pesticide of choice does not degrade at this temperature. Chlorpyrifos is known to be stable at temperatures up to 74˚C for 3 days (Mansour et al. 2018). Hence, in this case, exposure is not affected by maintenance at this temperature for the short period used. For alternative pesticides, a check on stability is needed, although it can be expected that few active ingredients and formulations would be affected by this treatment given their designed stability for on-farm storage.

All diet preparations were mixed thoroughly to ensure an even distribution of the insecticide and then 1ml was added to each well of a 12-well plate. This amount of diet provided *ad libitum* feeding for larvae of this size but may need increasing for later instars of larvae or larger lepidopteran species. In total there were 24 wells per treatment, per test, each well contained a single individual larva. Each plate was left to set at room temperature for 15 minutes, this period allows the large proportion of the added acetone to volatilise from the dosed diet. Remaining acetone can be checked by smell for the removal of solvent odour. Exposures were not initiated until the plates had cooled to room temperature to prevent heat stress to larvae.

All tests were conducted using 4th instar larva weighing 20 mg ± 10 mg from the same clutch of eggs. Individual larvae were placed onto the artificial diet in each well to initiate the exposure (Figure 2.1). This design gave a total of 24 individuals per treatment for survival and growth monitoring. Mortality was measured at four-time points over the 96 hours of exposure (after 6, 24, 72, and 96 hours) by stimulating the 2nd segment behind the head of the larvae with a fine paintbrush. If no movement was detected after 5 seconds, larvae were recorded as dead. All plates were kept incubated at a constant 20˚C for the duration of the test. This gave a measurement of larval survival over time at each exposure concentration used for the bioassays that can be used as input for TKTD-based GUTS modelling. Sublethal effects were measured by weighing surviving larvae at 0 and 96 hours.

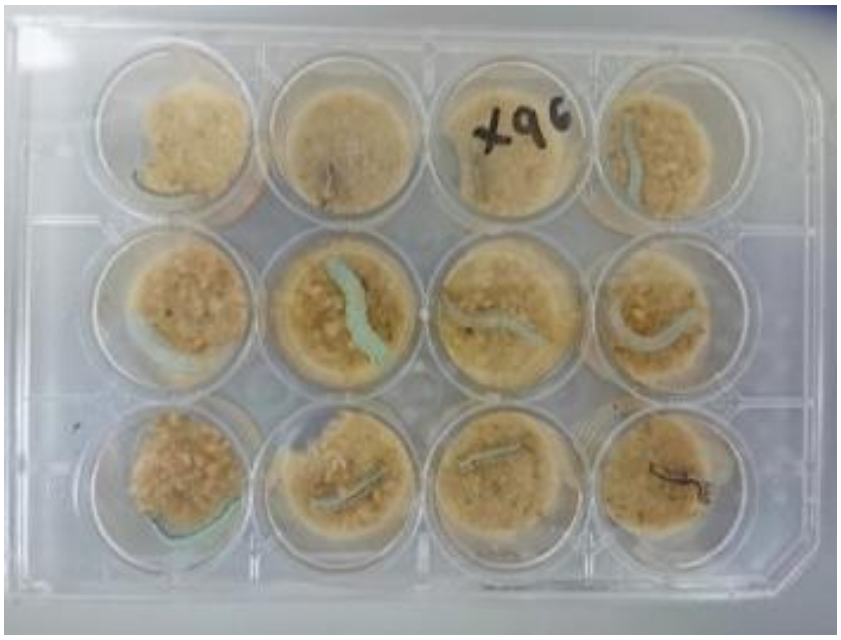


Figure 2.1 Bioassay plate containing 1 ml of spiked artificial diet and one 4th instar *Mamestra brassicae* larva.

2.2.4 Data Analysis

The GUTS model was used to assess survival in time through the use of fitted TK and TD parameters. The survival data obtained from each experiment was analysed with the openGUTS software (http://openguts.info/). The GUTS reduced (RED) model was used as it combines TK and TD damage dynamics and allows the linking of external concentrations to

the effect on survival over time. The GUTS-RED model is a relatively simple derivation of the classical GUTS TK/TD modelling framework including relatively few parameters. As such, it is an efficient model for the analysis of survival in time datasets because it requires relatively limited amount of time-dependent data. For example, no measurement of body residues is needed and no other information on the toxicokinetics is required. Indeed, the model can be adequately driven by the survival data in time alone.

The openGUTS software provides analysis with both the stochastic death (SD) and individual tolerance (IT) approach. Both of these approaches have been successfully used in the past and provide good fits and predictions of survival data over time. In this study, the GUTS-RED-SD model is selected because i) overall the GUTS-RED-SD model consistently gave a slightly better fit to the bioassay data based on the model efficiency values provided by the software, and ii) the individuals used were bred in laboratory conditions for more than 40 years and are genetically similar and therefore, do not fit well with the hypothesis made by the IT approach of a difference in internal threshold between individuals.

In addition, for comparison, the classic dose-response analyses with the conventional probit analysis statistic was carried out for survival measured at 48 hours and at the final 96-hour time point using SPSS v.27 (IBM, 2020), this was then plotted using R version 4.0.1 and the 'Ecotox' package *(Hlina et al. 2020)*. The relative weight change between the beginning and the end of the experiment was analysed with a Kruskal-Wallis and a post hoc Dunn's test was performed for significant changes in weight between treatments. Relative weight change was calculated as equation 2.1.

$$\frac{\text{Weight0} - \text{Weight 96}}{\text{Weight0}}$$

Equation 2.1. Calculation of body weight change index

2.3.0 Results

2.3.1. Assay Optimisation

2.3.1.1 Experimental Set up

The developed bioassay method was relatively easy to set up and monitor. Compared to other widely used methods such as leaf dip assay, the plates containing the spiked diet used for the exposure are more labour intensive to establish. This increased effort is, however, countered by the ability to easily generate survival data over time through continuous monitoring as needed as input for the TKTD models. This method also allows analysis of growth data throughout the experiment, which is run for a longer time than the leaf dip method. Assessment of larval survival for all individuals on a single plate can be done in little more than two minutes, meaning that within an experiment, replicate plates each with 12 individuals for a typical design using a control and six test concentrations can be checked for survival for all individuals in 14 minutes. In addition to measuring survival, at each time point assessed the larvae can be taken from the diet surface and weighed. This is the most time-consuming part of the process, as to carefully remove, clean and weigh all individuals from a plate can take 10 minutes; meaning that it will take at least one hour to sample and weigh all individuals in an experiment. Such weight change data is, however, useful for allowing assessment of the sublethal effects of exposure. However, as sublethal data is not needed for GUTS modelling, this step could be removed for studies focused purely on survival.

2.3.1.2 Mamestra brassicae survival and growth over the development period

To determine the optimal time for the collection of individual larvae for use in the toxicity bioassays, development was tracked from hatching to pupation. Larvae reached the correct size for testing (4^{th} instar larva weighing 20 mg ± 10 mg) between 12- and 16-days post-hatching (Figure 2.2). At this rate of development, if a bioassay was set up at 16 days post-hatch it would be expected that the larvae exposed on a control diet would reach an average weight of 0.297g after the bioassay four days later (corresponding to 20 days post-hatching).

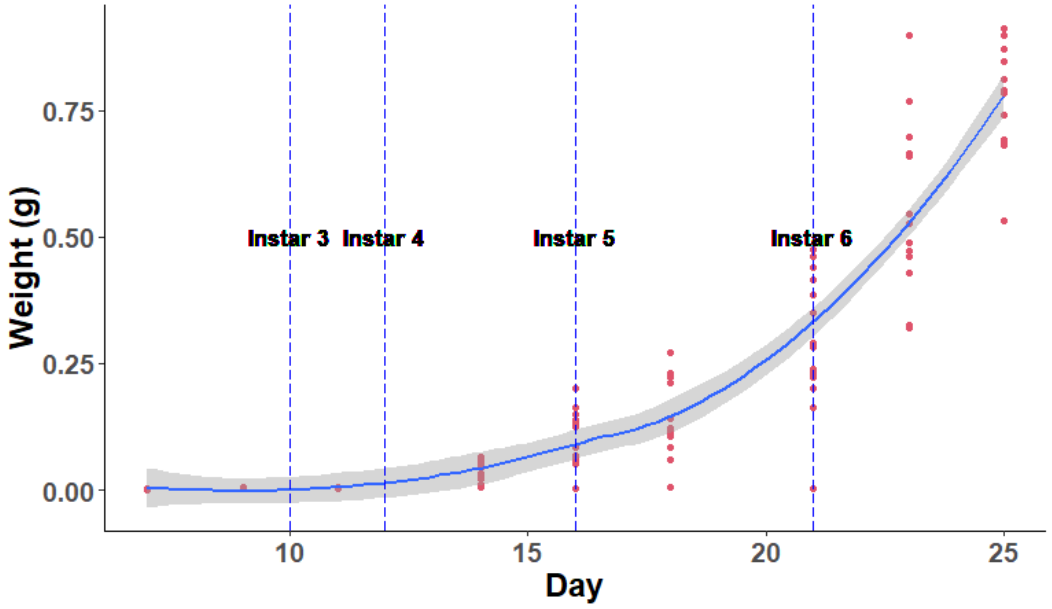


Figure 2.2. Growth pattern of *Mamestra brassicae*, fed ad libitum with an artificial diet, from instar 2 to pupation (N=15). Blue dashed lines represent the different moults. The points are the data and the line is the rolling average with 95% (confidence interval as grey shadow area)

2.3.2 Background Mortality

Optimised test systems for assessment of toxicity either through traditional concentration-response modelling or through the use of a TK/TD model such as GUTS should achieve, where feasible, a relatively low level of control (background) mortality. Within test guidelines, control mortality of <20% is usually seen as suitable criterion for acceptable test performance. Any test that achieved background mortality over 20% was abandoned and results were discarded. For the developed bioassay, 4th instar larvae were selected for testing at day 12-16 post-hatching, because in initial trials larvae at this stage showed lower background mortality compared with earlier instars and also an extended time between moults. To assess rates of background mortality within the assay, the data on survival over time of individuals added to the control treatment of each of the three conducted bioassays can be used.

For the three separate studies, the overall rate of survival in controls was 95.8%, 100% and 83.3% respectively. Thus, in all cases, control survival was above the 80% criteria accepted for most regulatory toxicity testing bioassays.

2.3.3 Exposure Mortality

The effects of exposure on survival could be readily monitored in time by a visual inspection allowing a high frequency of survival checks across the experiment. The use of 5 time point measurements (including 0 hours) gave a total of 192 survival checks at 8 concentrations for 5-time points (=960 individual assessments per test). The data-set could be used as time discrete data-sets for LC_{50} assessment through Probit analysis (e.g. using the 96 h data) for each exposure time or the whole time series data-set could be used as input for GUTS modelling.

The exposure to chlorpyrifos resulted in concentration and time-dependent effects on survival in all three of the experiments. The refining of the concentration range for testing allowed for both focussing the dose range to generate a more robust assessment of LC_{50} value from Probit analysis, TK and TD parameters from GUTS modelling and also checking the repeatability of the test in respect of these parameters. Dose-response curves (Figure 2.3) highlight full mortality was found by a concentration of 22.2 mg/kg and above 17.5 mg/kg across all three tests at 96 hours. Based on the three separate experiments, the Probit analyses returned LC_{50} values of 17.1, 20.4 and 10.6 mg/kg for studies 1,2 and 3, respectively at 48 hours (Table 2.2). The fact that the estimate LC_{50} fell within a factor of 3 indicates high repeatability of the test even when different concentrations of the same chemical are tested, as per criteria set out by Manfra *et al*. (2015).

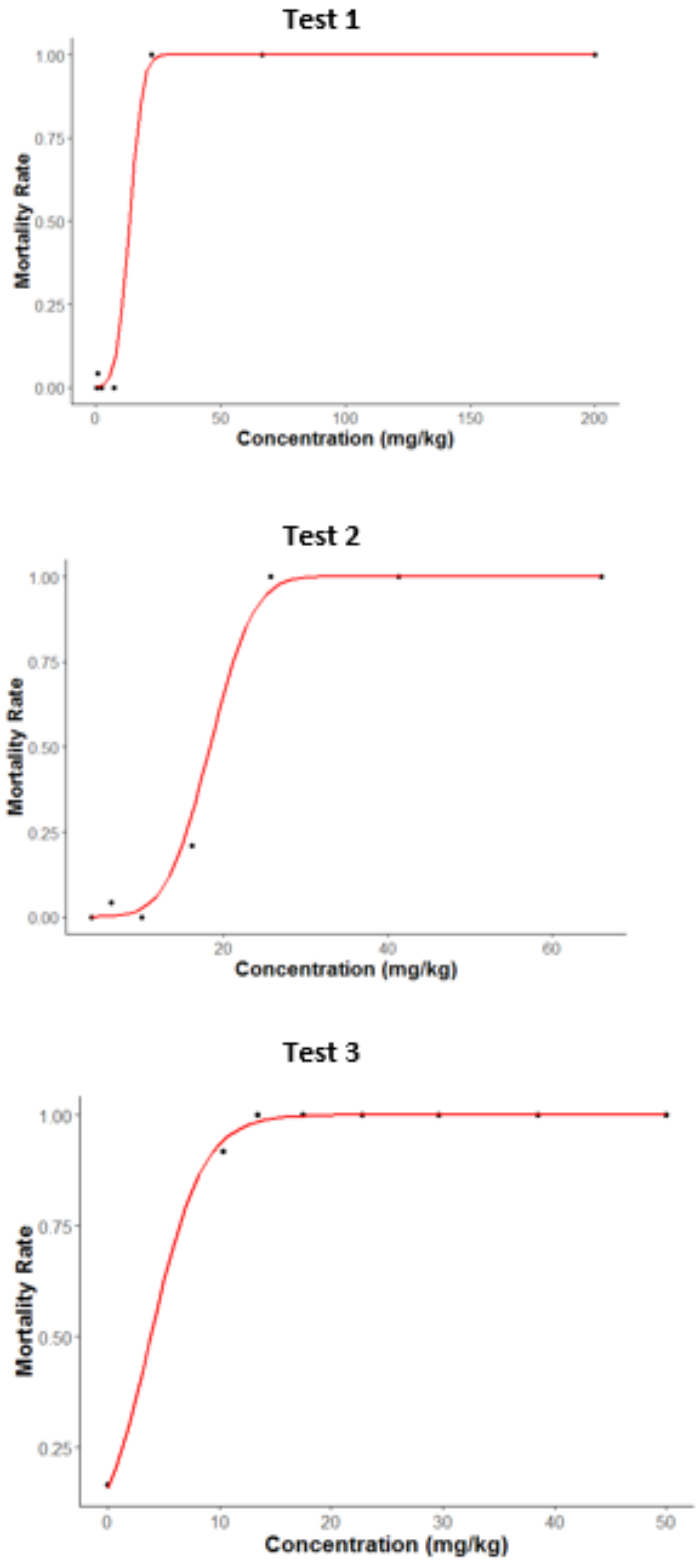


Figure 2.3 Dose-response curve for tests 1, 2 and 3 at 96 hours when modelled by Probit glm in R. Red line shows mortality rate per concentration.

GUTS-RED-SD models could be fit for all three experiments using the whole time series data set. As can be seen in Figure 2.4, the GUTS-RED-SD model fits almost all of the data points within the 95% CI. The no-effect concentration (NEC, i.e., median of the threshold distribution, m_w) was estimated at 7.4 (7.0-9.6), 15.6 (15.0-15.9) and 3.2 (0.003* – 5.84) mg/kg for test 1, 2 and 3 respectively (Table 2.3).

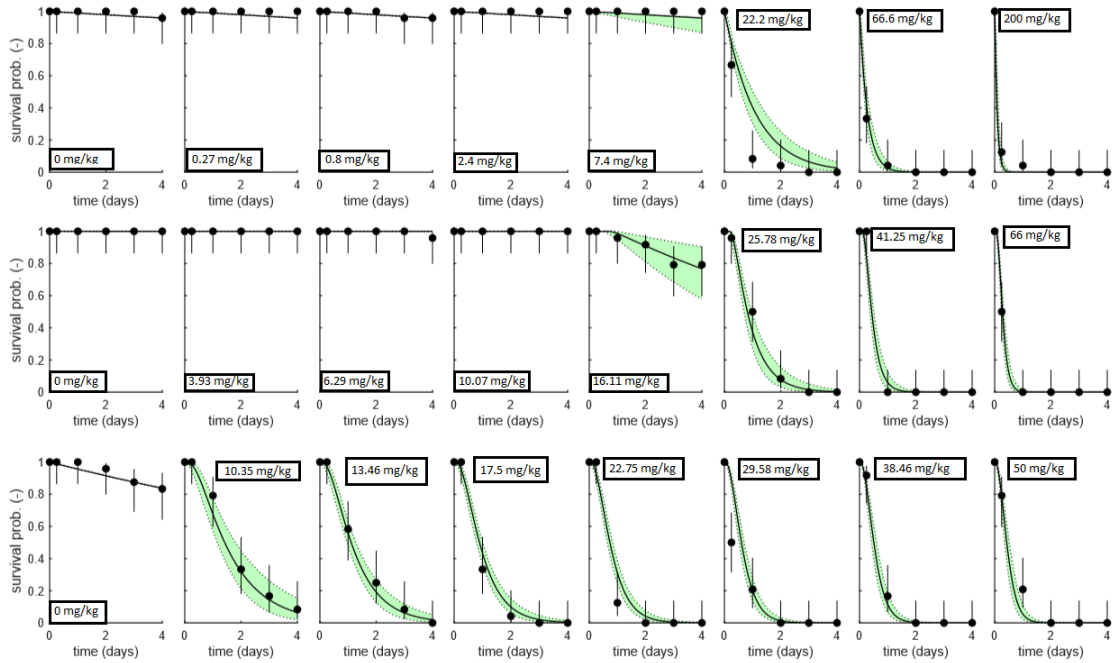


Figure 2.4 Observed and simulated survival over time of *Mamestra brassicae* larvae exposed to an artificial diet spiked with seven concentrations of chlorpyrifos and a control in separate laboratory bioassay repeats (the first row of plots is test 1, second is test 2 and third is test 3). The points are the data (bars show Wilson score confidence), and the lines are the GUTS-RED-SD model simulation (confidence intervals as green area).

As the GUTS model parameters are derived from the data for the full exposure concentration range over time, they can be used to derive LC_{50} value for any point in time in the experiment and simulated beyond the 96-hour exposure time (Figure 2.5). Using the calibrated GUTS model, the LC_{50} at 48 hours were 13.1, 18.7 and 8.1 mg/kg respectively in the three test replicates (Table 2.2). Thus, the LC_{50} values derived from the model are within a factor of 2 of those calculated with the discrete data for the 48-h sampling time of each of the three

independent experimental runs. The LC_{50} values derived from the 48-h period from the GUTS parameter vary between the three experiments by a maximum of 2.3-fold. This consistency between separate experimental study repeats indicates that the developed bioassay can provide a repeatable measure of pesticide impact on survival patterns in the lepidopteran species.

Table 2.2. LC_{50} values at 48h and 96h following the exposure of *Mamestra brassicae* to an artificial diet spiked with chlorpyrifos, calculated with probit, or derived with the GUTS-RED-SD model. N/A indicates the 95% confidence interval could not be calculated.

Test No.	LC_{50} 48h probit	LC_{50} 48H GUTS	LC_{50}96H probit	LC_{50} 96H GUTS
1	17.1 (N/A - N/A)	13.1 (11.7- 15.5)	13.4 (8.3-25)	10.3 (9.5 -12.5)
2	20.4 (18.5– 22.5)	18.7 (18-19.6)	18.3 (13.8- 28.3)	16.9 (16.4-17.4)
3	10.6 (0.5 – 15.8)	8.1 (5.1-10.2)	4.7 (2 – 6)	5.3 (2.12– 7.8)

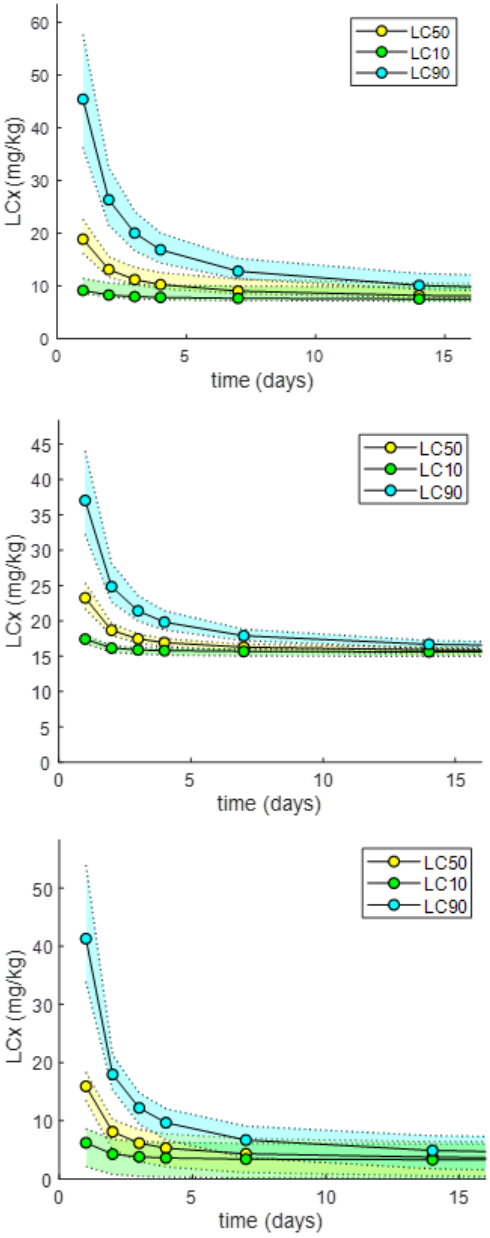


Figure 2.5. LC50, LC10 and LC90 values for *Mamestra brassicae* larvae exposed to chlorpyrifos in tests 1, 2 and 3 respectively. Calculated with the GUTS-RED-SD model.

Table 2.3. Parameter values (including 95% Confidence Intervals) of the GUTS-RED-SD model estimated for the 3 assays of *Mamestra brassicae* exposed to an artificial diet spiked with chlorpyrifos and used to simulate the survival curves presented in Figure 2.3. The * denotes where upper CI cannot be calculated because parameter CI has run into a boundary.

Test No.	Background mortality	Dominant rate constant	Median of threshold distribution	Killing rate
	h_b (d^{-1})	k_d (d^{-1})	m_w (mg kg^{-1})	b_w (kg mg^{-1} d^{-1})
1	0.01047	184.2 (13.7 – 184.2*)	7.4 (7.0-9.6)	0.061 (0.046-0.084)
2	1 10^{-6}	5.0 (4.0 – 7.4)	15.6 (15.0 – 15.9)	0.155 (0.108-0.215)
3	0.04445	2.3 (1.1-4.1)	3.214 (0.003* – 5.836)	0.106 (0.074-0.147)

2.3.4 Sublethal Effects

To assess sublethal effects, larvae were weighed at the start and end of the exposure in the second experiment conducted to assess the feasibility of adding a measure of weight change to the assessment of time-series survival effects. Weights at the end of the experiment could only be collected from larvae exposed to chlorpyrifos concentrations up to 16.1 mg/kg due to complete mortality found for all individuals exposed to the higher tested concentrations. Larvae grew in all measured treatments (Figure 2.6). Weight data was found to be not normally distributed with a Shapiro Wilk test for normality and therefore was analysed using the non-parametric Kruskal Wallis test. **L**arval weight change of larvae at each concentration showed significant variation in the magnitude of this weight increase between treatments ($H(4) = 21.263$, $p>0.001$). However, a post hoc Dunns test indicated no significant difference in weight change between the control larvae and those exposed to chlorpyrifos up to 16.11 mg/kg (Figure 2.6).

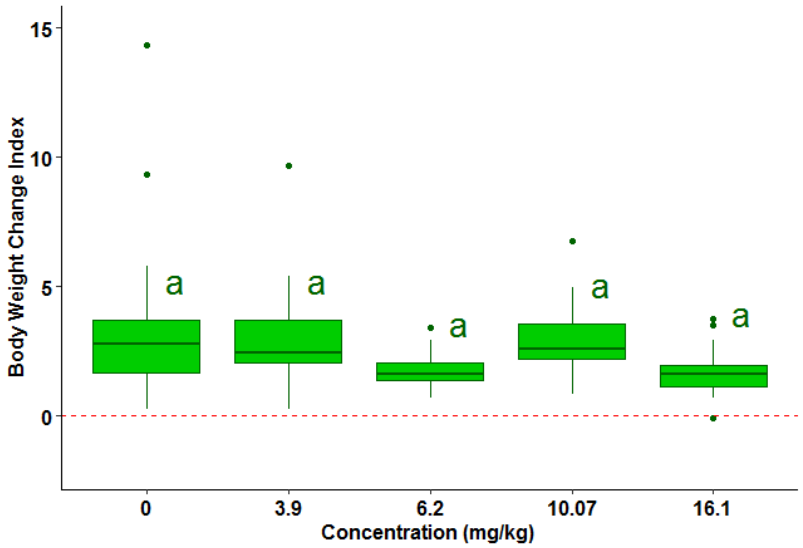


Figure 2.6. Box and Whisker plots showing mean (bold line) 75th percentile (upper and lower box limits) and 95% confidence intervals (vertical lines) for relative weight change of *Mamestra brassicae* larvae exposed for 96 hours exposed to different concentrations of chlorpyrifos. A different letter means significant difference (as analysed by Kruskal-Wallis and post hoc Dunn test).

2.4. Discussion

2.4.1 Bioassay Design and GUTS analysis

Here, a novel method for high-throughput sensitivity testing of lepidopteran species through dietary exposure was designed. Comparisons of survival data of *Mamestra brassicae* larva exposed to the organophosphate, chlorpyrifos from the traditional probit analysis and TKTD modelling was analysed across three separate replicates of different concentrations. The developed bioassay was found to be highly repeatable as across the three repeats studies there was no more than a threefold difference in LC_{50} values (13.1, 18.7 and 8.1 mg/kg). The bioassay was also able to cover a longer time period than the conventional leaf dip bioassay.

Maintained under the laboratory conditions used here at a temperature of 20^{o}C and a 16:8 light: dark photoperiod, *M. brassicae* eggs have an incubation period of approximately 5 days. The first instar larvae hatch as white on emergence, but then take on a green pigment from their first plant feed (Montagne 1977). In the culture population, as the moth larvae are reared on an artificial diet from birth, under this rearing regime this pigmentation is not seen in the first to third instar larvae. Instead, they are cream in colouration and reached the third instar at 10 days. The distinct morphological features of the larvae at each instar stage made it feasible to select individuals at the same life stage for testing based on simple visual analysis. This standardisation of the selected instars for testing reduced the potential for life-stage specific variation in sensitivity for survival and weight change for sublethal measurements.

While there are examples of previous toxicity studies with Lepidoptera conducted using artificial diet (Adamczyk et al. 1999; Knight et al. 2001; Morimoto et al. 2004), it is thought that the proposed assay is the first to use a design that specifically incorporates the toxicant into the supplied food. The value of exposure through application within the diet is that it provides a greater level of clarity on the added concentration and also allows the possibility to reliably track consumption which is not possible in such a quantitative manner in leaf surface application assays. Furthermore, the route of exposure to pesticides by larvae is both oral and contact, which provides a more realistic exposure scenario than oral exposure alone.

From initial assessments of background mortality rates and development over time (Figure 2.2), it was decided to use 4th instar larva for testing. However, given that larvae can develop

from egg to pupae within the test system, the overall approach could be used to test any larval instar and indeed for more extended testing of effects through the full larval development period. As, however, earlier instars are more prone to deaths arising from handling stress, the use of larger instar provides lower background mortality which is desirable to support both concentration-response modelling for single time point assessment and also for GUTS modelling of survival effects in time. If working with later instars of larger Lepidoptera, one change that can be introduced to the overall design of the test may be the use of a larger testing surface such as a 6-well plate, and a greater amount of added diet to allow them to continue with their development without space or food limitation. The potential to test across the full larval stage could be useful to identify susceptible stages. It has been proposed that the choice of instar used in bioassays may affect the sensitivity shown to pesticide exposure. Indeed, several studies have highlighted a greater sensitivity to chemicals at lower instars with the latter two stages often being the least sensitive to chemical toxicants (Knight et al. 2001; De Armas et al. 2020).

Organophosphate insecticides, such as chlorpyrifos, have become the pest management product of choice in many developed and developing countries due to their generic availability, low cost, limited potential for bioaccumulation and relative environmental persistence (Mitra and Maitra 2018). In the three repeat bioassays with chlorpyrifos, a high degree of repeatability of the results was found from the exposure, despite the use of different concentration series in each test. Probit analysis of the 48h time point survival data gave LC_{50} values between 10.6 and 20.4 mg/kg. Although this experiment included a novel experimental design, our results from the repeated bioassays mirror those from other Lepidopteran toxicity tests conducted with organophosphates (Morimoto et al. 2004). Morimoto et al (2004) who also used an exposure through artificial diet, found that the lepidopteran *Spodoptera litura* had an LC_{50} of 12.8 mg/L when exposed to another organophosphate, acephate. Greater toxicity of chlorpyrifos than found here was reported by Kalita *et al.* (2016) who found an LC_{50} of 3.35 mg/L at 96 hours in the silkworm (*Philosamia ricini*) indicating the potential for significant variation in species sensitivity among lepidopterans for chlorpyrifos.

The bioassay provided all the required data to calculate metlic use in risk assessment as well as the necessary data to calibrate a GUTS model. The difference in those metrics between the 3 replicates was low and support the robustness of the assay. The use of the LC_{50} value

to express sensitivity to a chemical as an LC_{50} value alone has limitations for comparison both between chemical and species. By their nature, LC_{50}s are derived from toxicity data for a single time point. Hence, they do not account for the dynamics/time dimension of the toxic effects and as a result, cannot be immediately compared between assays of different durations (Jager 2011). TKTD models, such as the GUTS framework account for the time dimension, and, once calibrated, can be used to calculate LC_{50} values at any time point for regulatory purposes. In addition, the LC_{50} derived with the GUTS calibrated model are more robust and reliable than the LC_{50} calculated with the classic Probit approach. This is further highlighted by the inability of probit analysis to calculate a confidence interval of the LC_{50} in some tests at 48 and 96 hours (Table 2.2). The GUTS model is parameterised with data from all time points, and therefore, is potentially less influenced by any specific value than for parameters (i.e. LC_{50}s) generated from the data for only a single (i.e the final) time point. These results support the advantages of TKTD modelling over the traditional probit analysis to obtain an LC_{50}, and its uncertainties (i.e. confidence intervals). Further, all of the GUTS-RED model parameters (i.e. m_w and k_d) were all in the same range of value when comparing the three tests. The similarity of these values highlight the high repeatability and reliability of the overall bioassay method and its suitability to generate the required data to calibrate a robust GUTS model. A further advantage of the GUTS-RED-SD model is that the derived parameters provide a wider insight into the nature of the toxic effect than possible from concentration-response model fitting. The threshold parameter m_w (i.e. the NEC: the no-effect concentration) is not time-dependent, and therefore provides a more valuable and comparable perspective of hazard than LC_{50} values which can vary with time (Figure 2.4). In addition, the dominant rate constant, k_d, can represent the damage recovery if it is slow relative to the toxicokinetic elimination, or which can represent the toxicokinetic elimination if, damage recovery is fast. If both rates are of similar size, k_d will represent the one-compartment approximation of ’he `true' two-compartment behaviour (TK and TD). Here the estimated k_d is not fast (except for test 1, but the CI is large so is not well characterized for this test) nor slow, which indicates chlorpyrifos is excreted (or metabolised) at a certain rate, and/or the damages are repaired at a certain rate as well. Finally, it is good to stress that in all 3 tests, the GUTS model provided a very good simulation of the data (Figure 2.3), indicating the data generated by the experimental design were of good quality, and once calibrated, the model could be used to predict new scenarios with time-varying concentrations, for instance.

2.4.2 Assessment of the sublethal effects

The sublethal data did not highlight any significant differences in relative weight in comparison to the control for larvae exposed to up to 16.11 mg/kg. However, the median weight change of control larvae exceeded that of all chlorpyrifos treatments. Weight change associated with the exposure to chlorpyrifos could occur for several reasons. The first could be a change in metabolic activity caused by ingestion of the chemical leading to an increase in maintenance costs to eliminate the chemical and repair any resulting damage due to toxicant effects. Such trade-offs between growth and maintenance are included within the wider concept of Dynamic Energy Budget Theory (DEB) (Baas et al. 2010; Jager and Zimmer 2012). A second hypothesis is that chlorpyrifos has an antifeedant effect. Previous studies have found that aside from the high invertebrate toxicity of organophosphates, compounds from this class can have strong antifeedant effects (Kalita et al. 2016). However, given that the lowest median body weight change index was seen at 6.2 mg/kg but not at the two higher concentrations, further work would be needed to link feeding or resource allocation changes to an effect of larval growth in an exposure-dependent manner.

The potential to obtain sublethal data for effects on growth from this bioassay provides the potential to expand the complexity of TKTD models for GUTS-based survival modelling into the use of the full life-cycle Dynamic Energy Budget toxicity (DEBTox) Model. Use of a DEBTox modelling approach would allow predictions to be made on the TKTD effects chemicals have on multiple traits relating to survival, growth, and potentially, development and reproduction. This would allow predictions to be made on the effects of exposure on energy allocation and toxicity for growth and repair, rather than just simply for survival. However, as this bioassay is optimised for use on larvae, effects on reproduction are not possible to obtain within the test. It may, however, be possible to extend the length of the test to get the growth data up to pupation and calibrate only the growth part of a DEB model. To measure reproduction, a different feeding strategy of adult moths must be considered. This could potentially include spiking the 10% honey solution that is used for adult culturing to obtain larvae for testing. This would mean that the morphologically distinct life stages of Lepidoptera would require separate methodologies during a whole lifecycle test and potentially internal residue data to calibrate such models.

2.5. Conclusion

Lepidopterans are a speciose rich and diverse order of insects that can be both beneficial and pest organisms in agriculture. Currently, there is a relative lack of knowledge of the effects of pesticides on life-cycle endpoints and TKTD traits in the lepidopteran species. This paucity of information is due in part to the lack of a bioassay method capable of generating necessary data to apply to TKTD models, such as GUTS. Here we develop and test a novel bioassay suitable for toxicity testing for the effects of pesticide exposure in time. The new bioassay can generate highly repeatable results over a longer range of time and with a high frequency of survival assessment for multiple individuals across multiple exposure levels. For testing here, we used *M. brassicae* larvae due to their ease of rearing and rapid reproduction rates. However, it is expected that, with diet optimisation, the bioassay would be suitable for alternative lepidopteran species. While tested for single chemicals, the overall method could be applied for mixture toxicity testing as the chemicals are added into the diet while in a liquid state, allowing multiple chemicals to be added in a single treatment. Further, it would also be possible to adapt the protocol to allow for pulsed exposures, by removing larvae from spiked plates and transferring them to clean artificial diet. Therefore, simulating many different exposure scenarios which could include different larval stages. Overall, the bioassay system presents a robust and reliable approach to measuring the potential impacts of chemical exposure in lepidopteran larvae.

CHAPTER 3 – SCREENING FOR TOXICITY

3.1 Introduction

The need for pest control is widely recognised. Indeed, management strategies involving pesticides are almost ubiquitous in agricultural settings, with use still increasing. For example, there has been a 7% rise in the total area of UK vegetable crops receiving pesticide treatment in the years between 2011 and 2019 (FERA 2019). It is not only the area of crops treated with pesticides that is growing, but also the number of active ingredients used. In the UK, between 2000 and 2015, the average number of pesticide active ingredients used at the farm level increased from 11 to 17 (FERA 2019). Modern pest control applications in agriculture often explicitly consist of the use of multiple pesticide active ingredients applied as a predefined program, within which pesticide applications are used at key points throughout the growing season. Therefore, non-target species living within and potentially outside of pesticide treated areas will typically be exposed to a cocktail of chemicals, rather than a single compound. This extent of this pesticide cocktail exposure varies according to crop and local soil and climate conditions. Such differences mean it may be difficult to model any combined effects within a generalised exposure scenario. Despite the potential threat to ecosystems of the diversity of pesticide combination in use, the effects of these mixtures are poorly characterised. As a result, mixture effects are currently not routinely considered within risk assessment practices.

Historically, mixture toxicity effects have been predicted using the models of Concentration Addition (Bliss 1939) for similarly acting chemicals and Independent Action (Loewe and Muischnek 1926) for dissimilarly acting chemicals. These models have been used successfully in ecotoxicology for many years and have repeatedly shown their ability to predict the toxicity of mixtures for these specific mode of action combinations (Altenburger et al. 2000; Arrhenius et al. 2004). However, these models are also limited in their scope, as they cannot predict anything beyond the null hypothesis of additive effects governed by the chemical working separately without interaction (synergy or antagonism) between them. In other words, the joint effect of the chemicals used in combination predicted by these models is the

same as the sum of each individual effect combined accounting for the different potencies of the chemicals of probabilities of effect. Therefore, the use of concentration addition and independent action results in predictions that cannot explain the presence of synergy or antagonism between chemicals.

Meta-analyses have been conducted to assess the frequency of non-additive (synergistic or antagonistic) effects in published mixture studies (Belden et al. 2007; Cedergreen 2014; Carnesecchi et al. 2019). These reviews have consistently concluded that synergistic or antagonistic effects are found in a relatively small, but nonetheless significant proportion of studies (10-25% depending on the definition used to identify non-additive effects). Models that can identify and characterise non-additive effects between chemicals within mixtures are consequently needed. A recent advancement has been the development of toxicokinetic-toxicodynamic (TKTD) models, which may provide an improved method for identifying synergistic and antagonistic interactions in mixtures (Cedergreen et al. 2017; Bart et al. 2021). Through both TKTD modelling and experimental studies, recent work has begun to develop hypotheses regarding the mechanisms that underlie synergism or antagonism. Such mechanisms include alterations of the pathways in which pesticides enter the body, the way that they are located within the body and/or the means by which they exert their toxic effects once taken up (Holmstrup et al. 2010). As perhaps the best-known example, synergism or antagonism in mixtures can occur when one chemical may decrease the metabolism of another by disrupting phase I metabolism processes, leading to an increase or decrease in the toxicity of the mixture (Cedergreen 2014; Gong and Diao 2017).

As a tool for characterising mixture effects and identifying synergistic or antagonistic interactions TKTD models such as the General Unified Threshold model of Survival (GUTS) provide a powerful approach (Jager et al. 2011). GUTS models can be used to improve understanding of chemical internalisation and damage leading to toxicity for both single chemicals and mixtures (Bart et al. 2021). Reduced versions of this model (GUTS-RED) do not require data on internal concentrations. Therefore, these approaches are useable for studies that observe effects on survival in time alone (Jager et al. 2011). TKTD models are designed not to be time dependent. This means that their predictions are more applicable than single time point dose-response models that give a toxicity metric (e.g., LC_x values) for only a single point (usually the final sampling occasion) in time. In GUTS models of mixture effects, the data for multiple single chemical effects can be combined to provide a prediction of the time

course of mixture effects assuming additivity. Measurements of mortality response to mixture exposures that are under-predicted by the GUTS mixture models (i.e., higher mortality than expected) can then be identified as synergistic and cases where mortality is less than that predicted by the model can be identified as antagonistic. This potential is indicated in Figure 3.1, where hypothesised synergistic or antagonistic effects are shown compared to additive predictions made based on the known effects of the two single chemicals.

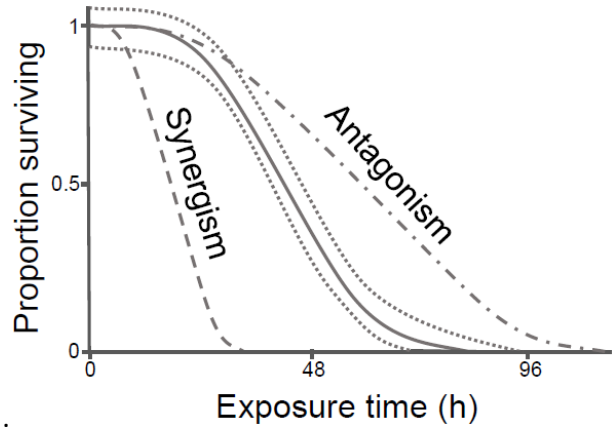


Figure 3.1. Schematic of TKTD interactions between chemicals. Concentration addition is shown as the solid line and 95% CIs are shown as close dashed lines. Synergy is simulated as a more rapid mortality effect and antagonism as a delayed response.

In this chapter, the GUTS mixture model developed by Bart *et al.* (2021) was used to characterise the effects of binary mixtures on survival in time of larvae of the lepidopteran *Mamestra brassicae*. To identify interactions between chemicals, first, the effects of the individual chemicals must be known. Hence, 9 pesticides, 5 insecticides and 4 fungicides are initially screened for their effects on acute (survival in time) and sublethal (relative weight change) endpoints in exposed *M. brassicae* larvae. The insecticides used for the study were selected from a range of available substances to represent a range of different modes of actions and target a range of neurological receptors; while the fungicides were selected for their previously reported synergistic interactions or their widespread use. Chemicals were selected as representatives of many of the major classes of both insecticides and fungicides ensuring the exposure scenarios assessed are likely to be common in agricultural settings for non-target species.

The toxicity of the 9 selected pesticides was screened using the dietary exposure-based assay optimised in Chapter 2. Selected mixtures of these pesticides were then tested for synergistic and antagonistic interactions. Pesticide combinations taken forward to mixture testing were investigated using the same assay adjusted and optimised for testing mixture effects on survival and weight change. Mixtures were chosen based on whether they displayed clear concentration dependent toxicity in the single chemical assays and/or have been previously reported to interact synergistically. The GUTS-RED model used in Chapter 2 was used to analyse the single chemical studies and the GUTS mixture model developed by Bart *et al.* (2021) used to predict survival to compare with observed data to identify interactions between chemical pairs. The overall hypothesis for this work was that due to their target-specific mode of action, insecticides will affect survival and growth of larvae, but fungicides will not show these biomarkers of toxicity. Further, it is anticipated that certain classes of substance, notably those that can plausibly be associated with changing the activities of biochemical pathways known to be involved in xenobiotic metabolism, will be anticipated to result in more frequent and larger magnitude synergistic or antagonistic effects in mixtures.

3.2. Methods

3.2.1. Test animal husbandry

Mamestra brassicae larvae were collected from the same strain as in Chapter 2. These animals have been kept in culture for over 40 years at the UK Centre for Ecology and Hydrology. For culturing, the larvae have been raised on a modification of Hoffman's tobacco hornworm diet (Smith 1966), referred to hereafter as 'artificial diet'. The larvae cultures were maintained in a controlled room at a constant 20^{o}c under a 16:8 light: dark pattern.

To breed larvae for testing, adult *M. brassicae* were maintained in flight cages with continuous access to 10:90% honey: water solution. Eggs were collected three times a week to ensure that the selected cohort used for each study consisted only of individual hatched from eggs laid only within the previous 24-hour period. Collected eggs were either added to rearing boxes containing artificial diet, until the caterpillars were approximately 16 days old, at which time they were used for the bioassays or raised to adults to breed the next generation.

3.2.2. Preparation of Test Chemicals

Two types of experiment were conducted using the single pesticides and binary mixtures. The set of single chemical studies were conducted to assess the sensitivity of the test species to each pesticide. These studies provided important insights into the sensitivity of the larvae to the different substance MoAs, and also provided data on the nature of concentration-responsive relationships for effects on weight change and survival that could be used to design the mixture tests. These 'single chemical bioassays' were conducted for nine pesticides, five insecticides; cypermethrin, imidacloprid, clothianidin, cyantraniliprole and sulfoxaflor, and four fungicides; azoxystrobin, propiconazole, chlorothalonil and prochloraz. These active ingredients were selected either because of their widespread use, known synergistic potential or because of their position as emerging pesticides with unknown synergistic potential (Table 3.1). All bioassays were conducted using high purity analytical standards of each chemical (>99%). All substances were purchased from Sigma Aldrich

(Poole, UK), except sulfoxaflor which was purchased from Greyhound Chromatography and Allied Chemicals Ltd (Birkenhead, UK).

Table 3.1. Chemicals selected for *M. brassicae* bioassay tests and the rationale behind their selection

Pesticide	Rationale for Selection
Cypermethrin (pyrethroid insecticide)	Widely used pyrethroid, known synergism with azole fungicides (Cedergreen 2014)
Clothianidin (neonicotinoid insecticide)	Widely used neonicotinoid worldwide, known synergism with azole fungicides (Haas and Nauen 2021)
Imidacloprid (neonicotinoid insecticide)	Widely used neonicotinoid worldwide, potential for synergism with azole fungicides
Cyantraniliprole (diamide insecticide)	Ryanoid-acting insecticide, limited existing data on synergism potential
Sulfoxaflor (Sulfoxamine insecticide)	Sulfoxamine insecticide active for nAChR; limited existing data on synergism potential
Propiconazole (Triazole fungicide)	Representative of commonly used class of azole fungicides, synergism potential through CYP P-450 inhibition (Kretschmann et al. 2015)
Prochloraz (Imidazole fungicide)	Representative of class of azole fungicides, frequently reported as synergist in literature through CYP P-450 inhibition (Kretschmann et al. 2015)
Azoxystrobin (Strobilurin fungicide)	Representative strobilurin fungicide; mode of action induces oxidative stress which could underlie synergism
Chlorothalonil (Aromatic fungicide)	High worldwide used fungicide: reduces glutathione, metabolism which may affect phase 2 metabolism (Song et al. 2017)

M. brassicae larvae were exposed to a range of concentrations of each of the 9 chemicals. The upper concentration for testing was in some cases determined by the maximum solubility of the chemical in acetone used for dosing (Table 3.2). However, in some cases where preliminary data was available, a lower maximum concentration was selected based on an expectation of mortality at this concentration. Where a mortality effect was found, concentrations were refined to target testing in the responsive concentration range. The data from this final defined test across the concentration-responsive range were analysed to determine an accurate LC_{50} value and to calibrate a GUTS-RED model for that pesticide.

Table 3.2. Maximum Solubility of Pesticides in Acetone

Chemical Name	Maximum Solubility in Acetone (mg/L)
Cypermethrin	450,000
Imidacloprid	67,000
Cyantraniliprole	6540
Sulfoxaflor	217
Clothianidin	15,200
Propiconazole	Miscible
Prochloraz	600,000
Azoxystrobin	86,000
Chlorothalonil	18,000

Each chemical stock was prepared as a dilution series in acetone (as in Chapter 2), except sulfoxaflor as this chemical has low acetone solubility and was, therefore, dissolved in water. Each single chemical bioassay measured both acute effects on survival in time and also sublethal effects by measuring relative weight change. Seven treatments and a control were used for all studies; except chlorothalonil, which was tested with only 6 treatments and a control due to larvae availability at the time of testing. Table 3.3 shows the concentrations tested for all compounds. Fungicides were diluted in series by a factor of 3 and insecticides a factor between 2 and 3 depending on range finder results.

Table 3.3 Concentrations of chemical treatments used in toxicity testing of *Mamestra brassicae*. The concentration shown is final, i.e., once acetone is removed leaving the final 1%.

Chemical Name	Concentrations Tested (mg/kg)
Cypermethrin	0, 1.12, 2.12, 3.81, 6.86, 12.35, 22.22, 40
Imidacloprid	0, 5, 8.81, 15.54, 27.39, 48.27, 85.1, 150
Cyantraniliprole	0, 4.38, 6.58, 9.87, 14.81, 22.22, 33.33, 50
Sulfoxaflor	0, 0.08, 0.26, 0.8, 2.4, 7.22, 21.66, 65
Clothianidin	0, 0.14, 0.41, 1.23, 3.7, 11.11, 33.33, 100
Propiconazole	0, 1.37, 4.12, 12.34, 37.04, 111.11, 333.33, 1000
Prochloraz	0, 1.37, 4.12, 12.34, 37.04, 111.11, 333.33, 1000

Azoxystrobin	0, 1, 3, 9, 27, 83, 250, 750
Chlorothalonil	0, 0.62, 1.85, 5.56, 16.67, 50, 150

3.2.3. Preparation of Bioassay Plates

3.2.3.1. Single Chemical Testing

All exposures were conducted using the same spiked artificial diet as described in Chapter 2. Each chemical was mixed into the media while the diet was liquid (at approximately 50°C) to ensure an even pesticide distribution. Heat stability of chemicals at 50°C was confirmed for all substances, except sulfoxaflor and cyantraniliprole (F A O and W H O 1983; Garau et al. 2002; Lin et al. 2005; Leng et al. 2012; Liu et al. 2017; Gao et al. 2019). Imidacloprid heat stability could only be found for aqueous solutions, in which the degradation process is known to intensify with heat (Zheng and Liu 1999). However, for the short amount of time imidacloprid is exposed to 50°C in this bioassay (<10 minutes) degradation still remains unlikely. Sulfoxaflor and cyantraniliprole heat stability requires verification, although there is no reason to expect this would not be the case.

The spiked diet was pipetted into each well of a 12-well plate to give a 1 ml volume. Individual 4th instar *M. brassicae* larva weighing 20 mg ± 10 mg were added to the diet surface in each well. Two full plates were prepared for each treatment, giving a total of 24 larvae (two 12 well plates) for each test concentration. Each larva was exposed for 96 hours at a constant 20˚C under constant darkness. At 6, 24, 48, 72, and 96 hours from the start of the test, survival was checked by stimulating the second segment behind the head with a paintbrush for 5 seconds. Larvae that did not respond were recorded as dead. To measure weight change, larvae were weighed at the start and end of the 96-hour exposure to determine effects on growth. Any larvae reported as dead during the 96-hour exposure were not weighed.

3.2.3.2. Mixture bioassay preparation and procedure

The high-throughput bioassay used for the single chemical testing was also used for the mixture studies. Although it is feasible to develop the approach to test more complex mixtures, here binary mixtures were tested, as they have the greatest potential to identify the dominant interactions between chemicals (Cedergreen *et al.* 2012). It has been shown that in environmental mixtures, most of the overall effects can often be attributed to the effects of only a small number of the chemicals present (Spurgeon *et al.* 2022). Hence, binary mixture effects are also relevant to many environmental mixture scenarios.

The majority of mixture tests were conducted to assess the joint effects for combinations of an insecticide and a fungicide or of an insecticide pair (Figure 3.2). The selection of the combinations of mixtures for assessment used mechanistic information about the mode of action of the chemical to develop hypotheses about the potential for interactions leading to synergism. It was hypothesised that occurrences of synergy may be caused by the presence of chemicals that could act as cytochrome-P450 enzyme inhibitors. Such chemicals would be expected to reduce rates of xenobiotic metabolism affecting the breakdown of co-exposed chemicals. Therefore, bioassays initially concentrated on identifying synergy in the testing of binary mixtures with a known *CYP450* inhibitor. Previous work has shown that azole fungicides, such as prochloraz, have the potential to inhibit cytochrome P450 activity (Cedergreen 2014). Hence, mixture tests included several combinations that included members of this fungicide class. In addition to the predicted potential synergistic concentrations, mixtures with unknown effects were also included, with these hypothesised to be additive to provide a test of the model to analyse different types of joint effects.

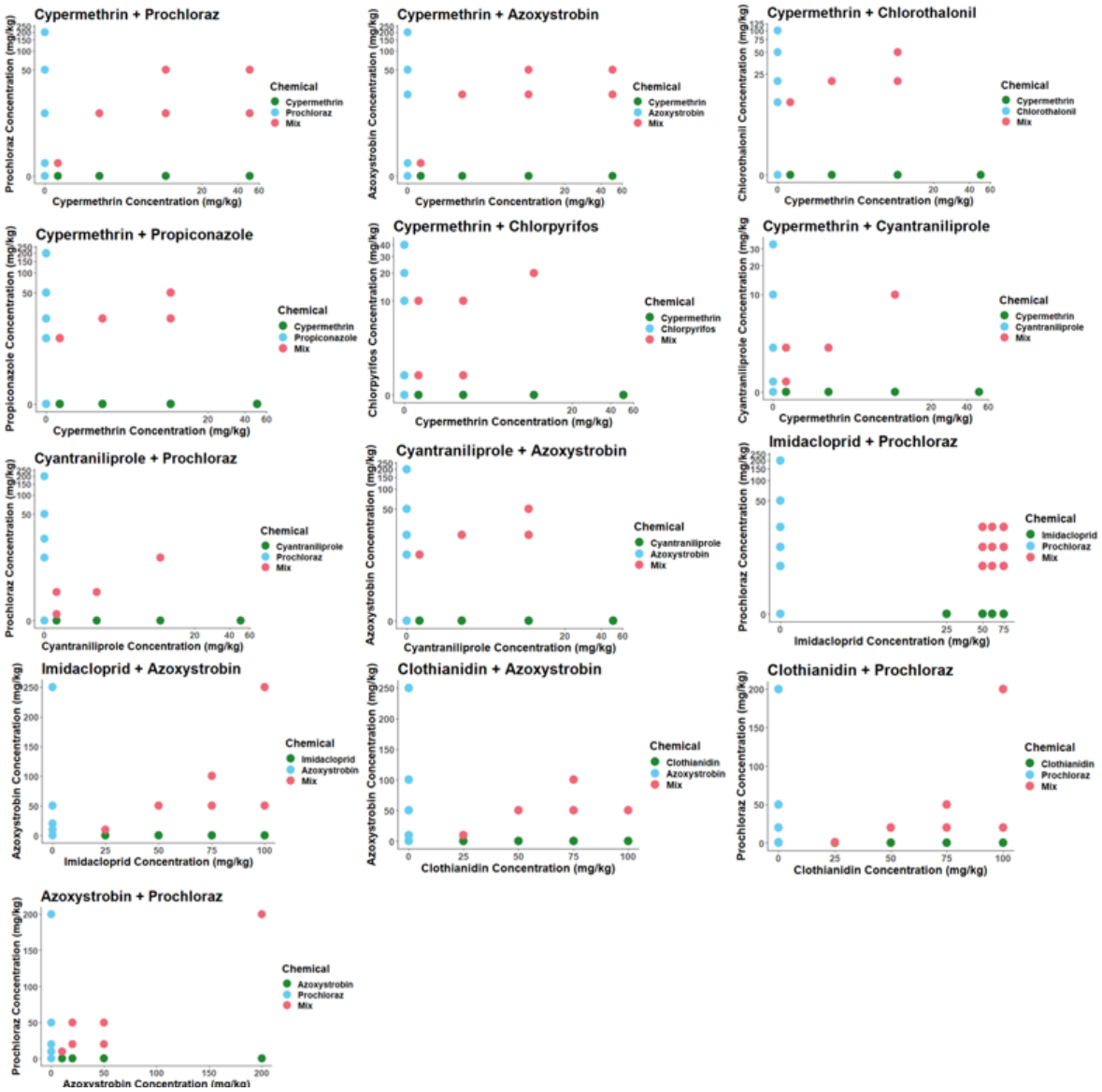


Figure 3.2. Binary mixture pairings and concentrations used of each chemical in single and binary mixture treatments used for each experiment.

Mixture tests used the same method as for the single chemicals. Each mixture study consisted of an exposure conducted in plates with a range of concentrations of chemical A and chemical B and a set of combinations of mixture of concentrations of the two chemicals (Figure 3.2). Single chemical treatments were included in the assay to allow both an assessment of inter-experimental variability and also to allow the calibration of the GUTS mixture model used for data analysis against effects measured for the single chemicals with the same exposure. Due to the increased amount of treatments and larvae required; instead of the 24 individuals used for single chemical testing, exposure for mixtures 12 individuals (one 12 well plate) were used. This reduction in replication was compensated for by the

increased number of treatments, meaning a greater number of larvae were included in each mixture experiments compared to each single chemical test.

While the exposure method used broadly followed that for the single chemical studies, some slight modifications were needed. The most pronounced difference was in the chemical spiking. Whereas for single chemicals, artificial diet was spiked with a dilution factor of 100, i.e., pesticides accounted for 1% of the diet; the mixtures studies used a dilution factor of 200, i.e. exposure to half of the pesticide concentrations used in single chemical exposure. This allowed single chemical treatments to be combined to produce the binary mixtures following addition to wells in a 1:1 ratio. Exposures thereafter, mirrored those for the single chemical bioassays with the exception that larvae were weighed at 48 hours as well as at 96-hours. All other test conditions and steps remained unchanged from the single chemical studies.

3.2.4. Statistical Analyses

3.2.4.1. Single Chemicals Bioassays

As with the chlorpyrifos bioassays detailed in Chapter 2, the single chemical effect data for each chemical was analysed with the GUTS-RED model, using the openGUTS software (http://openguts.info/). As all larvae are genetically similar due to their extensive years in culture, the stochastic death model was chosen over individual tolerance, as the latter model assumes variation in threshold effects concentration between individuals. Fitting of the GUTS-RED-SD model provided LC_x values and GUTS parameter estimates for each chemical. For chemicals without a clear effect on survival, no analyses or GUTS-RED calibration was performed.

The sublethal effects of all chemicals on body weight were used to determine an EC_x value for each tested chemical for effects on relative weight change. Values were determined using the $MOSAIC_{growth}$ tool (Charles et al. 2022). Weight data at 0 and 96 hours was input into this software which determines either the ER_x or EC_x on sublethal endpoints using a Bayesian framework. Weight change data was found to be not normally distributed by a Shapiro-Wilk test and so was tested with a Kruskall Wallis and post hoc Dunn's test. The weight change index was calculated using Equation 2.1 as found in Chapter 2.

3.2.4.2. Mixture Bioassays

All survival data calculations and analyses for the mixture studies were performed using Matlab 2021b and the BYOM modelling software platform (*www.debtox.info/byom.html*) using the specifically designed GUTS_MIX package, designed by *Bart et al. (2021),* explicitly for the analysis of time series survival data from mixture bioassays. Within the GUTS_MIX tool, the GUTS-RED-SD model as used for the same reasons as for the single chemical data can be combined with either a damage addition (DA) or independent action (IA) approach. Damage addition was used between pesticides sharing a similar mode of action, whereas the independent action model was used when pesticides had a different mode of action. More details on the structure of these models can be found in Bart et al. (2021). Briefly, in both models, each chemical keeps the parameter of its dominant rate constant. The DA model adds the scaled damage of chemical A and chemical B, with a weight factor that accounts for differences in toxicity between the chemicals, whereas in the IA model, each chemical keeps its GUTS parameters and the joint effect is determined by multiplying the survival probability of chemical A and chemical B.

The procedure for fitting the different GUTS_MIX tool models followed a stepwise approach. The model was first calibrated using the single chemical data from mixture experiments. Using the parameters obtained from these calibration results, a prediction of the mixture effect over time was performed and plotted against the observed mixture effects to allow comparison. All of the mixture pairings in the bioassays conducted were either a mixture of insecticide and fungicide or a pair of insecticides that interact with different target receptors. Therefore, all of the mixture analyses used the GUTS-RED-SD Independent action (IA) model for the main assessment.

Sublethal effects were analysed by modelling body weight change index with a GLM. A post hoc Tukey's HSD was carried out on matching concentrations to determine if there was a significant effect on weight change with the combination of chemicals in comparison to the single chemical alone, i.e., 0.5 mg/kg of chemical A was compared to 0.5 mg/kg of chemical A + 1 mg/kg of chemical B. For relative weight change, significant effects not seen in single chemicals exposure but present in the combination treatments were identified as possible synergism for sublethal effects in cases where the combined effect also exceeded additive effects.

3.3. Results

3.3.1. Single Chemical Toxicity

3.3.1.1 Insecticide Mortality

All exposures showed low background mortality after 96 h, with 100% control survival in all tested chemicals except for clothianidin at 96% (i.e. one death from 24 control individuals). These results confirm the robustness of the assay against background mortality effects. Something that helps in the fitting of robust GUTS-based models for survival effects.

Of the four insecticides, only cypermethrin caused 100% mortality after 96 h at any tested concentrations, with this effect seen at 22.2 mg/kg, but not at the top concentration of 40 mg/kg (Figure 3.3). Exposure at the highest concentration tested (150 mg/kg, 100 mg/kg, 50 mg/kg and 65 mg/kg respectively) for imidacloprid, clothianidin, cyantraniliprole and sulfoxaflor resulted in mortality after 96 hours of 16.6%, 79.16%, 29.16% and 0% respectively.

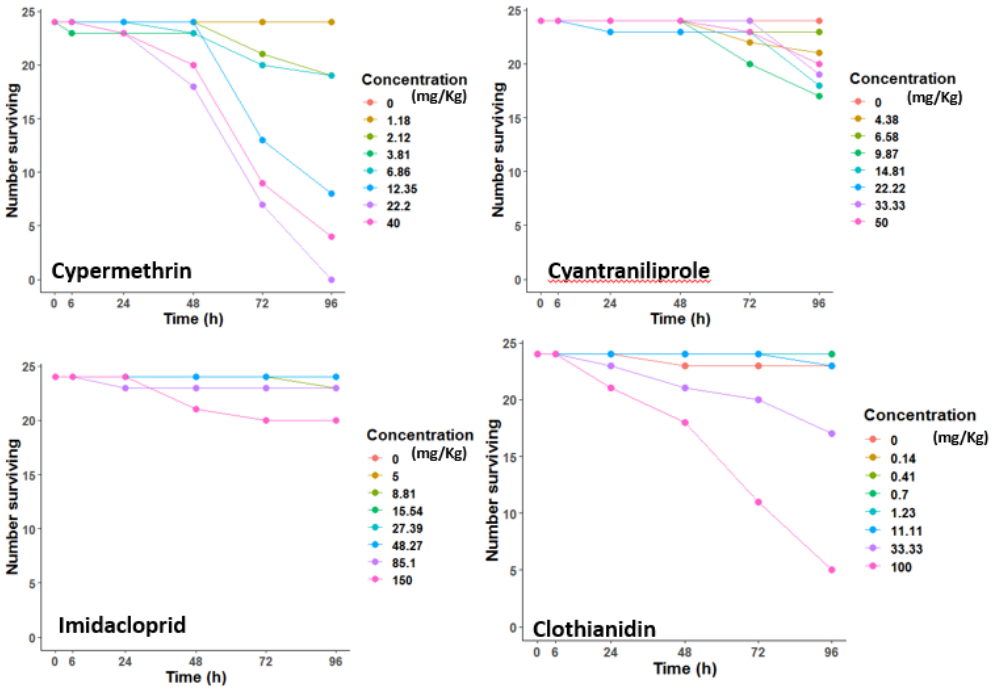


Figure 3.3. Survival in time for *Mamestra brassicae* larvae exposed to the four insecticides cypermethrin, cyantraniliprole, imidacloprid and clothianidin; sulfoxaflor did not affect survival and was, therefore not plotted.

GUTS-RED-SD models were fitted to the time series survival data-set for all chemicals for which an effect on survival was found (i.e. cypermethrin, *imidacloprid, clothianidin, cyantraniliprole*). The GUTS-RED-SD model provided accurate simulations of the observed data for cypermethrin, imidacloprid and clothianidin (Figure 3.4). Cyantraniliprole indicated some non-monotonic response, resulting from greater mortality at lower than higher concentrations. These effects meant that GUTS-RED-SD was unable to fully model survival for this insecticide, with the model fit over-simulating observed mortality, especially at the higher concentrations (Figure 3.4). The GUTS-RED-SD parameter values indicated slow toxicokinetics for cypermethrin and cyantraniliprole (Table 3.4). The toxicokinetic parameters for other insecticides, imidacloprid and clothianidin, could not be characterised from the data set as their values ranged from 0 to infinite, meaning that the true value could take any rate.

The mortality observed for all insecticides, except sulfoxaflor, allowed an LC_{50} to be calculated (Table 3.4) at both 48 and 96 hours. Cypermethrin was most toxic, with a 96 h LC_{50} value returned from the GUTS-RED-SD model of 11.6 mg/kg, compared to 891 mg/kg for imidacloprid, 54.6 mg/kg for clothianidin and 73.2 mg/kg for cyantraniliprole. Larvae showed such low mortality to imidacloprid and cyantraniliprole that the predicted LC_{50} value at 96 hours exceeds the maximum solubility for these chemicals. Therefore, it was not possible to complete an exposure with complete mortality.

Table 3.4 Parameter values (including 95% Confidence Intervals) of the GUTS-RED-SD model estimated for four insecticide assays of *Mamestra brassicae* exposed to a spiked artificial diet. The * denotes where upper or lower CI cannot be calculated because the parameter CI has reached a boundary. LC_{50} values at 48 and 96 hours as calculated by the model are also displayed. Sulfoxaflor is absent as no mortality was caused.

Chemical	Background mortality	Dominant rate constant	Median of threshold distribution	Killing rate	48h LC50 (95% CIs)	96h LC50 (95% CIs)
	h_b (d^{-1})	k_d (d^{-1})	m_w (mg kg^{-1})	b_w (kg mg^{-1} d^{-1})	(mg kg^{-1})	(mg kg^{-1})
Cypermethrin	1 10^{-6}	0.005 (0.002-0.18)	0.0003 (0.0003*-0.06)	1.52 (0.05-5.7)	46.3 (36.7 – 59.3)	11.6 (9.22-14.9)
Imidacloprid	1 10^{-6}	2.72 (0.002*-184*)	0.00137 (0.00137 – 8.23)	0.0002 (0.0002*-0.09)	1980 (865 - 6060)	891 (406 - 1890)
Clothianidin	0.0107	0.283 (0.002*-1.99)	4.838 (0.005-19.7)	0.009 (0.003-1.75)	164 (106 - 297)	54.6 (40.2 – 80.2)
Cyantraniliprole	1 10^{-6}	0.004 (0.002-0.27)	0.001 (0.001*-0.5)	0.28 (0.01-1)	292 (203-435)	73.2 (51.3 - 109)

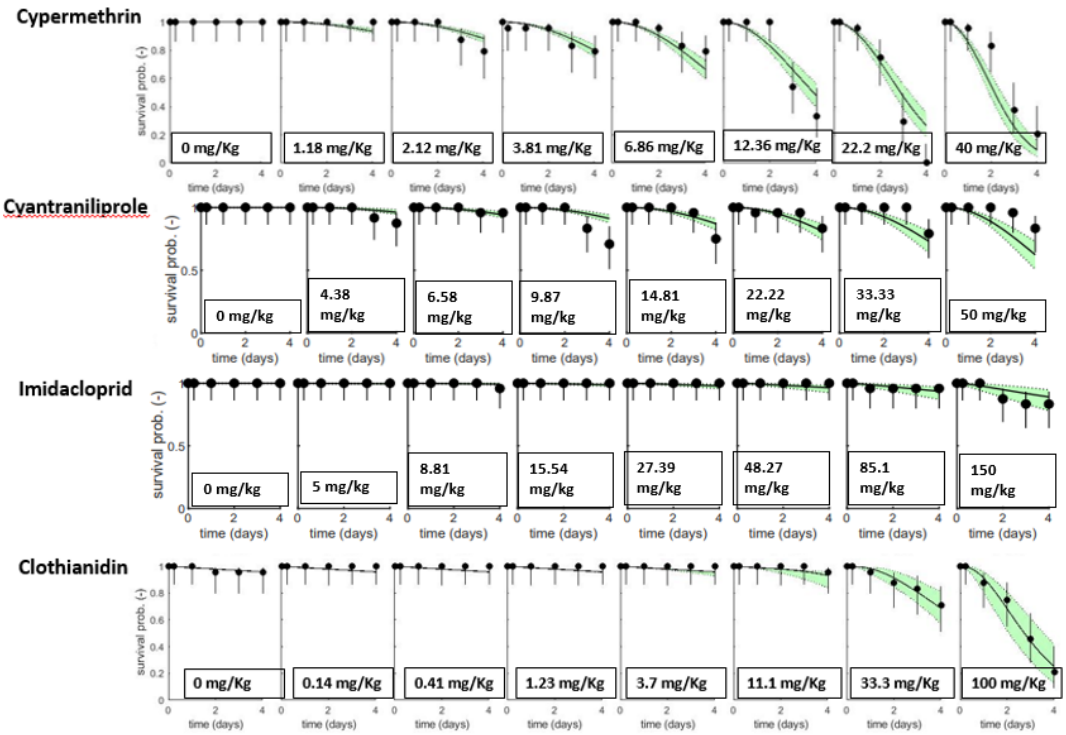


Figure 3.4. Observed and simulated survival over time of *Mamestra brassicae* larvae exposed to cypermethrin, cyantraniliprole, imidacloprid and clothianidin. Separate rows show different chemicals at each of the 7 concentrations tested and control. The points are observed data with error bars showing Wilson score confidence and solid black lines are the predicted survival using GUTS-RED-SD model, green areas show 95% confidence intervals.

3.3.1.2. Insecticide Sublethal Effects

Effects on growth, measured as body weight change index were measured over the 96-hour exposure period. Control larvae grew in all experiments. This consistent growth highlights the repeatability of the bioassay and its capability to allow normal larval growth under the test conditions. Sulfoxaflor did not affect relative weight change. The remaining four insecticides each had a significant impact on growth compared to controls (Kruskal-Wallis : cypermethrin $H(7)=52.64$ $p<0.001$; cyantraniliprole $H(8)=71.14$, $p<0.001$; imidacloprid $H(7)=77.08$, $p<0.001$; clothianidin $H(7)=58.392$, $p<0.001$). For three of these four insecticides (cypermethrin, imidacloprid, clothianidin), a clear concentration-response pattern was seen for the growth effect. The exception was cyantraniliprole, where an effect was observed whereby larvae showed reduced growth in all treatments (Figure 3.5). Kruskal-Wallis

indicated significant differences in growth for exposed larvae for all four insecticides at concentration below those affecting survival. This greater sensitivity highlights that lower concentration exposures can significantly impact weight change when compared to those affecting survival, particularly for cypermethrin (significant effect on weight index at 2.1 mg/kg) and cyantraniliprole (concentrations of 4.3 mg/kg and above).

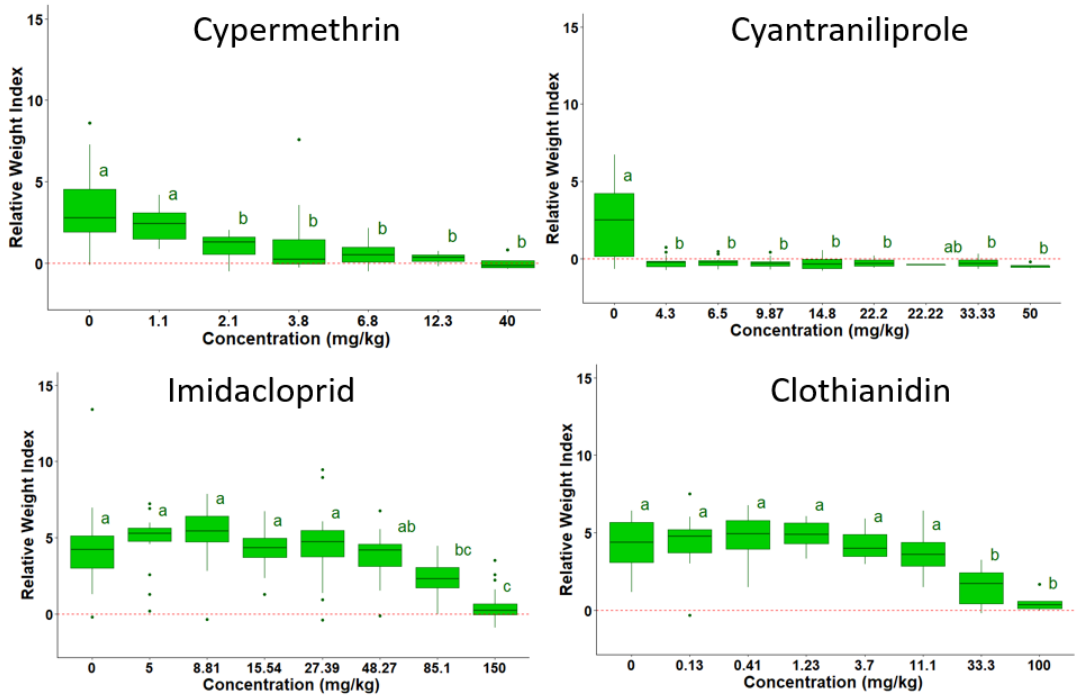


Figure 3.5. Box and whisker plots showing the mean (bold line) 75th percentile (upper and lower box limits) and 95% confidence intervals (vertical lines) for relative weight change of *Mamestra brassicae* larvae exposed for 96 hours exposed to cypermethrin, cyantraniliprole, clothianidin and imidacloprid, treatment with different letters are significantly different (Kruskal-Wallis and post hoc Dunn's test). The red dashed line is at 0 mg/kg for all plots, values below 0 indicate weight loss.

Fitting dose response curves to the relative weight change data indicated that, despite causing only a limited effect on mortality, cyantraniliprole had the lowest EC_{50} (Table 3.5), followed by cypermethrin, which also had high toxicity for mortality. The EC_{50}s for relative weight change index for the three chemicals that share the MoA of targeting nicotinic acetylcholine receptors, imidacloprid, clothianidin and sulfoxaflor, were all higher than for

the pyrethroid and ryanoid at least 8-fold. This suggests *that M. brassicae* may have low sensitivity to a range of different insecticides that are active against this target.

Table 3.5. EC_{50} values of insecticides at 96 hours exposure to *Mamestra brassicae* larvae. Values and 95% confidence intervals are calculated using $MOSAIC_{growth}$ (Charles *et al.* 2022).

Chemical	EC_{50} at 96h (mg/kg)
Cypermethrin	3.32 (1.32 – 13.8)
Imidacloprid	101 (84.2 – 123.8)
Clothianidin	26.6 (18.4 - 43)
Cyantraniliprole	0.96 (0.49 – 1.68)

3.1.3 Mortality and weight change effects of fungicides

For all four tested fungicides: azoxystrobin, chlorothalonil, prochloraz and propiconazole, no clear dose-related effect on mortality was seen (Figure 3.6). Thus, although some low level mortality (up to 20%) was seen in some bioassays, deaths were distributed throughout the treatments and can, therefore, be considered stochastic and so unrelated to the exposure concentration. As a result, it was not possible to fit GUTS-RED-SD models to calculate parameters or LC_x values for any time-point.

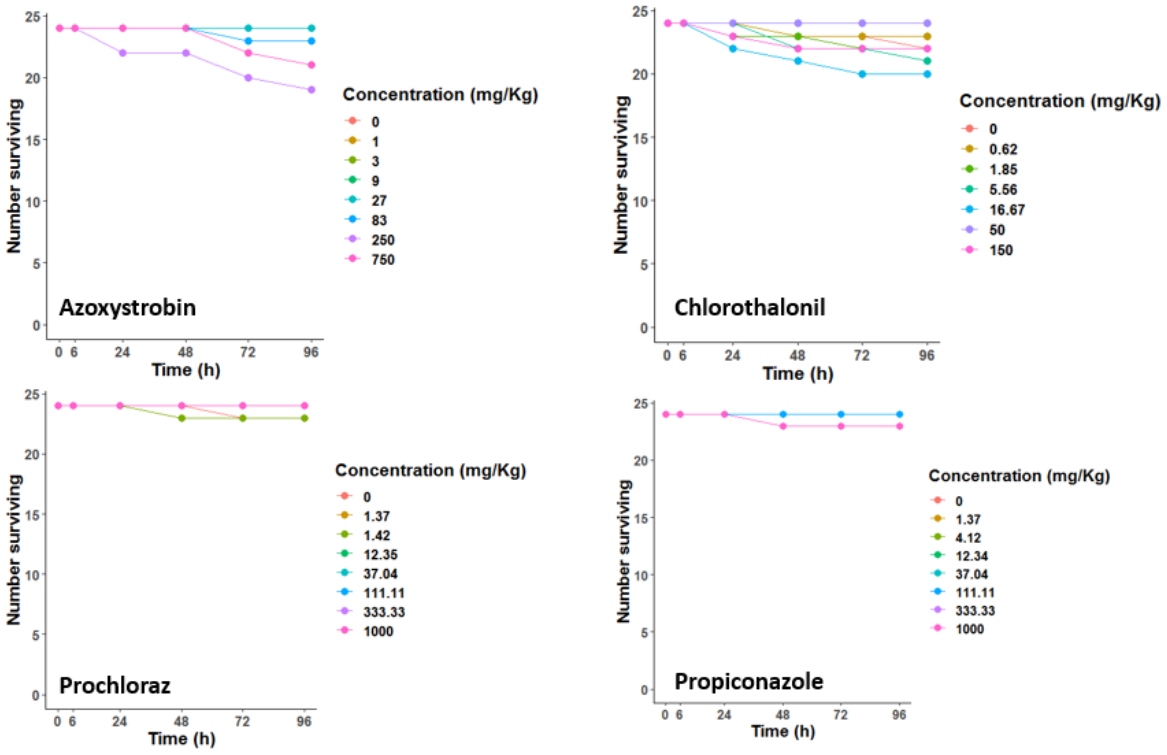


Figure 3.6. Survival in time curves for the *Mamestra brassicae* larvae exposed to the four fungicides: azoxystrobin, chlorothalonil, prochloraz and propiconazole.

Larval growth was seen in the controls of each of the fungicide similar to that of the insecticide, although there were greater variations in control growth rates for the fungicides. A significant sublethal effect on relative weight change rate was seen for two fungicides, azoxystrobin (Kruskal-Wallis: H (7) =61.69, $p<0.001$) and propiconazole H (7)=35.68 $p<0.001$). EC_{50}s for the effects of azoxystrobin and propiconazole were 53.3 (5.63-363) and 132 (42.1 – 456), both values being within the solubility limit for these two chemicals. No clear concentrations related effect on growth was seen at any exposure concentration for prochloraz or chlorothalonil, although there were some effects at intermediate concentrations compared to controls (Figure 3.7) (1.85 and 5.56 mg/kg of chlorothalonil). EC_{50}s could not, therefore, be determined for these fungicides.

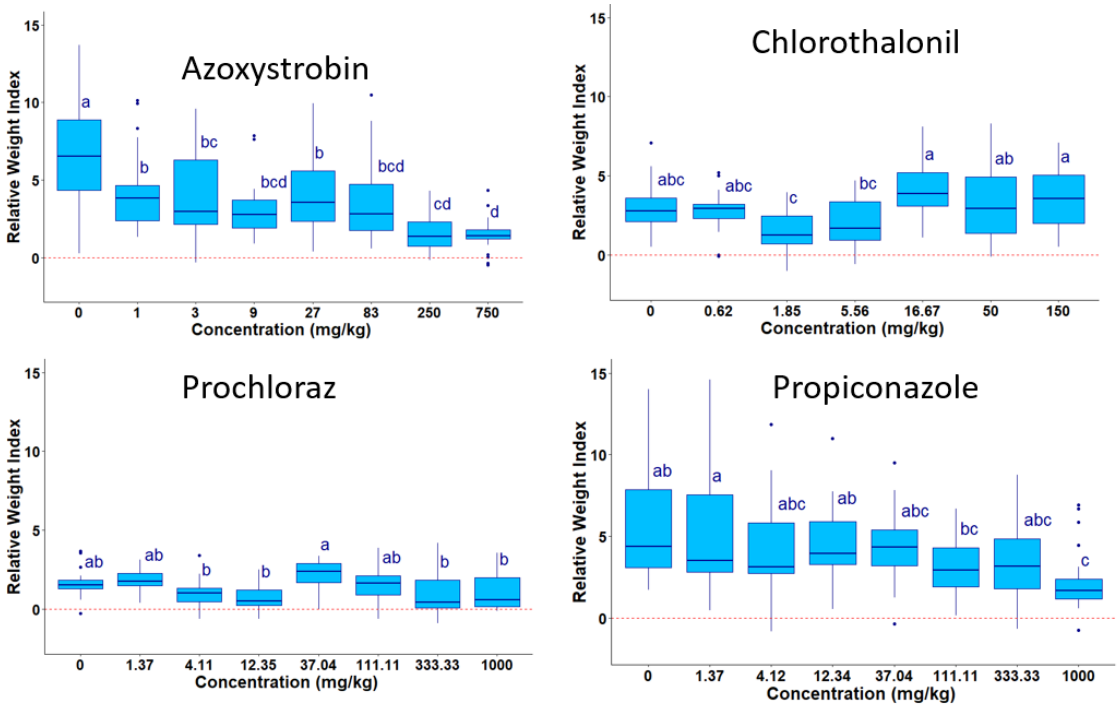


Figure 3.7. Box and whisker plots showing the mean (bold line) 75th percentile (upper and lower box limits) and 95% confidence intervals (vertical lines) for relative weight change of *Mamestra brassicae* larvae exposed for 96 hours exposed to, azoxystrobin, chlorothalonil, prochloraz and propiconazole, treatment with different letters are significantly different (Kruskal-Wallis and post hoc Dunn's test). The red dashed line is at 0 mg/kg for all plots, values below 0 indicate weight loss.

3.3.2 Mixture Toxicity

The survival in time data for the mixture studies was modelled using the GUTS-RED-SD IA mixture model developed by Bart *et al*. (2021). This model is specifically designed for the analysis of mixture effect experiments. The model is designed based on the assumption that the chemicals tested in the mixture exert their toxic effect by acting on different target sites. Thus, the model is consistent with known information about the mechanisms of action of the binary combinations of the pesticides tested here, which are drawn from different classes that have different known biological targets.

3.3.2.1 Control survival

In all mixtures tested, control survival was above the 80% validity criteria commonly used for toxicity bioassays (see Chapter 2). The low levels of control mortality meant that it was possible to fit GUTS-RED-SD IA models to datasets to observed mortality effects in those cases where effects were observed.

3.3.2.2 Mixture showing no concentration related effects

Three mixtures showed no concentration related effects. This absence of mortality in these tests meant that these mixtures could not be analysed using the GUTS-RED-SD IA mixture model.

Imidacloprid and azoxystrobin showed no concentration-related effects on mortality showing only stochastic mortality. Thus, there was no mortality effects in any treatment to the maximum concentration tested, e.g., imidacloprid up to 100 mg/kg. The absence of any mortality effect in the mixture indicated the absence of any synergism for this mixture.

Mortality in the imidacloprid and prochloraz mixture was also not concentration dependent. As a result, this mixture could also not be fitted by the GUTS-RED-SD IA mixture model. The absence of mortality at the tested concentrations was consistent with the results of single chemical imidacloprid and prochloraz single chemical tests. Again, the absence of any mortality effect in the mixture indicated the absence of any large magnitude synergism of resulting from the mixture exposure.

Prochloraz and azoxystrobin could not be fitted using the GUTS-RED-SD IA mixture models, as no mortality was also observed for either chemical or the mixture. Findings of no effects in the mixture for this combination are consistent with the absence of a synergistic effect between chemicals in this mixture despite the presence of prochloraz, as a potential synergist.

3.3.2.3 Cypermethrin and prochloraz

The GUTS-RED-SD IA mixture model for the mixture of the pyrethroid cypermethrin and azole fungicide prochloraz indicated the presence of a synergistic interaction. The greatest under-prediction of observed survival by the was found between the concentrations of 5 and 10 mg/kg of cypermethrin and 5 and 50 mg/kg of prochloraz (Figure 3.8). To confirm the consistency of the synergism between these two compounds, a second larger-scale test was carried out (Figure 3.8, fourth row). GUTS-RED-SD IA modelling of this second study confirmed synergism in the mid-range of cypermethrin and prochloraz concentration mixtures, although with a smaller scale of deviation from predicted additive effects at concentrations containing 10 mg/kg of cypermethrin and higher. This consistent nature of the synergistic response for the cypermethrin and prochloraz mixtures confirms the presence of a synergistic response pattern for these two chemicals.

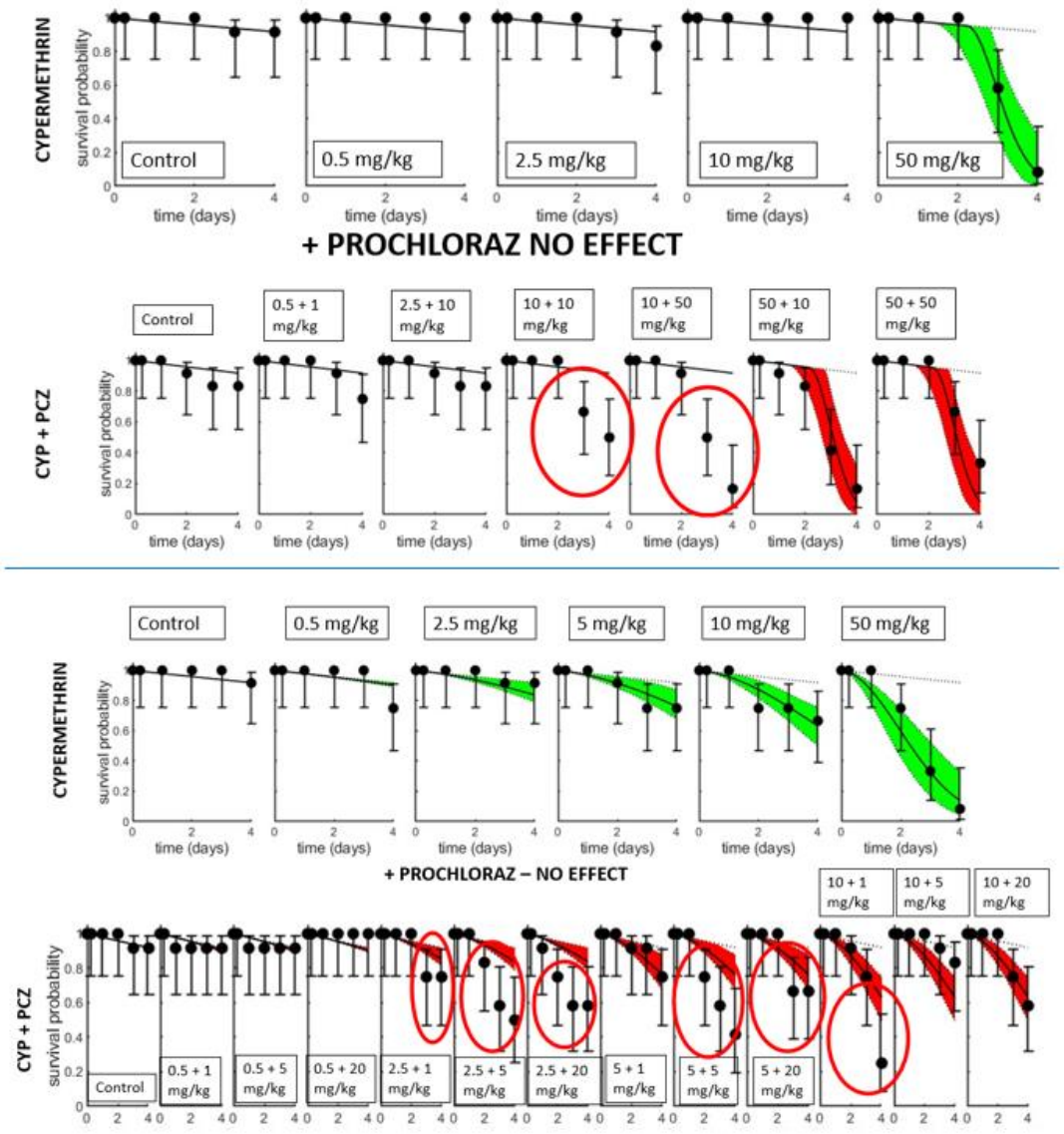


Figure 3.8. Observed and predicted survival over time for *Mamestra brassicae* exposed to mixtures of cypermethrin and prochloraz separately (first row of plots for cypermethrin, prochloraz had no effects), and the mixture of both (second row of plots). Mixture effects (second row of plots) are predicted with the GUTS-RED-SD independent action (IA) model, calibrated on the results from the single exposures (first row of plots). The dashed line is background mortality, and the solid line is simulated mortality. Under-predictions of mortality due to synergism are shown as points that fall below the 95% confidence interval (red area) and are highlighted by the red ring. The third and fourth row of plots shows the second cypermethrin and prochloraz experiment, over a wider range of treatments. The third row of plots is observed survival to cypermethrin, and the 4th is predicted survival with the GUTS-RED-SD independent action model.

Weight of the control larvae increased between 0 and 96 hours (Figure 3.9). A GLM fitted to the relative weight change data indicated that cypermethrin, prochloraz and their interaction term each had a significant effect on relative weight change (cypermethrin, $F(4)=3.96$, $p<0.01$, prochloraz, $F(4)=10.1$, $p<0.001$, cypermethrin*prochloraz, $F(6)=11.1$, $p<0.001$). Tukey's post hoc test was used to investigate the significant differences between treatments with the same concentration of cypermethrin, but different concentrations of added prochloraz. Observations indicated that within all treatments with animals exposed to the cypermethrin alone, these individuals always weighed more than animals exposed to that concentration in the presence of prochloraz, except at the highest concentration where mortality was seen. This greater effect on relative weight change in the presence of prochloraz was, however, not significant according to Tukey's test in any case. Therefore, although indicated by the repeated observation of an effect of prochloraz addition to reduce relative weight change, the presence of synergism could not be statistically verified. Further, given that prochloraz had a clear effect on larvae relative weight change over 96 hours, this reduced weight gain could be due to an additive effect of the presence of prochloraz, rather than any synergistic interaction.

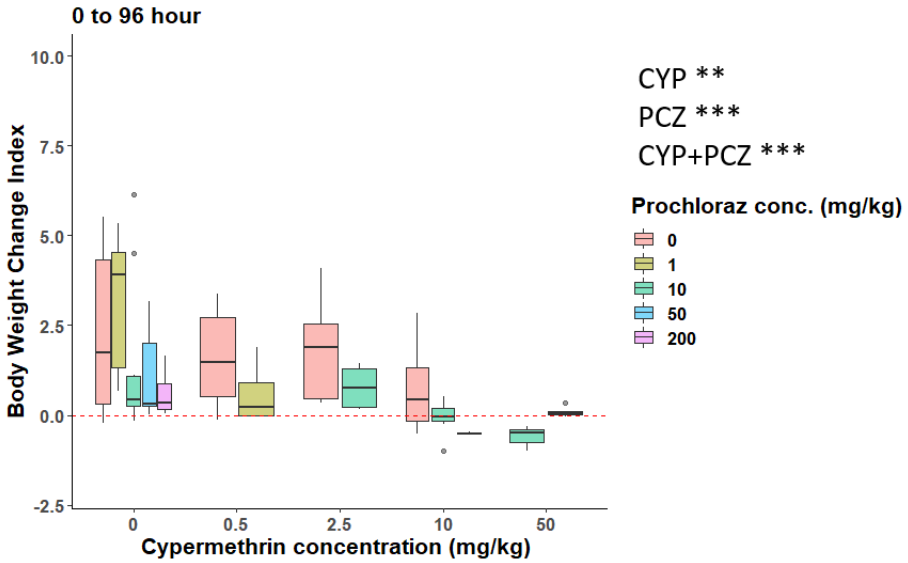


Figure 3.9. Relative weight change between 0 and 96 hours. Significance of cypermethrin, prochloraz and the mixture of both on weight as determined by GLM are shown '***' $p<0.001$ '**' $p<0.01$ '*' $p< 0.05$, NS no significance difference.

3.3.2.4 Cypermethrin and azoxystrobin

Cypermethrin had a concentration-dependent effect on survival consistent with the results from the single chemical study with this compound. No concentration-related response was, however, found for azoxystrobin. Therefore, a GUTS-RED-SD IA model could not be parameterised for this fungicide. For the cypermethrin and azoxystrobin mixture, observed effects on survival were consistent with GUTS-Mix additive model predictions, based on the minimal deviation of the observed response from model simulated survival (Figure 3.10). The consistency of predicted and observed effect indicates that the effects of this mixture were dominantly additive.

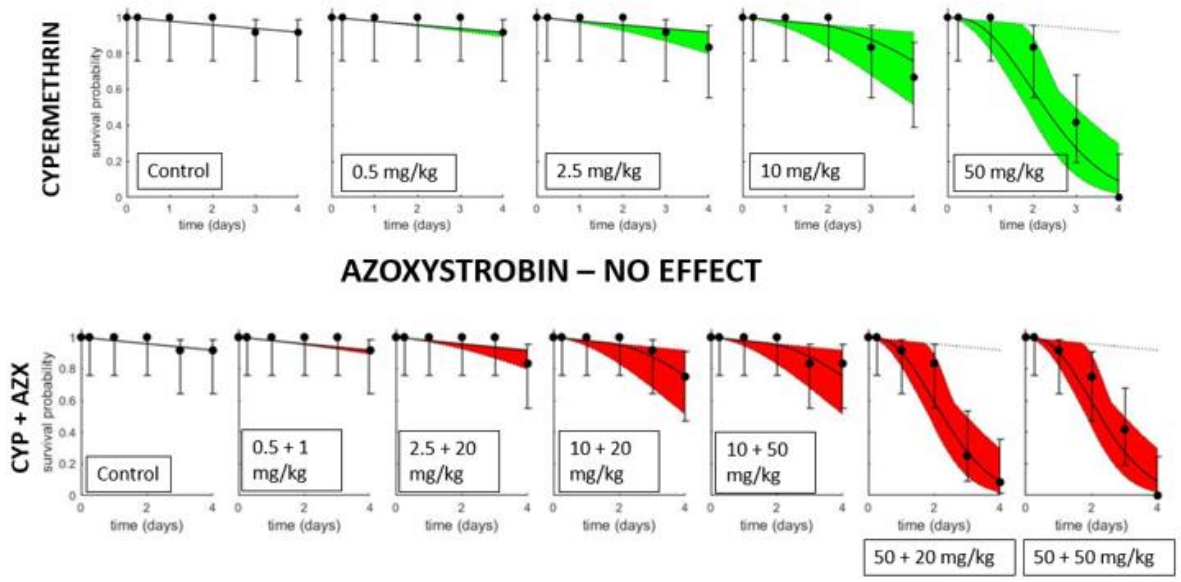


Figure 3.10. Observed and predicted survival over time of *Mamestra brassicae* exposed to mixtures of cypermethrin and azoxystrobin separately (first row of plots for cypermethrin, azoxystrobin had no effect), and the mixture of both (second row of plots). Mixture effects (second row of plots) are predicted with the GUTS-RED-SD independent action (IA) model calibrated on the results from the single exposures (first row of plots). The dashed line is background mortality and solid line is simulated mortality. In this mixture, there is no deviation from the simulated survival and so this mixture is identified as dominantly additive.

Sublethal effects for this mixture show growth of all control larvae over the 96-hour test period, but a loss of weight in the 50 mg/kg of cypermethrin single and cypermethrin 50 mg/kg plus 50 mg/kg azoxystrobin treatments (Figure 3.11). GLM showed a significant effect

of cypermethrin (F (4)=9.91 p<0.001), but not azoxystrobin or the mixture treatment on relative weight change of larvae. Tukey's post hoc test was used to identify significant differences between treatments consisting of the same concentration of cypermethrin but different levels of additional azoxystrobin. At 96 hours, all cypermethrin single chemical only treatments weighed more than larvae exposed at the same concentrations in the presence of added azoxystrobin. However, no treatment of mixture treatments was significantly different to the single exposed cypermethrin treatment at the same concentration. Therefore, this data is in agreement with the survival data, that this binary mixture is additive. Furthermore, as cypermethrin and azoxystrobin had a significant difference on weight over 96 hours in the single chemical testing, it is likely that weight reductions are the result of an additive presence of azoxystrobin in the mixture treatments.

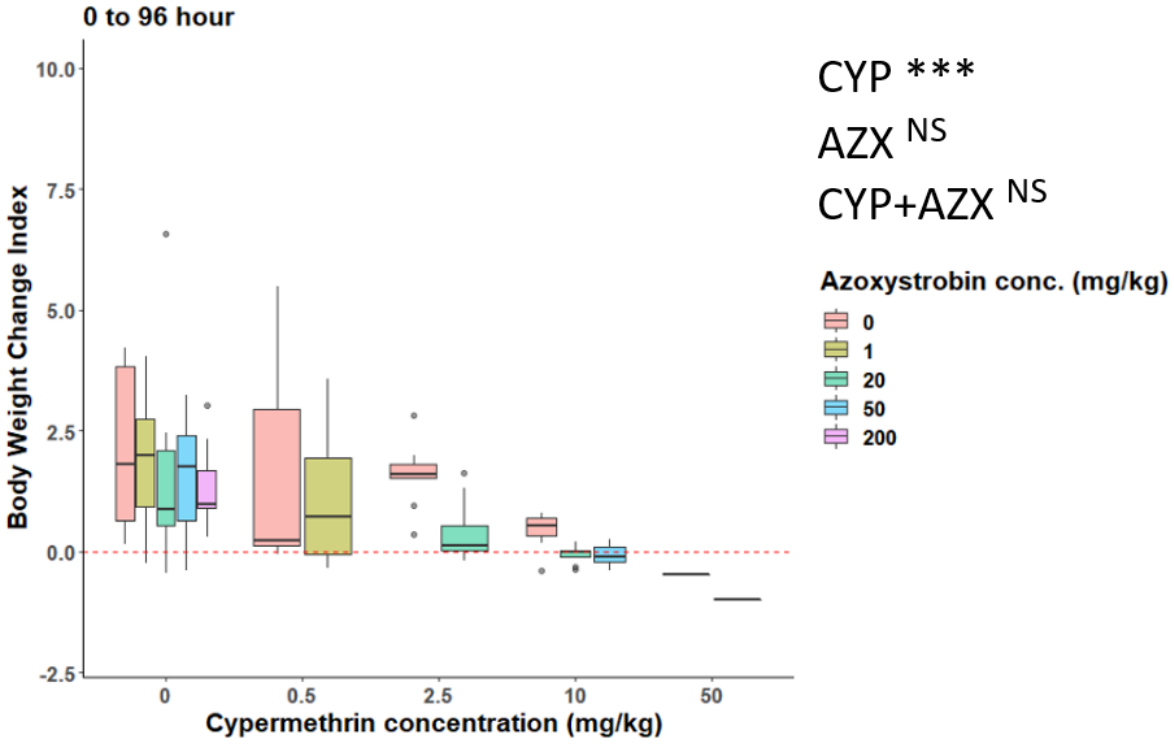


Figure 3.11. Relative weight change of *Mamestra brassicae* larvae between 0 and 96 hours. Significance of cypermethrin, azoxystrobin and the mixture of both on weight change by one-way GLM are shown as '***' p<0.001, '***' p<0.01 '**', p< 0.05*, NS no significance difference.

3.3.2.5 Cypermethrin and chlorothalonil

GUTS-RD-SD IA modelling of the single cypermethrin exposure indicated a clear concentration-related effect on mortality at the 50 mg/kg concentrations. Chlorothalonil did not impact larvae survival and mortality was not modelled as a single chemical. When exposed to cypermethrin in conjunction with chlorothalonil, the GUTS-Mix model predicts larval survival well at levels up to the mixture treatment involving 10 mg/kg of cypermethrin and 50 mg/kg of chlorothalonil. For this combination, the model under predicts survival from 72 hours onwards indicative of synergism in these treatments (Figure 3.12). These results suggest that the binary mixture of cypermethrin and chlorothalonil is synergistic at higher exposure levels and over longer time.

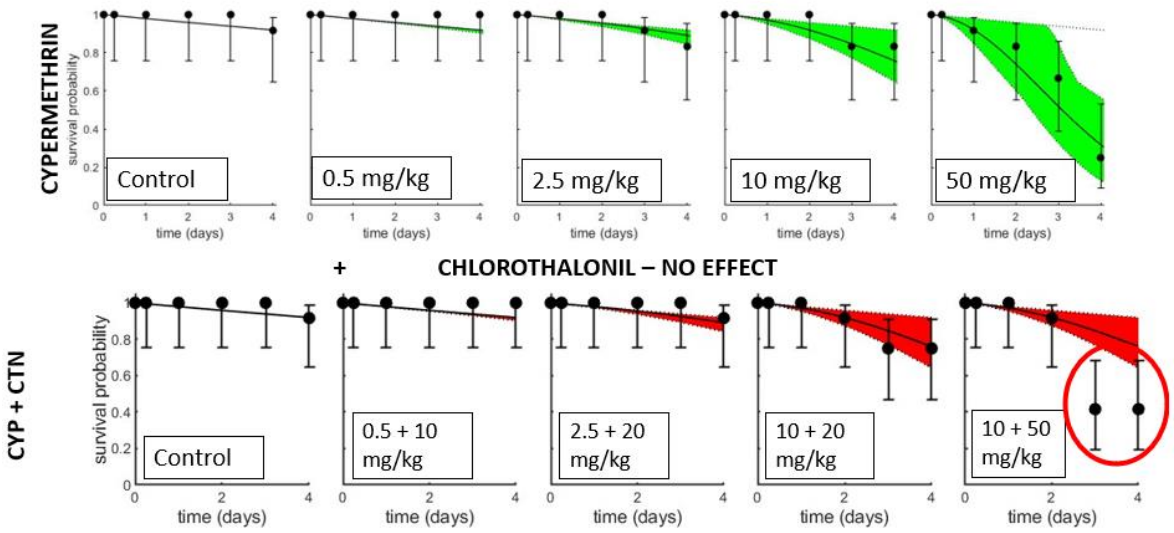


Figure 3.12. Observed and predicted survival over time of *Mamestra brassicae* exposed to mixtures of cypermethrin and chlorothalonil separately (first row of plots for cypermethrin, chlorothalonil had no effects), and the mixture of both (second row of plots). Mixture effects (second row of plots) are predicted with the GUTS-RED-SD action model calibrated on the results from the single exposures (first row of plots). The dashed line is background mortality and solid line is simulated mortality. In this mixture, there is some deviation from simulated survival in the 10 + 50 mg/kg treatment and so this mixture is identified as synergistic.

Controls and all chlorothalonil-only treatments showed an increase in weight over the duration of the exposure in agreement with the single chemical study (Figure 3.13). Larvae exposed to 50 mg/kg cypermethrin showed weight loss. Relative weight change of larvae

was lower in all co-exposed treatments of cypermethrin and chlorothalonil in comparison with the same concentration of cypermethrin alone. However, this difference was not significant in any treatment. Accordingly, the GLM showed that there was a significant difference between weight in larvae exposed to cypermethrin, but not chlorothalonil or the mixture ($F(4)=34$ $p<0.001$). Tukey's post-hoc test was used to investigate significant differences between relative weight of cypermethrin and the same concentrations of cypermethrin with added chlorothalonil. Although animals exposed to cypermethrin single treatments always weighed more than the same concentration of cypermethrin with chlorothalonil, no significant differences were recorded in relative weight change between the single and combined chemical treatments. Thus, although survival data for this mixture indicated synergism, the statistical analysis of sublethal data does not indicate a synergistic interaction between these chemicals.

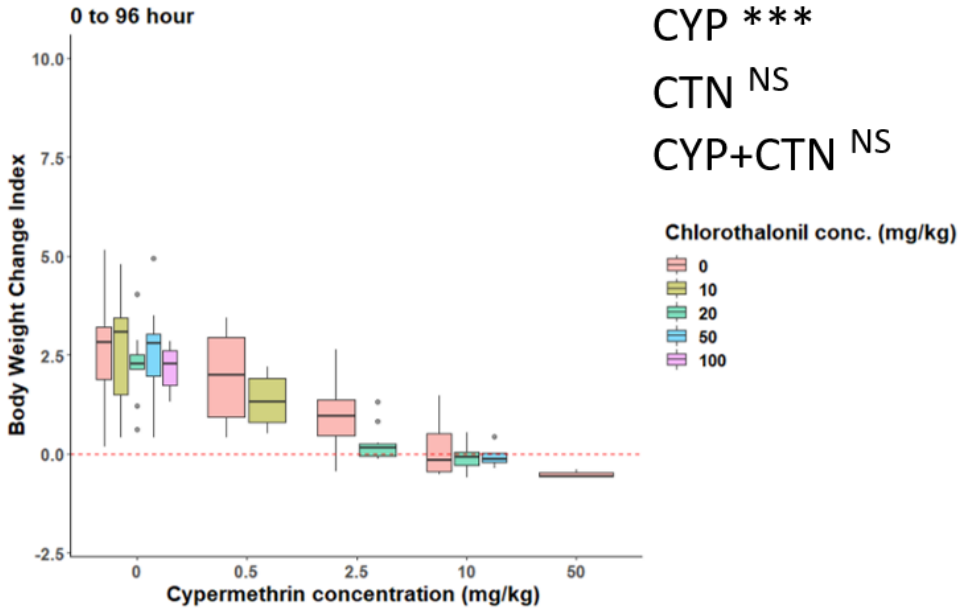


Figure 3.13. Relative weight change between 0 and 96 hours. Significance of cypermethrin, chlorothalonil and the mixture of both on weight as determined by GLM are shown as '***' $p<0.001$, '**' $p<0.01$, '*' $p< 0.05$, NS no significance difference.

3.3.2.6 Cypermethrin and propiconazole

The fungicide propiconazole had no effect on survival. Hence, only the single cypermethrin treatments and the mixture treatments were analysed using the GUTS-RED-SD IA model. For

cypermethrin alone, an effect on survival in the 50 mg/kg treatment was seen consistent with previous studies. Cypermethrin and propiconazole was identified as synergistic for effects on survival using the GUTS-RD-SD IA mixture model. Thus, in the 10 mg/kg + 50 mg/kg mixture treatment related effects on survival of co-exposure of cypermethrin with propiconazole were found. This interaction resulted in the under predicted of observed effects by the GUTS-Mix additive model (Figure 3.14).

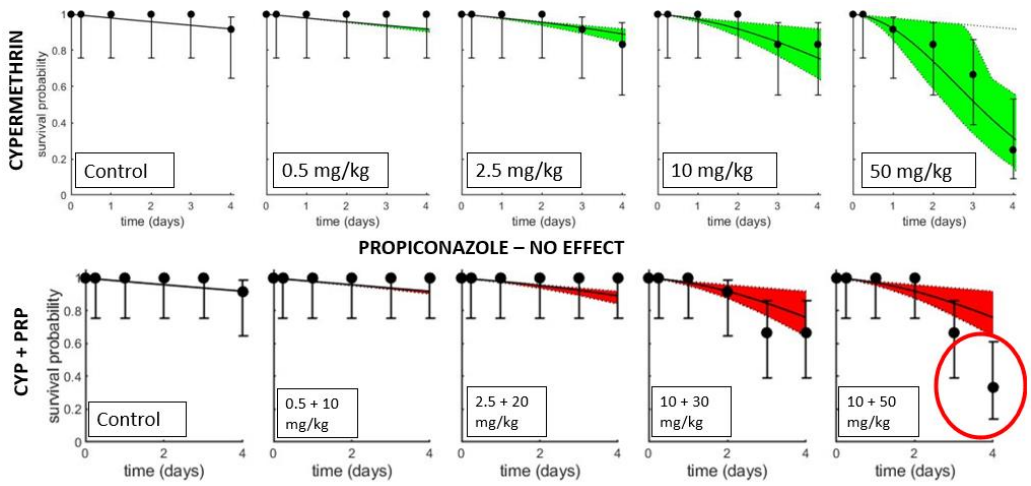


Figure 3.14. Observed and predicted survival over time of *Mamestra brassicae* exposed to a mixture of cypermethrin and propiconazole separately (first row of plots for cypermethrin, propiconazole had no effect), and the mixture of both (second row of plots). Mixture effects (second row of plots) are predicted with the GUTS-RED-SD independent action (IA) model calibrated on the results from the single exposures (first row of plots). The dashed line is background mortality and solid line is simulated mortality. In this mixture, there is no deviation from the simulated survival and so this mixture is identified as dominantly additive.

Weight increased in all control and propiconazole-only treatments (Figure 3.15). Cypermethrin had a significant effect on the weight of larvae consistent with the results for this chemical ($F(4)=39.1$ $p<0.001$), while propiconazole and the mixture did not significantly affect weight change. Tukey's post hoc test investigated differences between the concentrations of cypermethrin and the same concentrations of cypermethrin with added propiconazole. No significant differences were found between cypermethrin single treatments and the mixture treatments of the same concentration of cypermethrin in the presence of propiconazole. Thus, also survival results indicate that this mixture was synergistic, sublethal results indicated additivity.

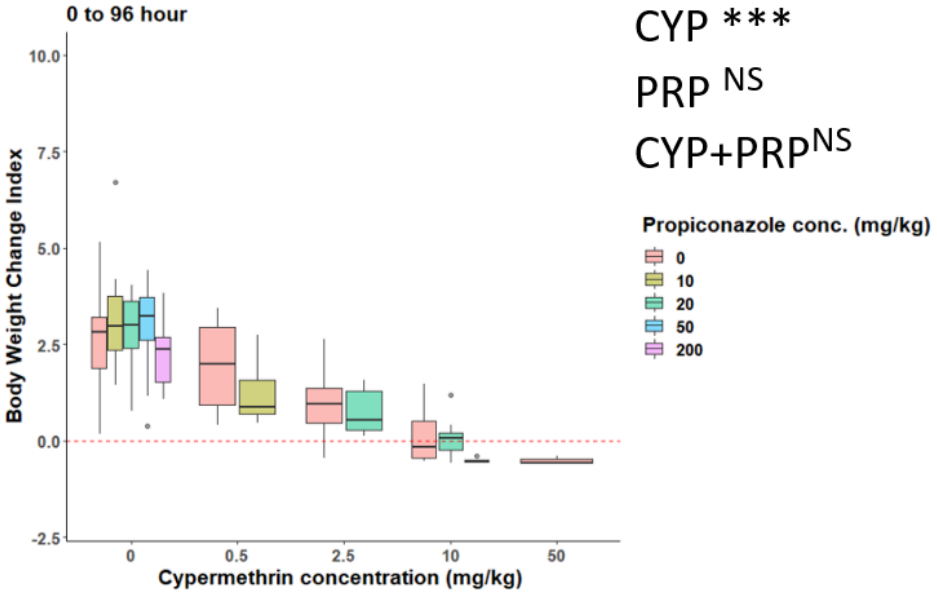


Figure 3.15. Relative weight change between 0 and 96 hours. Significance of cypermethrin, propiconazole and the mixture of both on weight as determined by GLM are shown '***' 0.001, '**' 0.01, '*' 0.05. NS No significance.

3.3.2.7 Cypermethrin and chlorpyrifos

Cypermethrin and chlorpyrifos was the only study in which control survival of below 80% was found. This relatively high level of background mortality means that the outcomes of the modelling for this mixture should be treated with some caution. Despite this high background mortality, it was still possible to successfully simulate the effects of both single chemicals using the GUTS-RED-SD IA model (Fig. 3.16). The response pattern seen in the tested mixtures was complex. The measured data indicated synergism compared to the additive model predictions in the 2.5 + 1 mg/kg mixture, and antagonism in the 0.5 + 10, 2.5 + 10, and 10 + 20 mg/kg cypermethrin and chlorpyrifos mixtures. This mixture, thus, could not be classified as synergistic or antagonistic and so can be categorised as likely dominantly additive, although with the potential for treatment-specific responses dues to a dose dependent deviation effect (Jonker et al. 2005).

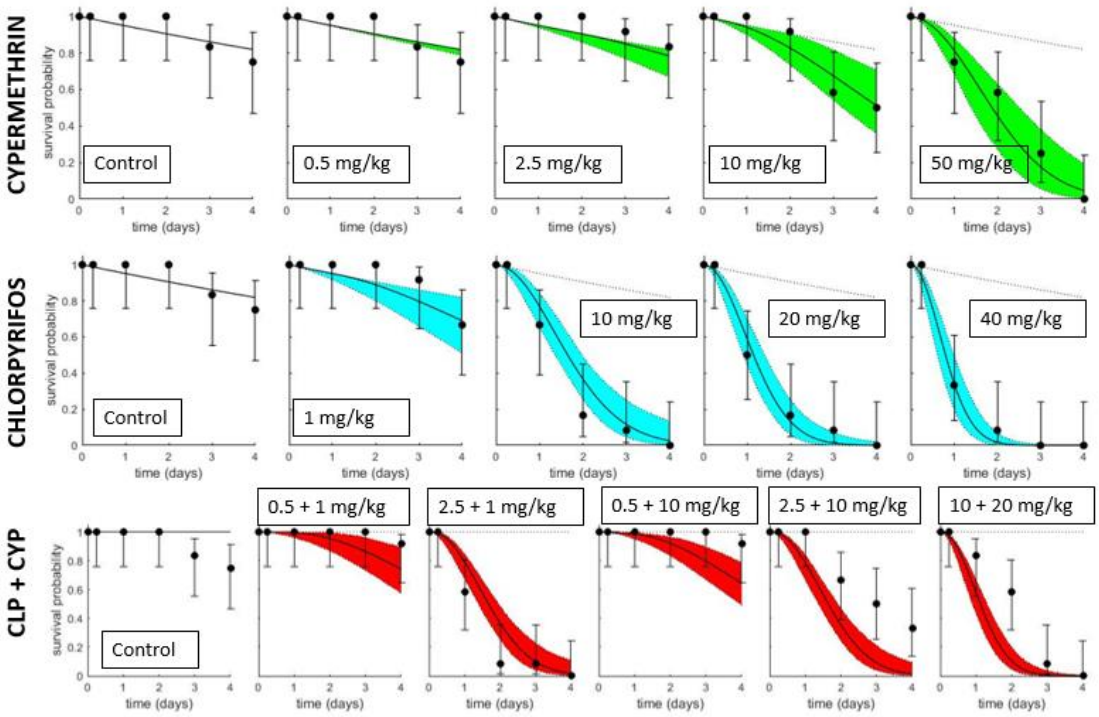


Figure 3.16. Observed and predicted survival over time of *Mamestra brassicae* exposed to mixtures of cypermethrin and chlorpyrifos. separately (first row of plots for cypermethrin, second for chlorpyrifos, and the mixture of both (third row of plots). Mixture effects (third row of plots) are predicted with the GUTS-RED-SD independent action (IA) calibrated on the results from the single exposures (first and second row of plots). The dashed line is background mortality and solid line is simulated mortality. In this mixture, there is no deviation from the simulated survival and so this mixture is identified as dominantly additive**.**

Control weight did not increase in line with the other tests conducted. Therefore, conclusions based on the data for all treatments should be treated with some caution. Weight change data indicated that at 96 hours, all larvae co-exposed to cypermethrin and chlorpyrifos had a higher total weight than those exposed to cypermethrin alone (Figure 3.17). In Chapter 2, results showed that chlorpyrifos exposure did not significantly affect weight of larvae, which is reflected in the sublethal results seen here. However, in both the bioassay in Chapter 2 and in these results, larvae at concentrations over 20 mg/kg did not survive. As a result, sublethal effects on growth could not be measured in all concentrations. Neither cypermethrin, chlorpyrifos or the combination of both caused any significant sublethal effect

on relative weight change when analysed with GLM. Tukey's post hoc test also found that there were no significant differences in relative weight change between cypermethrin single treatments and the same cypermethrin concentration with added chlorpyrifos. Therefore, sublethal data was classed as additive.

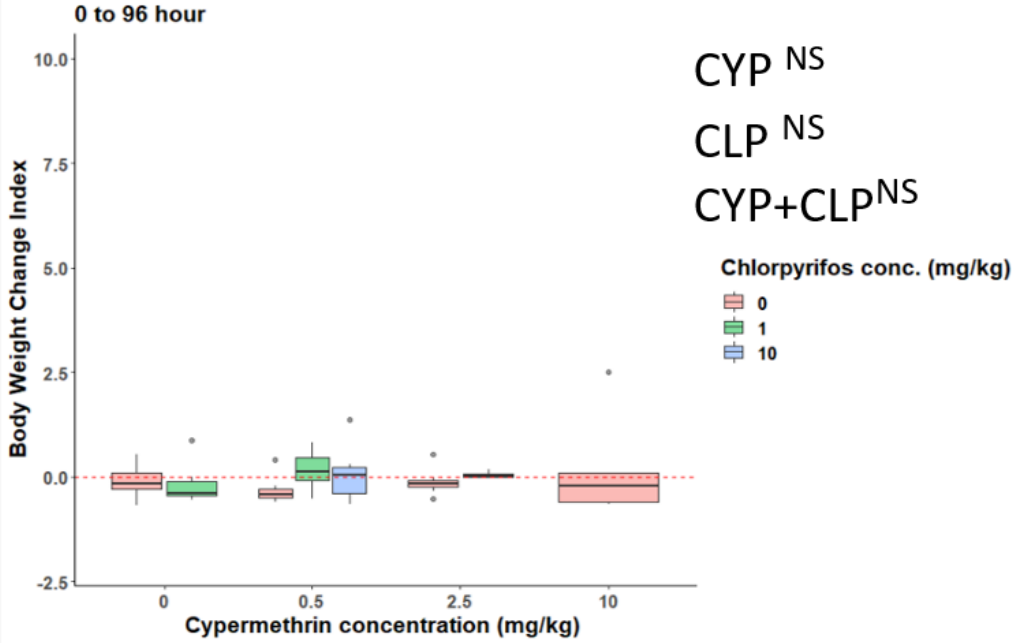


Figure 3.17. Relative weight change between 0 and 96 hours. Significance of cypermethrin, chlorpyrifos and the mixture of both on weight as determined by GLM are shown as '***' 0.001, '**' 0.01, '*' 0.05. No significance is denoted as NS.

3.3.2.8 Cypermethrin and cyantraniliprole

Both chemicals in this mixture had a dose-dependent effect on mortality that could be modelled using the GUTS-RED-SD IA model (Figure 3.18). Survival was clearly affected in the 50 mg/kg cypermethrin and 33 mg/kg cyantraniliprole treatments. In the mixture treatments, synergism was identified by the under prediction of mortality in the 10 + 10 mg/kg of cypermethrin and cyantraniliprole treatment. Thus, mortality greatly exceeded that of predicted joint effect of 33.3 mg/kg cyantraniliprole when larvae were exposed to 10 mg/kg of cyantraniliprole and 10 mg/kg cypermethrin. In this mixture, the observed mortality response was outside of the GUTS-Mix model prediction.

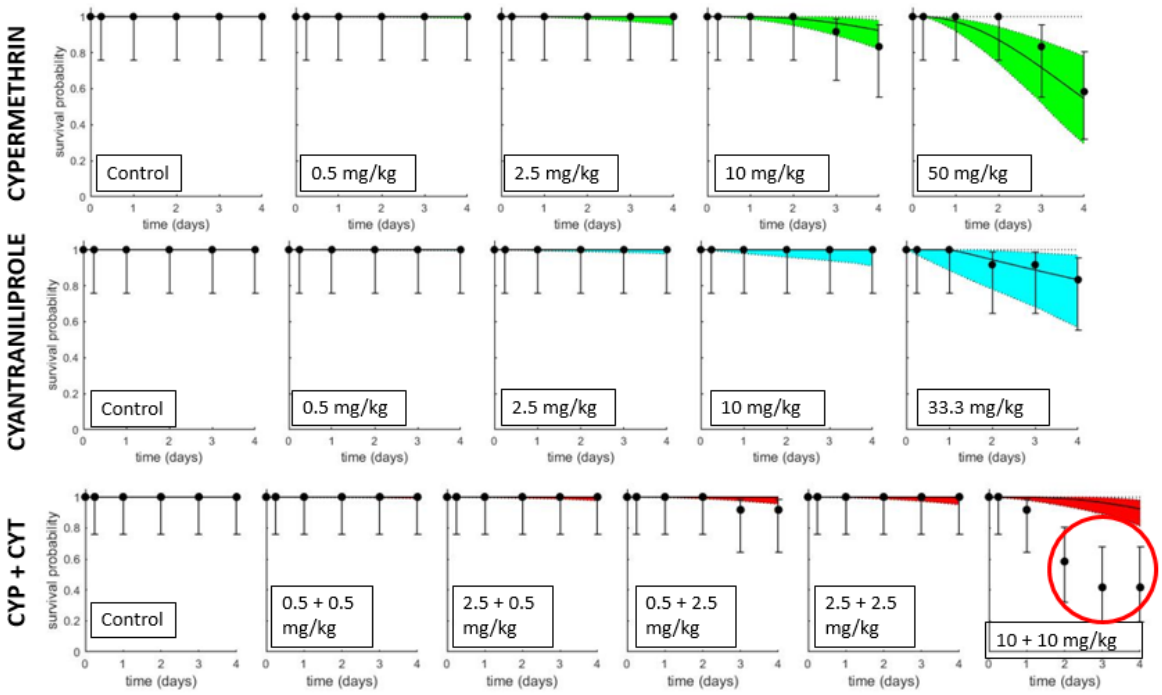


Figure 3.18. Observed and predicted survival over time of *Mamestra brassicae* exposed to a mixture cypermethrin and cyantraniliprole separately (first row of plots for cypermethrin, second is cyantraniliprole), and the mixture of both (third row of plots). Mixture effects are predicted with the GUTS-RED-SD independent action (IA) model calibrated on the results from the single exposures (first and second rows of plots). The dashed line is background mortality and solid line is simulated mortality. Synergy in the mixture is highlighted with a red ring in the 10 + 10 mg/kg cypermethrin and cyantraniliprole treatment.

Weight change measurements in the mixture bioassay (Figure 3.19), indicated similar control and cyantraniliprole exposed larvae growth to those in the single chemical tests. For cyantraniliprole, a significant effect of exposure on weight change was found. Tukey's test indicated that larvae exposed to cyantraniliprole single chemical treatments were significantly different from the controls. The mixture exposures indicated a concentration-dependent response to cyantraniliprole with increasing exposure concentration (GLM ($F(4)=83.1$, $p< 0.01$). Cypermethrin and the mixture of cypermethrin and cyantraniliprole had a significant effect on the relative weight change of the exposed larvae (cypermethrin ($F(4)=17.1$, $p< 0.01$, cypermethrin + cyantraniliprole $F(5)24.9$, $p< 0.01$). For example, larvae in the 0.5 mg/kg cyantraniliprole + 2.5 mg/kg of cypermethrin weighed significantly less than those exposed to 0.5 mg/kg of cypermethrin alone ($p<0.001$). This sublethal result implies

that cypermethrin and cyantraniliprole interact synergistically, as reflected also by the survival data.

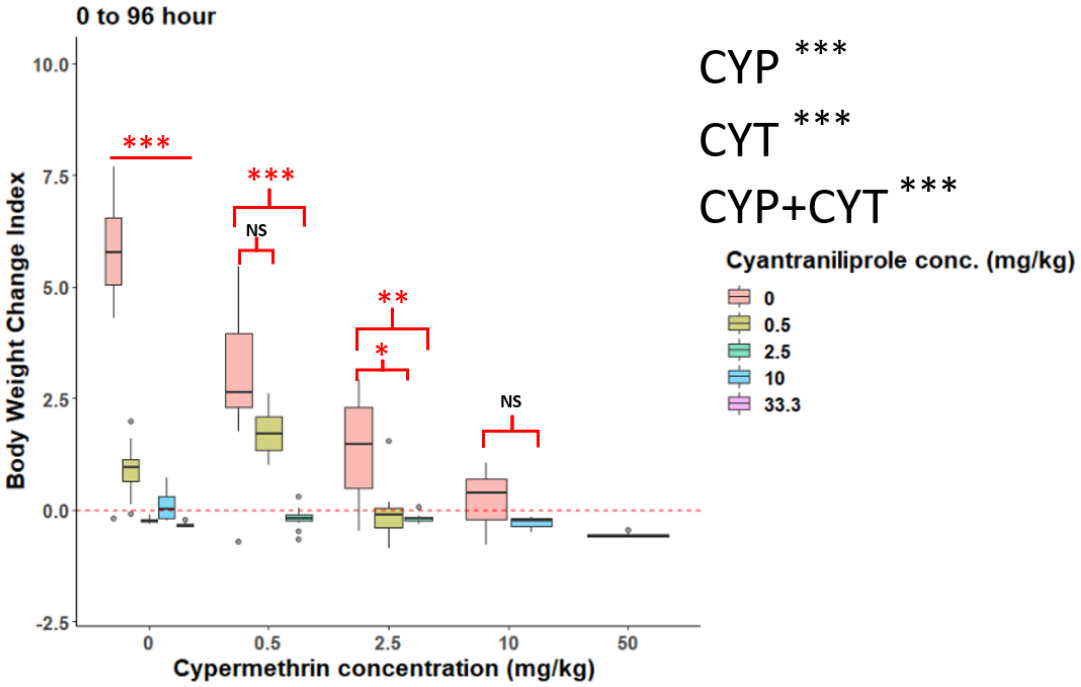


Figure 3.19. Relative weight change between 0 and 96 hours. Significance of cypermethrin, cyantraniliprole and the mixture of both on weight as determined by GLM are shown as '***' 0.001, '**' 0.01, '*' 0.05. No significance is denoted as NS. Line over 0 mg/kg of cypermethrin treatments indicates the weight of all cyantraniliprole-only exposed larvae is significantly less than control larvae. Pairwise Tukey HSD test highlighted significant differences in the weight of larvae in 0.5 and 2.5 mg/kg of cypermethrin treatments, red brackets highlight these differences.

3.3.2.9 Cyantraniliprole and prochloraz

In the cyantraniliprole single chemical exposure, total mortality could not be reached within maximum acetone solubility. Survival for this insecticide was slightly under predicted by the GUTS-RED-SD IA model (Figure 3.20). Prochloraz did not affect survival as consistent with single chemical testing, hence, no model was parametrised for this compound. For the mixture treatments, the GUTS-Mix model underpredicted larval survival in the 2.5 + 20, 10 +

20 and 10 + 50 mg/kg treatments. Therefore, this mixture is identified as being slightly synergistic, with this synergy more pronounced in the higher exposure level 10 + 20 mg/kg and 10 + 50 mg/kg mixture treatments.

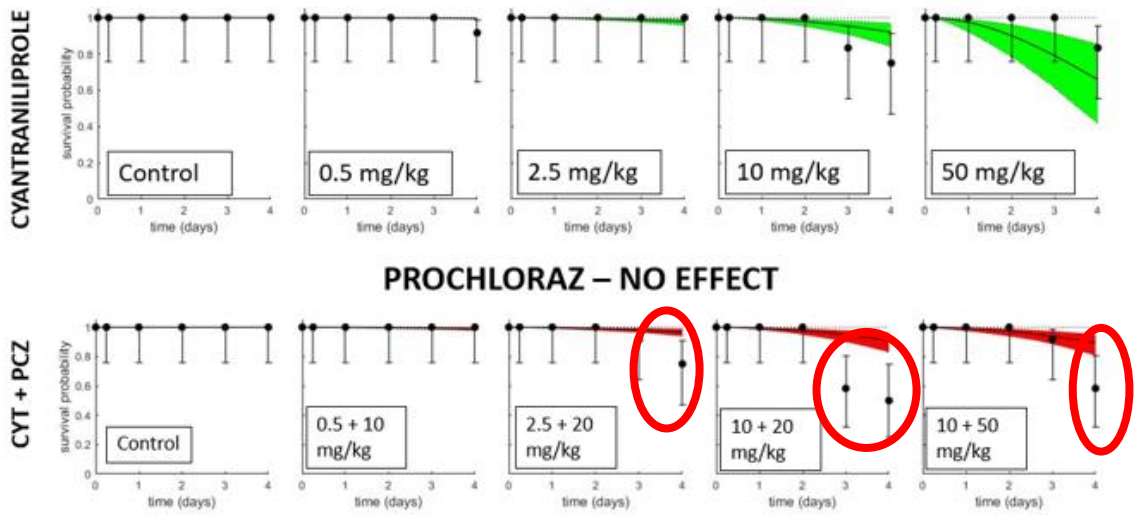


Figure 3.20. Observed and predicted survival over time of *Mamestra brassicae* exposed to a mixture of cyantraniliprole and prochloraz. separately (first row of plots for cyantraniliprole, prochloraz had no effect), and the mixture of both (second row of plots). Mixture effects (second row of plots) are predicted with the GUTS-RED-SD independent action (IA) calibrated on the results from the single exposures (first row of plots). The dashed line is background mortality and solid line is simulated mortality. Synergy in the mixture is highlighted with a red ring.

Control larvae showed relative weight increase between 0 and 96 hours (Figure 3.21). GLM showed that cyantraniliprole change ($F(4) = 60.5$ $p<0.001$), prochloraz ($F(4)=22.6$ $p<0.001$) and the mixture ($F(4)=22.8$ $p<0.001$) each had a significant effect on larval weight. Tukey's post hoc test was used to identify significant differences between treatments with the same concentration of cypermethrin but with different concentrations of added prochloraz. However, the only significant effects found were prochloraz treatments compared the control.

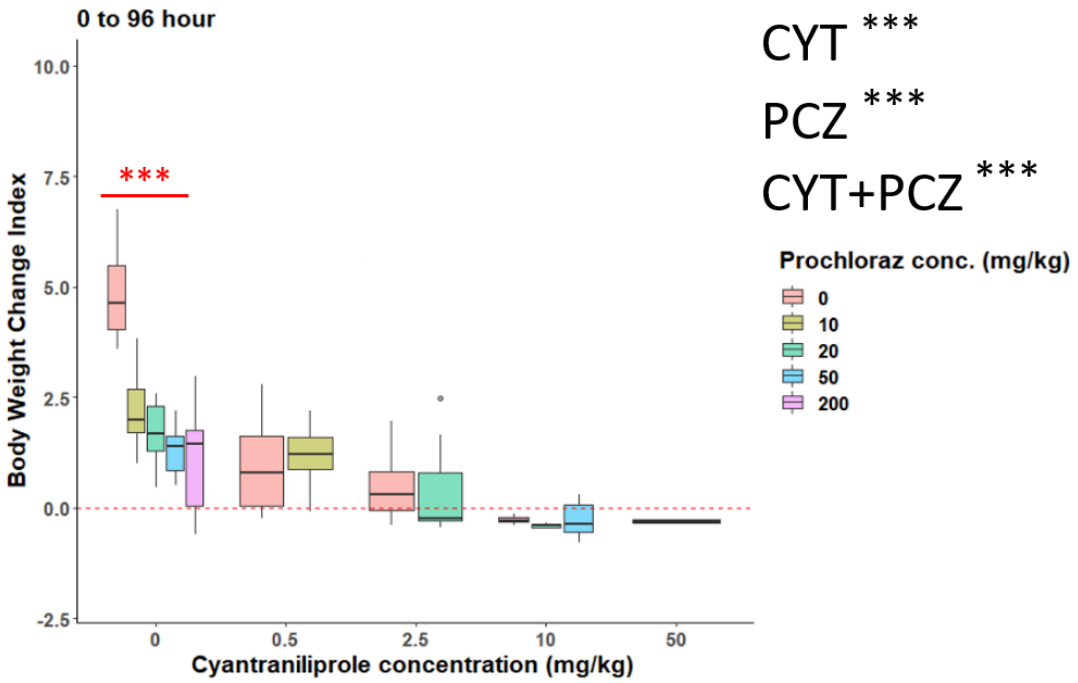


Figure 3.21. Relative weight change between 0 and 96 hours. Significance of cyantraniliprole, prochloraz and the mixture of both on weight as determined by GLM are shown as '***' 0.001, '**' 0.01, '*' 0.05. No significance is denoted as NS. Red line over 0 mg/kg of cyantraniliprole treatments indicates relative weight of all prochloraz single chemical exposed larvae is significantly less than control larvae.

3.3.2.10 Cyantraniliprole and azoxystrobin

The GUTS-RED-SD IA model survival fit for the cyantraniliprole single chemical exposure indicated a concentration-dependent effect of exposure on mortality, although full mortality was not reached within the maximum acetone solubility. Azoxystrobin did not affect survival, mirroring the single chemical testing, hence, a GUTS-RED-SD IA model could not be fitted for this chemical. Larvae exposed to a mixture of cyantraniliprole and azoxystrobin did not display any mortality until 96 hours in the 10 mg/kg + 20 mg/kg treatment (Figure 3.22.). Synergy was seen in the 10 mg/kg + 50 mg/kg treatment indicated by mortality greater than the GUTS-Mix model additive prediction. All other mixture treatments either had no mortality or were identified as additive, as observed effects were within the prediction window.

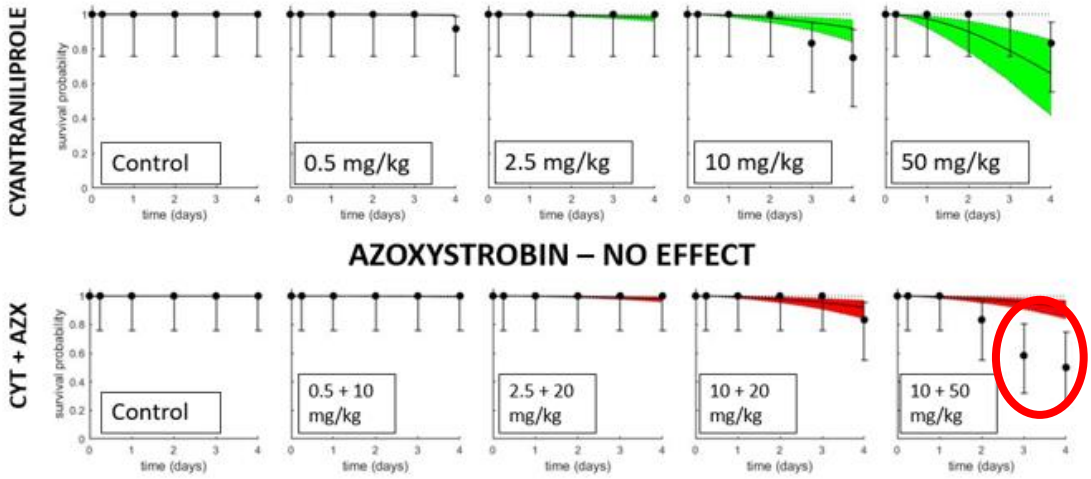


Figure 3.22. Observed and predicted survival over time of *Mamestra brassicae* exposed to cyantraniliprole and azoxystrobin in mixture separately (first row of plots for cyantraniliprole, azoxystrobin had no effect), and the mixture of both (second row of plots). Mixture effects are predicted with the GUTS-RED-SD independent action (IA). The predictions are simulated with the GUTS-RED-SD IA model calibrated on single exposures, with the stochastic death approach (first row). The second row is the mixture simulation. Synergy is seen only in the 10 + 50 mg/kg of cyantraniliprole and azoxystrobin and highlighted with a red ring.

Growth was seen in control and all azoxystrobin-only treatments consistent with the results of the single chemical studies (Figure 3.23). GLM highlighted the significant impact of azoxystrobin on relative weight change as seen in the single chemical bioassay ($F(4, 128)=9.84$, $p<0.001$) and the significant difference between relative weight of larvae exposed to cyantraniliprole compared the mixture of cyantraniliprole and azoxystrobin ($F(4, 128)=2.95$ $p<0.05$). Larvae in the 2.5, 10 and 50 mg/kg of cyantraniliprole single chemical treatments decreased in weight throughout the exposure; cyantraniliprole significantly impacted the weight of larvae ($F(4, 128)=88.8$ $p<0.001$). Tukey's post hoc analysis between cyantraniliprole and mixtures of azoxystrobin and the same concentration of cyantraniliprole indicated no significant interactions between cyantraniliprole and azoxystrobin on relative weight change.

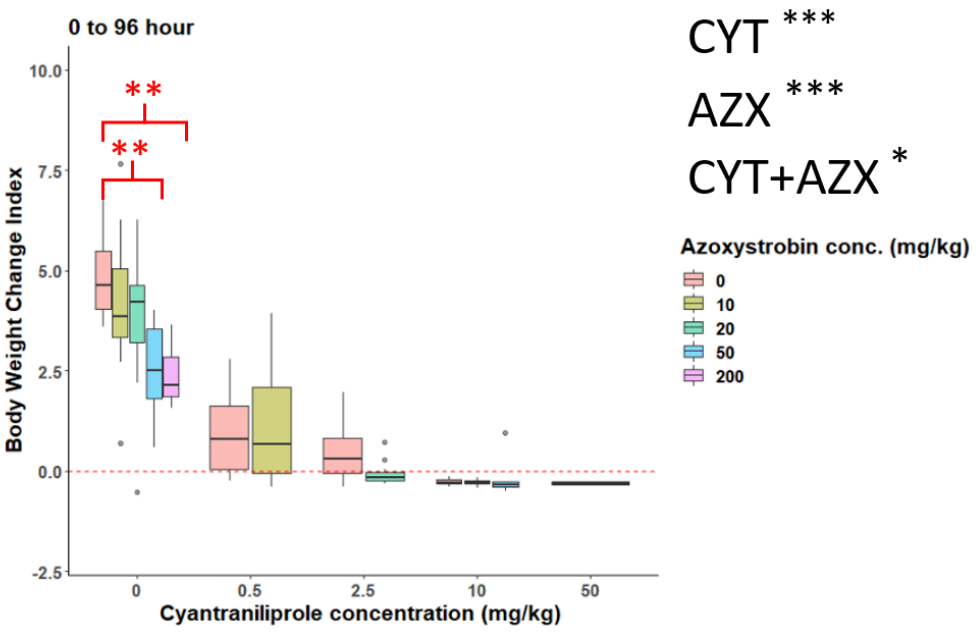


Figure 3.23 Relative weight change between 0 and 96 hours. Significance of cyantraniliprole, azoxystrobin and the mixture of both on weight as determined by GLM are shown as '0.001 ***' 0.01'**' 0.05 '*'. No significance is denoted as NS. Bracket in 0 mg/kg of cyantraniliprole treatments indicates weight of 50 and 200 mg/kg azoxystrobin exposed larvae was significantly less than control larvae.

3.3.2.11 Clothianidin and azoxystrobin

As for the single chemical azoxystrobin exposure, in this mixture bioassay, azoxystrobin exposure at the tested concentrations did not affect mortality. For clothianidin, a concentration-dependent mortality was observed, especially at the 100 mg/kg concentration that could be well described by the GUTS-RED-SD IA model. Within the mixture exposure, strong antagonistic interactions were seen, indicated by observed effects in the mixture is less than those predicted by the GUTS-RED-SD IA mixture model (Figure 3.24). This antagonism meant that while there was 58% mortality in larvae exposed to 100 mg/kg of clothianidin, when 100 mg/kg of clothianidin co-exposure occurred with 250 mg/kg of azoxystrobin only 9% mortality and no mortality was seen in the 100 mg/kg clothianidin treatment with 50 mg/kg azoxystrobin. No sublethal effects were measured for this mixture.

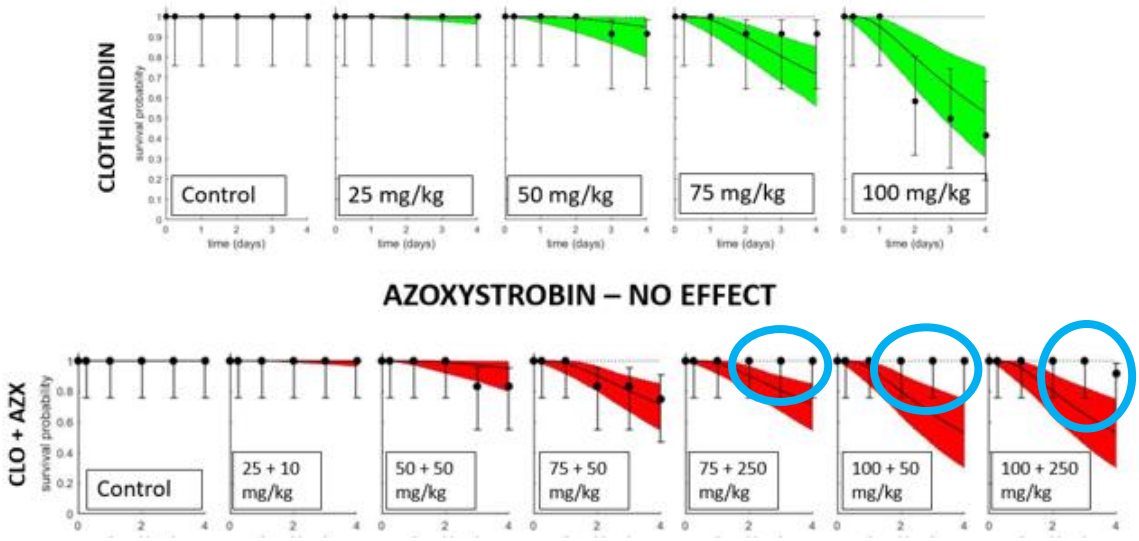


Figure 3.24. Observed and predicted survival over time of *Mamestra brassicae* exposed to a mixture of clothianidin and azoxystrobin in mixture separately (first row of plots for clothianidin, azoxystrobin had no effect), and the mixture of both (second row of plots). Mixture effects are predicted with the GUTS-RED-SD independent action (IA) calibrated on single exposures, with the stochastic death approach. Antagonism is indicated in the three highest concentration mixture exposures by a blue ring.

3.3.2.12 Clothianidin and prochloraz

Mortality of larvae exposed to clothianidin alone in the mixture study was non-monotonic, being 75% in the 75 mg/kg treatment, but only 17% in the 100 mg/kg treatment. These values were not well described by the GUTS-RED-SD IA model, hence observations for the mixture should be treated with some caution. No effect of prochloraz on survival was seen meaning that this chemical could not be modelled. For the mixture, survival was well predicted by the GUTS-RED-SD IA model in the mixture treatments indicating this mixture is predominantly additive with observed survival close to the simulated values. As the lack of interaction seen here was initially hypothesised, no sublethal data was collected from this mixture testing.

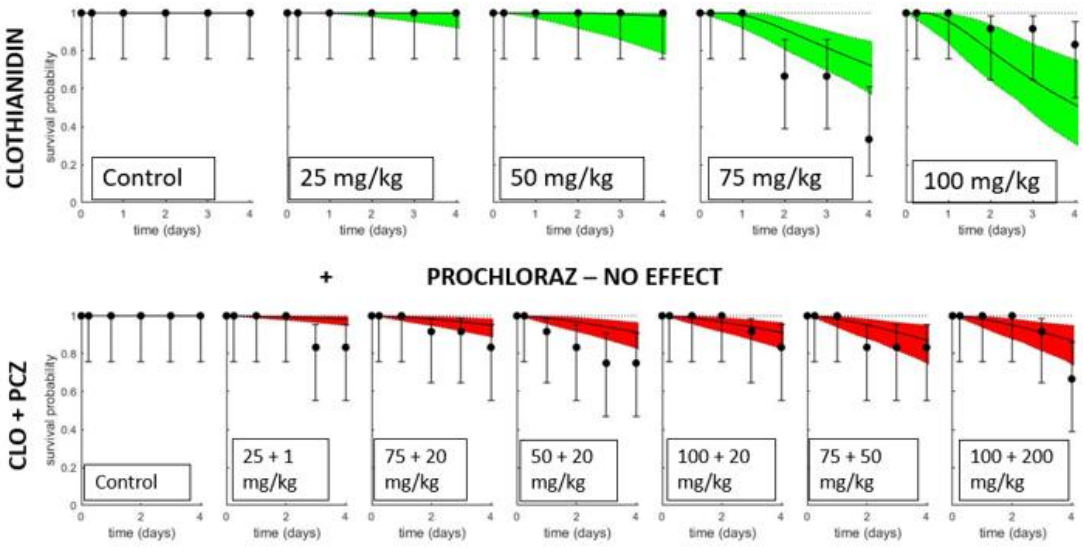


Figure 3.25. Observed and predicted survival over time of *Mamestra brassicae* exposed to a mixture of clothianidin and prochloraz separately (first row of plots for clothianidin, prochloraz had no effect), and the mixture of both (second row of plots). Mixture effects (second row of plots) are predicted with the GUTS-RED-SD independent action (IA). The predictions are simulated with the GUTS-RED-SD IA model calibrated on single exposures, with the stochastic death approach. In this mixture, no interactions were seen thus this mixture is identified as additive.

3.3.2.13 Summary

Of the 13 mixture combinations tested, seven were identified as synergistic for effect of (mainly mortality, but also in some cases relative weight change), three additive, one antagonistic and three could not be modelled using the GUTS-RED-SD IA model as only stochastic mortality occurred (Table 3.6). Of the seven synergistic mixtures, six were composed of an insecticide and fungicide and one was an insecticide and insecticide mixture. Four of the mixture involved cypermethrin and three cyantraniliprole. Every cyantraniliprole mixture reported synergistic interactions with another chemical, both fungicide and insecticide. Synergy was never identified in mixtures with a neonicotinoid insecticide; however, antagonism was reported in the mixture of clothianidin and azoxystrobin.

Table 3.6 Summary of mixture bioassay results. Results are classed as synergistic, antagonistic, additive or not modelled based on analysed using the GUTS-RED-SD IA mixture model. Sublethal effects were determined by GLM and here only the mixture data is presented.

Chemical A	Chemical B	Result	Survival Synergy	Sublethal Synergism
Imidacloprid	Azoxystrobin	Stochastic mortality - no model	Stochastic death up to 100 mg/kg imidacloprid	Not analysed
Imidacloprid	Prochloraz	Stochastic mortality - no model	Stochastic up to 75 mg/kg imidacloprid	Not analysed
Prochloraz	Azoxystrobin	No mortality	No mortality up to 200 mg/kg both chemicals	Not analysed
Cypermethrin	Prochloraz	Weak synergy	Synergy in middle range concentrations - between 2.5 mg/kg and 10 mg/kg cypermethrin	Significant effect on relative weight change $p<0.001$
Cypermethrin	Azoxystrobin	Additivity	No synergy seen at any concentration range	No significant effect on relative weight change
Cypermethrin	Chlorothalonil	Synergy	Synergy in 10 + 50 mg/kg treatment only	No significant effect on relative weight change
Cypermethrin	Propiconazole	Synergy	Synergy in 10 + 50 mg/kg treatment only	No significant effect on relative weight change

Cypermethrin	Chlorpyrifos	Additivity	Mild synergism at 2.5 + 1 mg/kg. Antagonism in 2.5 + 10 and 10 + 20 mg/kg treatments - not well characterised	No significant effect on relative weight change
Cypermethrin	Cyantraniliprole	Synergy	Synergy in highest tested concentration (10 + 10 mg/kg). Sublethal synergy seen in all but 50 + 10 mg/kg treatment	Significant effect on relative weight change $p<0.001$
Cyantraniliprole	Prochloraz	Slight synergy	Synergy in all mixture treatments above 2.5 mg/kg of cyantraniliprole.	Significant effect on relative weight change $p<0.001$
Cyantraniliprole	Azoxystrobin	Synergy	Synergy in highest tested concentration (10 + 50 mg/kg)	Significant effect on relative weight change $p<0.05$
Clothianidin	Azoxystrobin	Antagonism	Antagonism seen in top three treatments, 75 + 250, 100 + 50 and 100 + 250 mg/kg	Not analysed
Clothianidin	Prochloraz	Additivity	Mixture well characterised. Additive in every concentration tested except 50 + 20 mg/kg	Not analysed

3.4.0. Discussion

Pesticide applications expose organisms to mixtures of chemicals with a range of modes of actions targeting a variety of receptors and metabolic pathways that when perturbed can lead to toxic effects. The sensitivity of organisms to these agrochemicals can vary depending on the mode of action and the specific species exposed. In this chapter, sensitivity of *M. brassicae* larvae to a range of pesticides (insecticides and fungicides) representing pesticide classes with differing mode of actions was assessed. As hypothesised, fungicides did not effect larval mortality but in some instances (propiconazole and azoxystrobin) growth was impaired with increasing concentrations.

Of the 5 insecticides tested, the pyrethroid cypermethrin was the most toxic with an LC_{50} of 46.3 mg/kg at 48 hours and 11.6 mg/kg at 96 hours. The high potency of this insecticide to *M. brassicae* is consistent with findings of effects of other pyrethroids on Lepidoptera. For example, deltamethrin (another pyrethroid) was shown to be acutely toxicity to the Codling moth (*Cydia pomonella*) (Bouvier et al. 2002). An LC_{50} for a susceptible population of 4th instar moths of 9.89 mg/L was found after a 96-hour exposure which is consistent with our finding here for cypermethrin.

Within the bioassay and confirmed by the GUTS-RED-SD model, cypermethrin had a progressive, but slow, effect on survival in time. Thus, the majority of deaths on exposure to this chemical did not occur until at least 48 hours into the experiment. This pattern of delayed response can occur when either the internal steady state of the accumulated chemical is not achieved within the test duration due to slow toxicokinetics, or when an internal toxicokinetic steady state is reached before the first survival point, whereafter there is slow toxicodynamic damage leading to gradually increasing effects on survival, or (Jager and Ashauer 2018). Among the TKTD parameters for the GUTS-RED-SD, the dominant rate constant (k_d) value was low for cypermethrin,0.005 d^{-1}. K_d represents either the elimination rate k_e, or the damage repair rate k_r, or the combined effect of these two processes. Therefore, the very low k_d value for cypermethrin indicates either a very slow elimination of cypermethrin (via excretion or metabolisation), or a slow damage repair (TD), or slow rates for both. Slow toxicokinetics have been previously reported in studies of pyrethroids

(Kretschmann et al. 2015), indicating slow kinetics (i.e., elimination rate) may be the dominant process causing the delayed mortality as seen here.

Cypermethrin had a significant effect on relative body weight change index in all concentrations tested (>1.1 mg/kg). Although growth was reduced compared to controls, all treatments gained weight except in the 40 mg/kg treatment in some studies. Positive weight increase at lower concentrations suggests that the larvae continued to feed throughout the 96-hour exposure, meaning that not all effects may be attributed to antifeedant effects as reported previously for cypermethrin (Keng-Hong 1981; Pengsook et al. 2022). Instead, some sublethal effects on resource allocation that affect growth may also occur. The overall consequences of reduced growth and their impacts on development and pupation on fitness would require further extended study of impacts over the full development time. Such studies could be expanded upon further in the context of dynamic energy budgets as energy is diverted from the growth of larvae into the cell and tissue repair (Baas et al. 2010).

The initial hypothesis was that all insecticides tested would be toxic to *M. brassicae* larvae. This was not, however, the case; Of the three insecticides that bind to the nAChR (imidacloprid, clothianidin and sulfoxaflor), only clothianidin was found to cause greater than 50% mortality at the highest tested concentration (100 mg/kg). Further, even for this neonicotinoid, only partial mortality was seen and then only at concentrations near the maximum solubility of the compound. Imidacloprid resulted in only 25% mortality after 96 h at the highest tested concentration (150 mg/kg) and significant effects on growth were recorded at concentrations of 85.1 mg/kg and above. For sulfoxaflor, no acute or sublethal effects were seen at any concentration tested up to 65 mg/kg. Variation in toxicity between neonicotinoids has been previously documented (Funayama and Ohsumi 2007) and the results obtained from these bioassays are consistent with that finding.

The relatively limited effects from the two neonicotinoids and absence of any sublethal or acute effect arising from sulfoxaflor exposure was largely unexpected. Clothianidin is recommended for lepidopteran pests including those from the *Noctuidae* family, such as *M. brassicae* (Uneme 2011). Applications of clothianidin are thought to be effective against Lepidoptera, even at low doses, suggesting that an effect would have been expected in contrast to the finding from the exposure conducted. There have been numerous previous studies of the effects of neonicotinoid insecticides on insects, including lepidopterans.

Compared to the large majority of these previous analyses, the LC_{50} values found here are generally higher. For example, this LC_{50} values of 194 and 54.6 mg/kg at 48 and 96 hours for clothianidin exceed any values reported before for a lepidopteran for any neonicotinoid. This suggests either bioavailability of neonicotinoids in the artificial diet is low or that *M. brassicae* are generally tolerant to neonicotinoid exposure through a species specific mechanism.

Sulfoxaflor is a sulfoximine insecticide. However, its mode of action is as a competitive modulator of the nicotinic acetylcholine receptor, the same target as for the neonicotinoids (Cutler et al. 2013; Simon-Delso et al. 2015). Previous research has suggested that sulfoxaflor can be equally toxic as imidacloprid for many non-target invertebrate species including earthworms and bees (Fang et al. 2018; Zhang et al. 2020; Yang et al. 2021). Sulfoxaflor is, however, not recommended for the control of lepidopteran and coleopteran pests (Corteva agriscience 2019). On this basis, it was expected that sublethal and acute results would be seen in clothianidin exposure, but it was unclear if sulfoxaflor would have any effect on larvae. Finding here of low toxicity for this compound suggest that it has a lower potential to interact with the nAChR to cause toxic effects.

The low toxicity observed for the two neonicotinoids and sulfoxaflor could be related to the toxicokinetics of these compounds, as governed by their ADME (Absorption, Distribution, Metabolism, Excretion) pathways. Many studies have highlighted the toxicity of neonicotinoids to aquatic invertebrates and phloem-feeding pests over other insect classes (Stamm et al. 2011; Uneme 2011; Cavallaro et al. 2017). Imidacloprid in particular is said to have a particularly high toxicity for aquatic species after entry into water courses. Presence in surface waters may result in a range of exposure through gills, the body wall and ingestion. Thus, such exposures are more complex than the mainly oral exposure achieved with the bioassay method used here. On feeding exposure, the tolerance shown by the *M. brassicae* larvae may indicate that when exposed in diet, these three chemicals are not readily taken up by the feeding caterpillars.

Toxicodynamic traits of *M. brassicae* may also explain the tolerance to neonicotinoids; in particular the potential of the compound to bind to the known target receptor. Imidacloprid is a partial agonist of nAchR subunits (Déglise et al. 2002; Brown et al. 2006; Cartereau et al. 2021). In other words, imidacloprid can bind to the nAchR, but only has a partial effect on

the receptor operation. However, clothianidin is a super-agonist and therefore, has a higher affinity for insect nAchRs (Brown et al. 2006). This could explain the higher mortality of larvae exposed to clothianidin in this test, but also similar findings of clothianidin toxicity exceeding that of imidacloprid in other studies (Stamm et al. 2011; Dai et al. 2019). Furthermore, neonicotinoids are thought to bind to multiple sites on membranes of neural tissues in various insect species (Simon-Delso et al. 2015). Such off-target binding could potentially mitigate the uptake of insecticide solely to the nervous system (Short et al. 2021). Further research on the expression and binding of nAchR proteins would be needed to confirm if this is the case in *M. brassicae* but it may perhaps explain the low sensitivity seen in the results of this chapter.

A further potential cause of the relatively low toxicity observed for the two neonicotinoids and sulfoxaflor may be the relatively short (96-h) duration of the bioassay. Studies of bees have found that chemical effects on mortality can increase when exposed past 96 hours (Suchail et al. 2001; Robinson et al. 2017). Therefore, a longer-term bioassay conducted for the two neonicotinoids and sulfoxaflor may have identified effects at lower concentrations. From the TKTD modelling, no kinetic information could be reliably calculated for either chemical due to the weak model fits because of the limited survival effects. The parameter m_w, which represents the NEC for clothianidin was 4.83 mg/kg, over 3000 times higher than that of imidacloprid. This higher value for clothianidin was because the stochastic mortality seen at lower concentrations of imidacloprid results in a low, and likely unrealistic, value for this chemical, compared to clothianidin for which deaths were monotonic.

The diamide insecticide cyantraniliprole has been previously reported to exert a toxic effect on lepidopteran species. However, at the highest concentration of cyantraniliprole (50 mg/kg) tested here, only 16.7% mortality was recorded. This low mortality meant that an LC_{50} could not be calculated for this chemical. The relatively low sensitivity of *M. brassicae* to cyantraniliprole, as indicated, differs from the results from previous studies. Thus, tests using Lepidopteran larvae of a similar size and instar found the LC_{50} of the oriental tobacco budworm (*Helicoverpa assulta*) 0.176 mg/L at 72 hours (Dong et al. 2017), indicating a >280-fold potential difference in sensitivity between this species and *M. brassicae*. However, further work is needed to determine whether this variation is the result of true differences between species or due to differences in bioavailability as a result of the different bioassay methods used in each study.

Although insensitive for effects on mortality, the EC_{50} for the effects of cyantraniliprole on growth at 0.96 mg/kg was the lowest of any tested insecticide. Sensitivity for this endpoint, is, thus more consistent with the known effects of this insecticide on lepidoptera. Cyantraniliprole was the only chemical to cause weight loss at all exposure concentrations and in almost every individual. Given this common effect of cyantraniliprole, one potential cause of weight loss could be through an antifeedant effect. This would be consistent with previous findings that cyantraniliprole significantly impairs consumption, efficiency of food ingestion, digestion, lipid and carbohydrate energy reserves and growth rates in the lepidopteran *Ostrinia furnacalis* (Xu et al. 2016; Xu et al. 2017). The inability to obtain nutrients from the diet resulted in reduced larval weight and prolonged larval duration in these studies, which reflects the weight loss seen in this bioassay exposure. From the bioassay sublethal data, it is likely that if exposure tests had continued mortality in all treatments would have been expected as weight loss continued to increase.

In agreement with the prior hypothesis, none of the four fungicides azoxystrobin, chlorothalonil, prochloraz or propiconazole affected *M. brassicae* survival at any concentrations tested. The survival data for all fungicides could, therefore, not be modelled with the GUTS-RED-SD model. Two fungicides, azoxystrobin and propiconazole did have a significant effect on relative weight change. Previous studies have demonstrated that azoxystrobin exposure can have a negative effect on lepidopteran species. For example, El-Kholy et al (2018) found azoxystrobin exposure reduced feeding rates and larval growth of the cotton leaf worm (*Spodoptera littoralis*), Not all studies with insects have, however, found and effect of azoxystrobin. For example, studies on survival of insidious flower bug (*Orius insidiosus)* nymphs noted had no effect of azoxystrobin (Rocha et al. 2006; Herrick and Cloyd 2017) and a study of adult rove beetles (*Atheta coriaria*) also reported no survival or feeding effects (Cloyd et al. 2009).

3.4.1 Additive Mixtures

Additivity is seen as the null hypothesis for mixture effects. It is based on the idea that chemicals exert their effects independently, but that this effect can be added in terms either of combined ratios of exposure concentration and effect potency (concentration addition) or as the product of null response (independent action). In the GUTS mixture analysis, additive effects according to the mechanistic assumptions of these simple approaches can

be modelled as a prediction of the joint effects of the two chemicals based on their respective single chemical TK and TD parameters. From this joint effect modelling, a prediction of mortality with 95% CIs over the exposure duration can be derived. The model predictions can then be used to appraise if the actual mixture effects accord with these assumptions of additivity based on whether the observed data lie within the model prediction window.

Three mixtures had no impact on survival: imidacloprid and azoxystrobin, imidacloprid and prochloraz and azoxystrobin and prochloraz, reflecting previous results that no effects were associated with the single exposures. As a result, these mixtures could not be analysed using the GUTS mixture modelling approach because no model could be calibrated based on the single exposures and, thus, no prediction could be made. That azoxystrobin and prochloraz had no effect in combination was unsurprising seeing that neither chemical had an effect on mortality in single chemical testing. The absence of any effect when these chemicals are exposed in combination does not allow any understanding of their joint effect, other than that co-exposure does not cause synergism that results in mortality that would not be predicted from the absence of effects for their singe chemical. The mixture of imidacloprid and azoxystrobin also did not show synergism as both imidacloprid and azoxystrobin did not cause a mortality effect as a single chemical and at the highest concentration used in the mixture (75 mg/kg). Here again the absence of any effect on survival points to an absence of synergism for this mixture.

The mixture of imidacloprid and prochloraz did not exhibit any effects on mortality and a second tested neonicotinoid azole fungicide mixture of clothianidin and prochloraz was also shown to be additive. The absence of any evidence for synergism in these studies, which both include an azole is counter to our prior hypothesis that mixtures that include this class of fungicide may show synergistic interactions. Azole fungicides such as prochloraz are known to inhibit phase I metabolism (Haas and Nauen 2021). Therefore, it would be hypothesised that such inhibition could lead to greater mortality in a mixture by targeting the metabolic pathways that detoxify the co-exposed substance. Imidacloprid in particular has been reported to have no synergistic interactions with fungicides (Iwasa et al. 2004; Thompson et al. 2014; Raimets et al. 2018), indicating that this mechanism of interaction may not be as relevant for this insecticide as for other insecticides, such as cypermethrin. Further investigation of pesticide metabolism would be needed to examine the extent to which prochloraz exposure affects neonicotinoid breakdown.

A further combination showing a dominant additive response was the mixture of the insecticide cypermethrin and fungicide azoxystrobin. This combination was the only mixture of a pyrethroid and fungicide in which synergy was not identified by the GUTS mixture model. Unlike the azole fungicides, prochloraz and propiconazole, there is no clear mechanistic hypothesis that would support a direct effect of the strobilurin fungicide azoxystrobin on any aspect of known phase I, II, or III cypermethrin metabolism. Instead, the main mechanisms of effect of azoxystrobin are thought to be by disrupting electron transfer and, therefore, mitochondrial respiration (Kumar et al. 2020; Li et al. 2021). As such, azoxystrobin may not interact synergistically because there is no competition of metabolic enzymes. However, it is possible that the metabolic effects of exposure to this fungicide may cause changes in energy dynamics that could affect responses to other chemicals.

3.4.2 Synergistic Mixtures

Synergistic interactions in mixtures were identified by cases of under-prediction of mortality by the GUTS-RED-SD IA model parameterised as an additive effect based on the single chemical responses. The identification of synergism using this approach does not provide a quantifiable prediction, e.g., it is not accurate to say that interactions between chemical A and chemical B showed a stronger synergistic effect than chemical B and chemical C, as in this model, predictions cannot be compared via a formal statistical test. However, using this approach it is possible to identify synergistic interactions between chemicals using the model as a predictive tool to compare to the observed data (considering confidence intervals) to assess whether actual effects concord with predictions.

In total, 6 of the 13 mixtures tested showed evidence of synergism according to the criteria of the underprediction of the observed effects by the GUTS-RED-SD IA mixture model assuming additivity. Evidence for synergism was found for: cypermethrin and chlorothalonil, cypermethrin and prochloraz, cypermethrin and propiconazole, cypermethrin and cyantraniliprole, cyantraniliprole and prochloraz and cyantraniliprole and azoxystrobin. As hypothesised, 4 of 6 of the synergistic mixtures included the pyrethroid cypermethrin. There was only one case, when exposed jointly with cyantraniliprole, where this synergism occurred when both chemicals had an effect on survival at the concentrations tested. Both of these two insecticides act on targets in the insect nervous systems, although different receptors. The primary mechanism of cypermethrin effects occurs through targeting the

voltage gated sodium channel, while cyantraniliprole acts on the ryanodine receptor. That both of these insecticides target the same tissue type (i.e nerves), potentially indicates a route of synergistic interaction. However, in addition to these main mechanisms of action, both insecticides also affect calcium metabolism. Cypermethrin can also inhibit calcium regulation (Glavan and Božič 2013), while cyantraniliprole is also responsible for uncontrollable release of calcium ions (Grávalos et al. 2015). This common effect on calcium regulation would be expected to result in the null hypothesis of additivity, however, there may be potential TK mechanisms that lead to a synergistic interaction. For example, cyantraniliprole exposure may deplete calcium in the neural cells and this cannot be replaced due to the presence of cypermethrin inhibiting calcium regulation.

In the other five mixtures showing evidence of synergism, in all cases, one of the compounds had no direct effect on survival alone, but instead acted as a potentiator by enhancing the toxicity of the co-exposed chemical. In each case, a fungicide acted as the potentiator for an insecticide. Azole fungicides accounted for 4 of these 6 cases, with the remaining one being for azoxystrobin and chlorothalonil. Synergistic interactions between pyrethroids and azole fungicides have been reported in many studies (Cedergreen et al. 2006; Cedergreen 2014; Kretschmann et al. 2015; Rösch et al. 2017). For this reason, before initial screening for synergism, it was hypothesised that synergistic interactions would occur between pyrethroids and azoles. This was confirmed to be the case for mixture containing cypermethrin and cyantraniliprole, although not those with either neonicotinoid. Hence, while synergism due on co-exposure with azole fungicides seem common, such response patterns are not ubiquitous for all chemicals.

A pattern seen in a number of the mixture experiments was of synergism most often present in the middle concentration range in the mixtures, when concentrations are compared to the single chemical exposures (i.e. concentrations that caused over 70% mortality in single chemical testing were not used in mixture exposures as any incidences of synergy would be difficult to model). This pattern was seen for cypermethrin and prochloraz and cyantraniliprole and prochloraz and was also seen in studies of the annelid *E. crypticus* and collembolan *F. candida* (personal communication, Robinson and Eagles 2022). This is potentially because at middle range concentrations, processes are disrupted and may result in mortality. Whereas at higher range concentrations, functions of one or many systems of the organism may be impacted, causing effects of inhibition to be overlooked by mortality

testing alone. However, in the present study mechanisms behind this are unknown and would warrant becoming a topic for future study.

The mechanism of synergism for azole fungicides are likely to be linked to their known potential to inhibit the action of the metabolic enzymes *CYP450s* (Cedergreen 2014; Rösch et al. 2017; Haas et al. 2021; Haas et al. 2022). Such inhibition can enhance the toxic effect of pyrethroid insecticides, as the organism cannot metabolise and detoxify the insecticide through the usual repertoire of biotransformation reactions. Prochloraz is reported to be a powerful synergist showing up to four times greater synergistic potential than other azoles such as propiconazole when co-exposed with a pyrethroid insecticide (Kretschmann et al. 2015). As prochloraz was found to have a significant effect on larvae growth in comparison to the control treatment in almost all of the mixture tests, this indicates a high degree of exposure to this chemical likely to underpin the synergistic effects seen.

A synergistic interaction with cypermethrin was also identified when co-exposed with the fungicide chlorothalonil. Synergy between these chemicals has not been previously documented, although synergy has been seen between chlorothalonil and the herbicide atrazine (DeLorenzo and Serrano 2003). Chlorothalonil may have the potential to inhibit the activities of phase II metabolic enzymes, through its effects on glutathione metabolism. An effect of chlorothalonil on metabolism of *M. brassicae* larvae was demonstrated by the sublethal effect seen in the single chemical bioassays. The synergism seen in the mixture bioassays may be the result of chlorothalonil reducing glutathione, which may in turn reduce the activities of glutathione-s-transferase enzyme systems (Song et al. 2017). However, confirming of this inhibition would require further investigation of enzymatic activity within the organism.

The strobilurin fungicide, azoxystrobin, when combined with cyantraniliprole, was also found to have synergistic effects as indicated by the under prediction of observed mortality by the additive mixture model. Indeed, cyantraniliprole had a synergistic interaction with every chemical with which it was tested (e.g. with cypermethrin, prochloraz and azoxystrobin). Hence, the ryanoid seems especially vulnerable to synergistic effects through a range of potential mechanisms. In the case of azoxystrobin, it is possible that the fungicide acts to restricts the stasis of larval physiological systems by inducing oxidative stress by reducing mitochondrial function and that this change in metabolic status in turn affect cyantraniliprole

toxicokinetics or toxicodynamics. Such an interaction has previously been reported in zebrafish (Kumar et al. 2020). The results seen here in both single and mixture bioassays points towards the hypothesis that cyantraniliprole has such a multifaceted physiological effect on larvae that any further inhibition of metabolism is sufficient to cause increased mortality beyond that expected due to non-interacting additive effects. It is possible that for this reason that cyantraniliprole is synergised by a range of chemical with different physiological effects, rather than through any specific mechanisms such as by the inhibition of biotransformation enzymes systems.

3.4.3 Antagonistic Mixtures

Antagonistic interactions have a less than additive effect on the target organism. Therefore, antagonism is not seen as an issue in risk assessment, since expectations are of reduced effects (Hernández *et al.* 2013). Antagonism can be seen between chemicals that share the same mode of action. This is possible, for example in those case where one chemical acts to block the target site interactions for another substance, thereby, preventing binding with target receptors (El-Masri, 2004). As a specific known example, exposure to organophosphate insecticides is known to result in antagonism when exposed in combination with chemicals that inhibit the action of voltage-gated calcium channels (VGSCs). Indeed, such cases account for 29% of previously reported antagonistic mixtures (Belden *et al.* 2007). The mechanism behind this effect is linked to the potential of the organophosphate in interacting with this target for other insecticides (Meijer et al. 2014). Alternatively, antagonism can occur when one chemical stimulates the metabolism of another (Hernández *et al.* 2017). For example, cadmium may accelerate hepatic degradation of chlorpyrifos, although the mechanism for this is so far unknown (Xu *et al.* 2017)

The strongest evidence for antagonism seen in the current study was observed for the mixture of clothianidin and azoxystrobin. Antagonism has been previously reported between azoxystrobin and thiamethoxam; of which clothianidin is a metabolite (Yang et al. 2021). It was assumed from this study that thiamethoxam triggered detoxification of isozymes, leading to antagonism. Thus, in this case rather than the primarily toxicodynamic mechanism

proposed for organophosphates, a primarily toxicokinetic mechanism is instead suggested. However, as is the case with all of the mixtures presented, without further insight into the molecular biology of the organism, it is impossible to determine the root cause of synergism or antagonism.

3.5.0 Conclusion

The increasing range of pesticide uses gives increasing cause for concern for the effects of mixtures of these active ingredients on populations of beneficial and non-target organisms. Although targeted pest management strategies like insecticide applications are effective against insect populations, it should also be recognised that fungicides too have sublethal effects on beneficial insect growth and fecundity. Thus, although crop yield may increase as pests are destroyed, pollinator populations may be affected which in turn in damage the agricultural economy.

Here, the bioassay developed in Chapter 2 was used to test the toxicity of a wide range of insecticides and fungicides, representing several modes of action on *M. brassicae* larvae. The single chemical bioassays demonstrated that, as hypothesised, insecticides were the only chemicals to have acute effects on the mortality of exposed larvae. Full mortality was, however, never reached for any single chemical, except for cypermethrin. Further, the neonicotinoid imidacloprid was ineffective at killing lepidopteran larvae, despite having a significant effect on growth. Further work to study bioavailability and mechanism of action would be needed to attribute these sensitivities to exposure within the assay or to the physiology of the test species.

Table 3.7 Summary of mixture exposure results. Mixtures labelled no model did not record mortality high enough for the model to predict any chemical interactions. This summary relates only to mortality.

Mixture	Result
Cypermethrin + Prochloraz	Synergy
Cyantroprilinol + Prochloraz	Synergy
Azoxystrobin + Prochloraz	No mortality
Imidacloprid/clothinidin + Prochloraz	No model for either
Cypermethrin + Propiconazole	Synergy
Chlorpyrifos + Cypermethrin	Additive
Cyantroprilinol + Azoxystrobin	Synergy
Cypermethrin + Azoxystrobin	Additive
Imidacloprid + Azoystrobin	No model
Cyantraniliprole + Cypermethrin	Synergy
Cypermethrin + Chlorothalonil	Synergy
Imidacloprid + Azoystrobin	Antagonism

The developed bioassay was used to assess the effects of binary mixtures of insecticides and fungicides in several combinations by comparing observed results against additive model predictions. Mixture testing successfully identified 6 cases of synergism (Table 3.7) . These included the combinations of azole fungicides and pyrethroids for which synergism has been previously observed, but also pairings for which synergism was previously undocumented, such as cypermethrin and chlorothalonil and cyantraniliprole and azoxystrobin. Such synergistic interactions of pyrethroid insecticides and the fungicides were likely to be caused by inhibition of one or more types of detoxification enzymes such as *GSTs* or *CYP450s* (Berenbaum, 2015; Liu 2017). The anthranilic diamide insecticide, cyantraniliprole, was synergistic in every tested combination. When paired with the growth data, this points to the conclusion that this chemical has widespread physiological consequences for exposed organism and any co-exposure alongside this pesticide that affects major aspects of metabolism could further accentuate such effects.

No case of synergism was found for in any mixture involving a neonicotinoid insecticide, including in exposures with prochloraz and azoxystrobin. A mixture of clothianidin and azoxystrobin was tested, but the results were indicative of strong antagonism.

Although the bioassay and statistical analysis formed a robust framework for highlighting cases of synergy in Lepidoptera, the model was somewhat hindered by the slow or uncharacterised kinetics of all insecticides used and low mortality for the majority of the chemicals. In pairings of fungicides and insecticides, invariably one chemical did not affect mortality and therefore, model predictions could operate solely on the insecticide survival. However, in other cases it is recommended that further research is undertaken into the long-term effects of exposure to pesticide mixtures, as the 96-hour bioassay was not indicative of field-relevant scenarios. Despite these limitations, survival of several mixture scenarios was predicted and verified by tracking joint effects over time. Such findings are testament to the design of the assay and modelling platform which allows the identification of such effects. Therefore, with modifications, this method could be suitable for risk assessment of joint toxicity of chemicals in mixtures and could potentially be applied to survival data of many lepidopteran species.

CHAPTER 4 - MIXTURE EFFECTS ON GROWTH ANALYSED WITH DEB-TKTD MODELLING

4.1. Introduction

Laboratory toxicity testing is often criticised for simulating exposure scenarios not applicable to the field situation. For example, in the field, pesticide exposure can occur over much longer timescales than the usual length of bioassay tests. Real word exposures may occur over the whole lifetime or life stage period of many species. An additional issue for environmental realism that that toxicity testing approaches often focus on dose-response analysis, focusing only on a single time point, which is not a true representation of exposure, as toxicity is a process in time (Baas et al. 2010). TKTD models such as GUTS, as used in previous chapters have been shown to successfully model the mortality of organisms with an added temporal dimension. While valuable for understanding the dynamics of survival effect, GUTS cannot simulate sublethal effects of chemicals that can also affect population viability.

While measuring the lethal toxicity of a chemical provides useful information to calculate risk from exposure, sublethal effects on parameters can also contribute substantially to the population level effects of chemical exposures as they are more sensitive than survival data alone. A common sublethal endpoint reported in risk assessment is reproduction, given that fecundity is a major contributor to population fitness (Barascou et al. 2021). However, as with lethal toxicity testing, effects on this parameter are often measured as reproductive outputs at a single time point, and do not take into account growth and maturation of the organism and the effects this has on fecundity within a population. Effects on growth and development may have severe implications for population dynamics in wild populations. For example, in terrestrial invertebrates, longer development times between instars can change the date of emergence from pupa and therefore the period of occurrence for populations, and reduction and delay in broods per year (Hassold and Backhaus 2009; Wang et al. 2009; Bart et al. 2020). In lepidopterans, insecticide exposure can thus, directly affect pupation. For example, increasing the frequency of unsuccessful pupation, reducing successful pupae emergence rates or lower pupal weight and subsequently emerged imago (Han et al. 2012; Dong et al. 2017; Wang et al. 2017; Krishnan et al. 2021). All of these effects may impact population dynamics and cannot be studied using single time point data taken from short-term exposure (Jager et al. 2011).

Measuring the effects of pesticide exposure over the course of an organism's life can be simulated and predicted using energy apportionment models. Energy apportionment models, such as Dynamic Energy Budget (DEB models), are designed to represent the rates at which an organism gain and use energy for vital processes; maintenance, growth and reproduction (Kooijman 2010). Models based on DEB are the most common and well established TKTD models that have been successfully used to understand the mechanistic basis of the sublethal effects of chemical exposure and their effects of multiple life-cycle traits (Kooijman 2010; Jusup et al. 2017). In ecotoxicology, DEB models can simulate how uptake of toxicants can disrupt processes by affecting accrual and use of energy in an organism. Further, use of DEB models can determine which process is affected by the toxicant: assimilation, growth, reproduction or maintenance (Ashauer and Jager 2018). This makes these models a valuable tool in risk assessment as they can explain which effects in which life stages are likely to be impacted most by pesticide application.

DEB models have several different variations ranging from the simplified models requiring limited data to the more complex full models. The most common model used for ecotoxicological testing is a variation of DEB called DEBTox. DEBTox assumes that stasis of an organisms' energy use can be altered by exposure to a toxicant (Jager et al. 2014a). Like other TKTD models, DEBTox accounts for changes in toxicity over time by simulating the TKTD processes that drive the toxic effect. The impact of these TKTD process on the acquisition and use of energy and how these effects impact on life-cycle traits over time. DEBTox has been successfully utilised to model mixture toxicity and can be used to gain an insight into interaction mechanisms of chemicals by simulating toxic effects on growth, reproduction and survival (Baas et al. 2010; Jager et al. 2014b; Margerit et al. 2016; Hansul et al. 2021; Vlaeminck et al. 2021).

Previous studies have shown that mixtures that include a pyrethroid and an azole fungicide are commonly associated with synergistic effects leading to greater than additive toxicity (Cedergreen 2014). Indeed in some tested species, azole fungicides can increase the toxicity of pyrethroid insecticides up to 50 fold (Kretschmann et al. 2015). This increase in pyrethroid toxicity on co-exposed with azoles has an obvious impact on mortality rates of exposed organisms, however, it also has the potential to disturb sublethal processes, such as growth and maturation, leading to greater than additive effects on these traits (Gottardi et al. 2017). There have been several studies of bees that infer synergism between pyrethroids and azole

fungicides including for effects on mortality and also for sublethal effects (Chalvet-Monfray et al. 1996; Papaefthimiou and Theophilidis 2001; Glavan and Božič 2013). However, to date there are no studies that detail this synergism in lepidopteran species, by measuring toxic effects at the sublethal level.

In the previous chapter, it was established that the combination of the synthetic pyrethroid, cypermethrin and the azole fungicide, prochloraz caused synergistic effects on the survival of *Mamestra brassicae* over a 4-day exposure period. Here this mixture is designed to assess effects on sublethal endpoints, more specifically on *M. brassicae* growth and development. Effects on growth and survival were measured from second instar up until pupation for larvae exposed to cypermethrin, prochloraz and different combinations of both chemicals. Further, moult time, pupation time and pupation success were also measured to provide an assessment of the effects of this pesticide combination on adult emergence. It is hypothesised that synergistic interactions that were seen for effects on larval survival between cypermethrin and prochloraz will be retained for effects on the measured sub-lethal effects. This synergy is anticipated to result in a slower development of larvae and increased incidences of arrested pupae ecdysis in mixture treatments than predicted from the additive effect of both chemicals. By combining statistical analysis with DEBtox modelling for impact on growth and survival, it was further possible to study the TK and TD parameters underlying these effects and their impact on energy budget traits.

4.2. Methods

4.2.1. Insect Culture

As with previous chapters, *M. brassicae* larvae were from the same population reared for 40 years on artificial diet. For the exposures, eggs were collected 12 days prior to the beginning of the experiment and raised in a rearing box. This allowed 5 days for hatching and a further 7 days of growth before they were used for testing. Previous pilot studies had shown poor survival across all treatments if larvae were handled before their moult to 2nd instar due to their small size and delicate physiology. Therefore, larvae were selected at 7 days post-hatch as second instars for use in the bioassay to ensure minimal background mortality in the early stages of the exposure as a result of any handling stress.

4.2.2. Chemical and Diet Preparation

The exposures to measure growth and development effects were conducted over a range of treatments of cypermethrin, prochloraz and for a mixture of these two pesticides. In both cases, the substance used high purity analytical standards (>99%). Both cypermethrin and prochloraz were purchased from Sigma Aldrich (Poole, UK). A pilot study with the two test chemicals highlighted a need to reduce concentrations from those used in mixture testing in Chapter 3. This was due to a heightened sensitivity in 2nd instar larvae compared to their 4th instar counterparts used in the 96-hour bioassay developed in Chapter 2 and used for testing multiple insecticides, fungicides and mixtures in Chapter 3. After this optimisation, a range of concentrations were selected for testing both single chemicals and mixtures that covered the sublethal response range for cypermethrin (Table 4.1).

To prepare stock solutions for dosing, the two chemicals were diluted with acetone to provide the range of concentrations needed for the study. For each exposure, a minimum of 30 ml of solution were made up per concentration. For the initial set up, a modified Hoffman's diet was heated, using the culture recipe detailed in Chapter 2. To control mould growth which proved problematic over the longer timescale used for the exposure, unlike in the previous bioassay exposures in Chapters 2 and 3, the artificial diet did not have the 25% extra water added. Aliquots were spiked with each of the treatments using the same method as previous chapters with 10 ml of dosed diet added to an individual round 25 ml plastic pot (53 mm diameter, 27 mm height). To ensure that effects were not related to any solvent

effects, the control and all lower concentration treatments were spiked with the similar volume of acetone as used for the highest concentration mixture exposure. In total, the full experiment included 17 treatments including a control, 5 cypermethrin only treatment, 5 prochloraz only treatment, and 6 mixtures.

Table 4.1. Concentrations of cypermethrin, prochloraz and the mixture cypermethrin and prochloraz in combination used in the growth assay.

Cypermethrin (mg/kg)	Prochloraz (mg/kg)	Cypermethrin and Prochloraz (mg/kg)
0.01	1	0.01 + 1
0.05	5	0.05 + 1
0.25	10	0.05 + 5
1.25	20	0.25 + 1
6.25	40	0.25 + 5
		1.25 + 10

4.2.3. Test Method

All larvae were exposed individually in plastic pots containing the spiked diet, with 15 replicates per treatment. To initiate the exposure, the 7-day old individual 2nd instar larvae were weighed to give a starting value for growth monitoring. Care was taken when selecting larvae for use in the test to avoid any larvae that had not yet moulted from 1st instar. Once the caterpillars were added to the surface of the diet, the exposure pots were kept in an incubator in the dark at a constant temperature of 20^{o}C. At the start of every week fresh diet was prepared and spiked with the relevant concentration of the test chemicals. This diet was used to prepare a new exposure replicate that was used to continue the exposure following transfer of the larvae to the new diet surface. This diet renewal prevented the opportunity for mould to grow that would affect larval movement and feeding. Further, changing the diet every 7 days meant that the exposure was maintained as degradation of the pesticide in the test could take place only over a maximum of 7 days. Finally, the regular supply of new diets

also ensured that larvae were fed with an *ad-libitum* feeding strategy and that diet restriction never affected larvae growth and development.

At day 0, 2, 4, 7, 9, 11, 14, 16, 18, 21, 23, 25, 28, 30, 32, 35, 37 and 40 d of exposure, the larvae were taken from the surface of the exposure diet and checked for survival and weight. Larvae were recorded dead if there was no movement after stimulation with a paintbrush for 5 seconds. Dead larvae were disposed of immediately after their day of death and instar at death was recorded. At each timepoint, all individuals were also weighed to monitor their growth until they reached 6th instar and thereafter their weight change as they began to lose weight in preparation for pupation. Instar status was also recorded to track developmental times through each stage of developed for all individuals in each treatment. Day and weight at pupation was recorded when reached and pupa were sexed, any unsuccessful pupations were also noted (n.b. unsuccessful pupations could be attributed to arrested pupal ecdysis, or incomplete shedding of the trachea cuticle). After 40 days of exposure, all control larvae had pupated for at least 10 days and the experiment was stopped. Any larvae that had not pupated by day 40 were excluded from analysis as no data on pupal success could be gathered for these individuals. All of the larvae excluded were in either the 1.25 cypermethrin or 1.25 + 10 cypermethrin and prochloraz treatments. These treatments were therefore, excluded from pupation analysis.

4.2.5. Data Analysis

4.2.5.1. Statistical analyses

The average day of moult to each instar was recorded and tested for significant difference between treatments using a one-way ANOVA. Significant differences between treatment were further investigated using the post-hoc Tukey's test. Average day of moult was compared between each treatment group for the difference chemicals used. For example, cypermethrin treatments were compared between themselves, as were prochloraz treatments. Mixtures were treated as a separate group for this analysis. Data on failure to reach pupation was analysed using a Chi-square non-parametric test. Amount of larvae reaching pupation varied per treatment group reflecting the fact that some larvae died during their growth phase. Therefore, sample sizes per group ranged between 7 and 13. Finally, analysis of time to pupation data was conducting with GLM and post hoc Tukey's HSD.

4.2.5.2. Toxicokinetic-toxicodynamic: DEBtox modelling

Modelling for the effects of exposure on the measured life-cycle traits was conducted using the simplified DEB model DEBkiss. This formulation of the DEBtox based DEBkiss model was developed and is fully presented in Jager and Zimmer (2012), and in Jager (2020) for application in ecotoxicology. In the model, food is taken up by the caterpillars and a fraction (κ) of the assimilated energy is used for the soma (growth and somatic maintenance), whereas the remainder (1 − κ) is used for maturation (in juveniles), maturity maintenance, and reproduction (in adults). Only the growth phase of the life cycle was considered and all model simulation ended once the larvae had entered into pupation. Therefore, only the DEB branch where assimilated energy is used for growth and somatic maintenance was considered. In contrast to other simplified DEB approaches, DEBkiss builds upon an explicit mass balance, and excludes the distinction of biomass in a structure and reserve compartment: all biomass is treated as "structure", which is an acceptable simplification for invertebrates (Jager et al. 2013).

The assimilation energy flux is written as follows:

$$J_A(t) = f\, J_{Am}^{a}\, L^2(t)$$

Where J_A [ma d^{-1}] is the mass flux for assimilation, J_{Am}^{a} [ma/($L^2 d^{-1}$)]is the area-specific assimilation rate at maximum food, f [-] is the scaled functional response which reflects food availability (between 0, no food, and 1, *ad libitum* feeding condition), L^2 [mm^2] is the volumetric length.

The maintenance energy flux is described as follows:

$$J_M(t) = J_M^{v}\, L^3(t)$$

Where J_M is the mass flux for maintenance, J_M^{v} [ma/($L^3 d^{-1}$)] is the volume-specific costs for maintenance, and L^3 [mm^3] is the volumetric length.

The mass flux for structure is described as follows:

$$J_V(t) = y_{VA}(kJ_A(t) - J_M(t))$$

Where J_V is the mass flux for structure (i.e., =growth), y_{VA} is the yield of structure on assimilates (growth), k (-) is kappa, the part of the energy used for growth and somatic maintenance. The remaining energy (1-kappa) is used for maturation in juvenile stage and egg production in the adult stage. This is not modelled here as only the larvae growth period was considered.

As presented in Chapter 2, *M. brassicae* present an exponential growth curve when measured either in biomass or structural length. This phenomenon, described in other insect species, has been explained by type M metabolic acceleration as presented in Kooijman (2014). It consists in the progressive increase of maximum food assimilation rate J_{Am}^a and energy conductance during this period thanks to their multiplication by an acceleration factor S_M. This factor evolves from S_M = 1 at birth to reach $S_M = \frac{L_p}{L_b}$ at pupation. Between these two stages, it evolves along with length: $S_M = \frac{L}{L_b}$ and the Assimilation flux is now described as follows:

$$J_A(t) = f\, J_{Am}^a S_M\, L^2(t)$$

Finally, the start of pupation depends on hormonal processes not related to energy processes. It is assumed the pupation starts once individuals reach a certain size, L_p, and the growth stops; if $L > L_p$, J_V = 0. A mortality background h_b (d^{-1}) is also added to account for mortality not related to the toxic compound. Together, J_{Am}^a, f, J_M^v, y_{VA}, S_M, k, L_p, h_b, form the physiological parameters of the DEBkiss-TKTD model.

The TK-TD component of the overall model accounts for the accrual of, and recovery from, damage (toxicodynamics, TD), which forms due to the bioaccumulation, distribution, biotransformation, and elimination of the chemicals in the organisms (toxicokinetics, TK). Here, there is no information on body residues (measurements or predictions); therefore, the TK and TD part are combined into a one compartment model linking the external concentration to the damage, over time). Written as follows:

$$\frac{dD_f}{dt} = k_d(C_f - D_f)$$

Here D_f (mg/kg) is the damage level (scaled by the external concentration in the food), C_f (mg/kg) is the total concentration in the food and k_d (d^{-1}) is the dominant rate constant describing the dynamics of the "scaled" damage, and represents the one-compartment approximation of the "true" two-compartment behaviour (TK and damage dynamics).

From the scaled damage level, a dimensionless stress level is calculated (Eqn 7):

$$s = b_b \max(0, D_s - z_b) \qquad \text{Eqn (7)}$$

Where s (-) is the stress level, b_b (mg/kg) is the effect strength on the energy budget, and z_b (mg/kg) is the damage threshold for effects on the energy budget. The stress modifies the value of one or more DEBkiss model parameters (Jager 2020). Only the growth phase of the life cycle was considered; therefore, three metabolic processes can be affected by the chemical: assimilation $J_{Am}^{a}(1-s)$, maintenance $J_M^v(1+s)$ and growth $y_{VA}/(1+s)$), or a combination of them. The affected metabolic process is generally referred to as a physiological mode of action (pMoA); A, M, G.

Finally, it is assumed that the same damage type affects both sublethal and lethal toxicity. To model survival, a module from the GUTS framework is added: the stochastic death version:

$$h = b_s \max(0, D_s - z_s) \qquad \text{Eqn (8)}$$

$$\frac{dS}{dt} = -(h + h_b)\, S \;\; with\; S(0) = 1 \qquad \text{Eqn (9)}$$

Where S (-) is the survival probability over time, h (d^{-1}) is the hazard rate, b_s (mg/kg d^{-1}) is the effect strength on survival (also known as killing rate, b_w, in the GUTS framework), and z_s (mg/kg) is the damage threshold for survival. Together, k_d, b_b, z_b, b_s and z_s form the five toxicological parameters of the DEB-TKTD model.

Once DEB-TKTD models are calibrated on single exposure, we can predict the mixture effects assuming additivity. For survival, it is modelled with a GUTS model, so the prediction of the mixture is performed as in Chapter 3 (i.e., addition of). For the growth, a stress level is calculated for each chemical and it modifies the value of one or more DEBkiss model parameters. If the two chemicals share the same pMoA, the stress levels are multiplied (e.g., for pMoA growth $y_{VA}/((1 + sA) * (1 + sA))$).

All model calculations were performed in Matlab 2021 with the BYOM v.6.0 modelling platform (http://www.debtox.info/byom.html), with the package DEBtox2019, modified to account for the metabolic acceleration of *M. brassicae*. As suggested in Jager (2018), first the physiological parameters of the model were fitted to the control growth. Next, the toxicological parameters were fitted to the entire dataset (i.e., on survival, growth and reproduction data together), keeping the physiological parameters fixed to their best value. Finally, with calibrated models (the best parameter set associated with the best pMoA; selected by lowest maximum log-likelihood (MLL) and Akaike information criterion (AIC)), the mixture effect was predicted and visually assessed; if the model under predicted the effect this could be interpreted as a synergistic effect.

Traditional DEB models require the parameter of length to determine growth. However, this measurement was difficult to obtain with *M. brassicae* larvae due to their tendency to curl when disturbed. Therefore, weight was determined to be a more reliable measurement endpoint in this species. *Mamestra brassicae* are isomorphic, meaning that their body shape does not change during growth. The implication of this is that volume is proportional to cubed length, therefore, the cubic root of the body weight can be used to calibrate the model (Bart et al. 2019).

4.3. Results

4.3.1. Mortality and Growth Effects of Single Chemical Exposure

4.3.1.1. Cypermethrin

Control mortality was 13.3%, below the desired upper limit of 20% mortality set out in Chapter 2 as the maximum level of background mortality that is needed for a test to be optimal for further interpretation and model fitting. At the highest concentration of cypermethrin tested, 6.25 mg/kg, all larvae were dead by day 7. A proportion of the exposed larvae, however, survived in all other test concentrations of cypermethrin for the full 40-day duration of the experiment. The treatments of cypermethrin selected, thus, represent a range of exposure levels covering full, partial and no additional mortality above background toxicity of effects on mortality. Effects across this range of effects levels provide a basis to assess and understand the effects of chronic exposure to cypermethrin on sublethal growth and development endpoints (Figure 4.1).

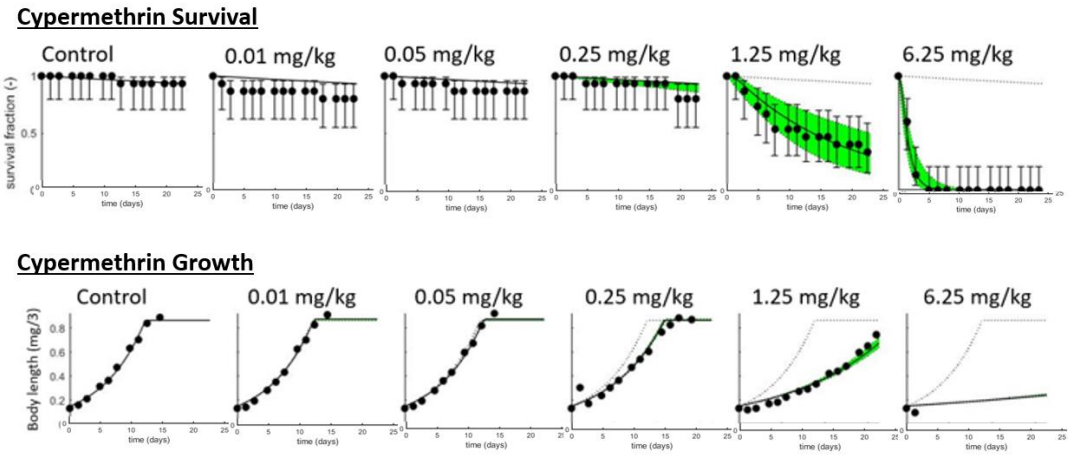


Figure 4.1. Survival and growth of larvae exposed to cypermethrin. For survival (first row), the dotted lines are the mortality background fitted to the concentration, bold lines are the model fits, and points indicate actual survival data (with error bars showing Wilson score confidence). For growth (second row), points indicated the measured data and the solid lines the model fits. The dotted lines are the mortality background fitted to the control treatment.

control growth for comparison to the treatment values. Green areas represent 95% confidence intervals of model fits.

The DEB-TKTD model was able to accurately simulate the growth in the control and exposed larvae over a total of 25 days exposure. The best pMoA to simulate the effect of cypermethrin on larvae growth was an increase of the growth energy cost (Table 4.2).By the end of the 25 days of exposure, all larvae in the control had stopped gaining weight in preparation for pupation by day 23 (Figure 4.1). Similarly, an arrest of growth by this time was also found for larvae exposed to 0.01 and 0.05 mg/kg of cypermethrin. Growth curves for 0.25 mg/kg and 1.25 mg/kg of cypermethrin were shallower than for the control treatment, indicating an impact of cypermethrin on growth rates of larvae. All larvae in the 1.25 mg/kg of cypermethrin treatment continued their growth after 40 days at which point all other treatments had reached pupation. Therefore, in this treatment growth was only modelled up to 25 days to provide consistency for the lower concentration treatments for which only growth data up to this time-point prior to pupation was available. In the highest cypermethrin exposure of 6.25 mg/kg, all larvae had died by day 7. Based on the weight change data available for larvae over this initial period, a small degree of growth was still predicted by the model, although data outside of the period where larvae were alive is not likely to represent a real case.

Table 4.2. Calibrations of pMoA's for *Mamestra brassicae* larvae exposed to treatments of cypermethrin and prochloraz. The blue bar represents the best fit, due to lowest MLL and AIC values. G is growth, M is maintenance and A is assimilation. The lighter blue bars show equal or similar MLL or AIC values.

Chemical	pMoA	MLL	AIC	R2 Surv	R2 Body Length
Cypermethrin	Growth	203.2	416.4	0.9604	0.9823
Cypermethrin	Growth + Maintenance	203.24	416.48	0.9604	0.9824
Cypermethrin	Assimilation + Growth	212.55	435.1	0.9604	0.9775
Cypermethrin	Assimilation + Maintenance + Growth	212.57	435.13	0.9601	0.9777
Cypermethrin	Assimilation + Maintenance	218.45	446.89	0.9604	0.9736
Cypermethrin	Assimilation	218.46	446.93	0.9606	0.9739
Cypermethrin	Maintenance	285.76	581.51	0.9601	0.6158
Prochloraz	Growth+ Maintenance	189.27	388.54	0.5723	0.9909
Prochloraz	Growth	189.65	389.3	0.534	0.9909
Prochloraz	Assimilation + Growth	190.41	390.82	0.6115	0.9906
Prochloraz	Assimilation + Maintenance + Growth	190.58	391.15	0.6239	0.9906
Prochloraz	Assimilation + Maintenance	191.36	392.72	0.6225	0.9903
Prochloraz	Assimilation	192.1	394.1	0.5385	0.9904
Prochloraz	Maintenance	230.01	472	0.6027	0.9682

4.3.1.2. Prochloraz

Unlike in the short-term exposures conducted in Chapter 3 where prochloraz had no effect on survival, an effect on larval survival was found here following exposure to 10 mg/kg and

above (Figure 4.2). The greatest effect on mortality was found in the highest concentration of prochloraz, 40mg/kg, these effects becoming clearly apparent only towards the mid-point of the assay. This observation of delayed mortality explains why these effects were not seen in the short-term exposure conducted in Chapter 2. No significant effect was found on mortality at exposure concentrations of 10 and 20 mg/kg. Exposure at the selected level, thus, provide an opportunity to assess how concentrations of prochloraz results in partial and no mortality affect growth and development traits.

The DEB-TKTD model was able to simulate accurately the growth in the control and exposed larvae over a total of 25 days exposure. The best pMoA to simulate the effect of prochloraz on larvae growth was an increase of the growth energy cost (Table 4.2). Growth was not as strongly affected by prochloraz exposure as was the case for cypermethrin (Figure 4.1 and Figure 4.2). All larvae exposed to prochloraz at any tested concentration reached 6th instar by day 22. However, deviation from rate of growth in the controls was still observed. Beginning at 10 mg/kg a comparison with the control growth rate (shown as a visible dotted line in Figure 4.2, second row) indicates an increasing deviation of measured growth with increasing prochloraz concentration.

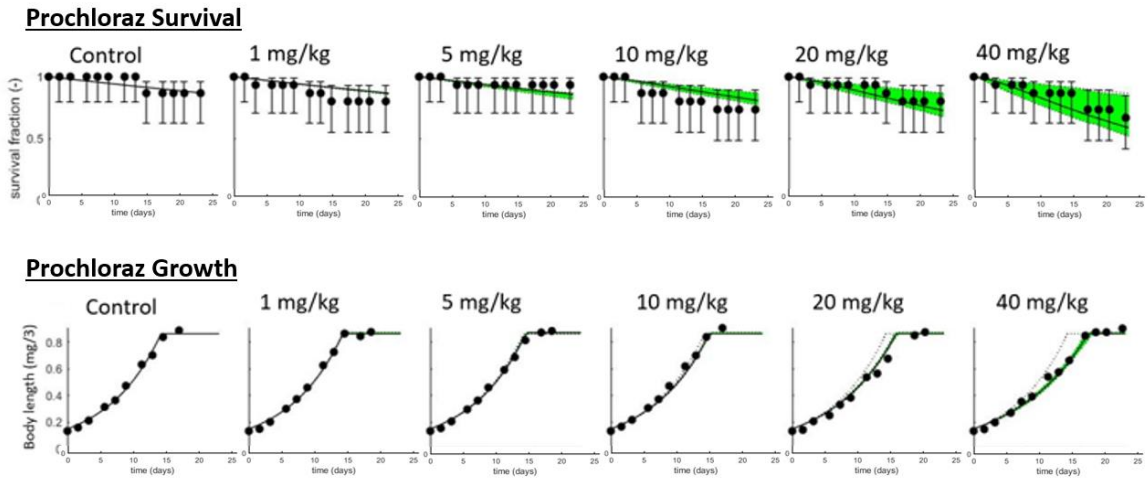


Figure 4.2. Survival and growth of larvae exposed to prochloraz. For survival (first row), the dotted lines are the mortality background fitted to the concentration, bold lines are the model fits, and points indicate actual survival data (with error bars showing Wilson score confidence). For growth (second row), points indicated the measured data and the solid lines the model fits. The dotted lines are the mortality background fitted to the control treatment. Green areas represent 95% confidence intervals of model fits.

These separate DEBKiss models were fitted to the observed survival in time, growth and pupation time data to provide a pMoA and associated set of TKTD and energy budget related physiological parameters for the effects of the chemical and mixture. Both cypermethrin and prochloraz exceed the threshold energy budget (z_b). This excess is predicted to result in effects on growth proportional to the amount the parameter value exceeds the z_b and the b_b (Table 4.3). The threshold for effects (z_s) on survival was lower for cypermethrin than prochloraz (0.92 vs 2.78 mg/kg respectively). However, the effect strength for survival (b_s) was below this in both chemicals (Table 4.3). This means that there was likely survival of larvae exposed to both chemicals.

Table 4.3. Parameter values, with 95% confidence intervals, of the DEB-TKTD models. Physiological mode of action for cypermethrin and prochloraz: increase of the growth energy cost.

Symbol	Description	value (CI)	Unit
DEBkiss parameters			
$\dot{J}^a_{Am}$	Maximum area-specific assimilation rate	0.0056 (0.0050 - 0.0117)	$mg/mm^2/d^{-1}$
$\dot{J}^v_M$	Volume-specific maintenance costs	0.0101 (0.0037 - 0.0996)	$mg/mm^3/d^{-1}$
L_0	initial structural length	0.036	$mg^{(1/3)}$
y_{VA}	Yield of structure on assimilates (growth)	0.8 (fixed)	-
f	Scaled functional response (0-1)	1 (fixed)	-
S_M	metabolic acceleration factor	-	$L(t)/L_0$
k	kappa	0.9 (fixed)	-
L_p	Structural length at which growth is ceased, start moulting	0.86	$mg^{(1/3)}$
h_b	mortability background	0.0021 (0.0018 - 0.0027)	d^{-1}
Toxicological parameters for cypermethrin			
k_d	Dominant rate constant	1.32 (0.67 - 10)	1/d
z_b	Threshold energy budget	1.7 10-5 ($1.7\ 10^{-5}$ - 0.008)	mg/kg
b_b	Effect strength energy-budget	0.95 (0.86 - 1.03)	kg/mg
z_s	Threshold survival	0.92 (0.20 - 1.08)	mg/kg
b_s	Effect strength survival	0.11 (0.05 - 0.19)	kg/mg/d
Toxicological parameters for prochloraz			
k_d	Dominant rate constant	0.21 (0.03 - 10)	1/d
z_b	Threshold energy budget	0.002 (0.002 - 2.846)	mg/kg
b_b	Effect strength energy-budget	0.007 (0.005 - 0.027)	kg/mg
z_s	Threshold survival	2.78 (0.002 - 37.77)	mg/kg
b_s	Effect strength survival	0.0004 ($9.2\ 10^{-5}$ - 0.01)	kg/mg/d

4.3.2. Mortality and Growth Effects of Mixture Treatments

For survival, based on the models calibrated on the single chemical exposures, the joint effects of cypermethrin and prochloraz on larval survival were predicted to assume no

interaction as both chemicals have different modes of action and target receptors. Compared to the model prediction of the joint effects of cypermethrin and prochloraz, the observed data indicated no consistent evidence of synergism in the 1.25 cypermethrin + 10 mg/kg prochloraz treatment, with the observed data closely according with the model predictions (Figure 4.3). Evidence for synergism is, however, seen in the 0.25 mg/kg of cypermethrin mixture exposed with both test concentrations of prochloraz (5 mg/kg and 10 mg/kg). This presence of synergism is increasingly pronounced between the 0.25 CYP + 1 PCZ and the 0.25 CYP + 5 PCZ treatments. Further, the 0.05 mg/kg of cypermethrin and prochloraz treatments show slight synergism when their survival curve is compared to that of cypermethrin alone.

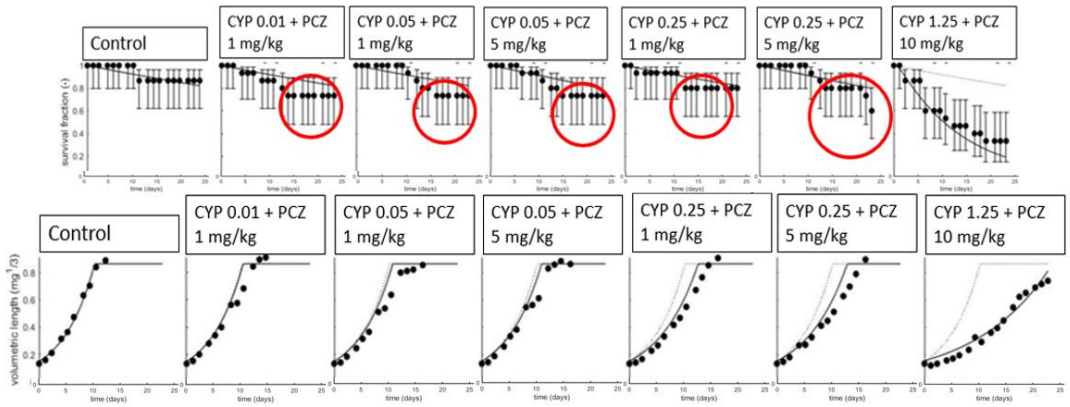


Figure 4.3. Predicted survival of *Mamestra brassicae* larvae exposed to a combination of cypermethrin and Prochloraz. Dotted line shows control treatment, solid lines are the predictions of the additive DEB-TKTD model, points are the observed data (with error bar) Synergy is highlighted by red rings and is identified as observed survival below additive effects. The bottom row plot shows predicted growth rates for combinations of prochloraz and cypermethrin treatments. Dotted line shows control treatment. Solid black line shows predicted growth of *M. brassicae* larvae.

The DEB-TKTD model predictions of the mixture effect on growth (using calibration of single exposures and based on an assumption of additivity) showed a marked underestimation of the observed effects for all treatments except the treatment of 1.25 of cypermethrin + 10 mg/kg of prochloraz (Figure 4.3). This increased effect resulting from synergism can be observed by the common overestimation of growth by the model when compared to the observed data. This underestimation of the effects can be further visually highlighted by

plotting the correlation between the predicted and observed effect (Figure 4.4). This plot shows how the observed growth effects consistently lie above the model prediction based on additive effects on growth. This result showed the mixture effects were stronger than the additivity of both chemicals, indicating synergism.

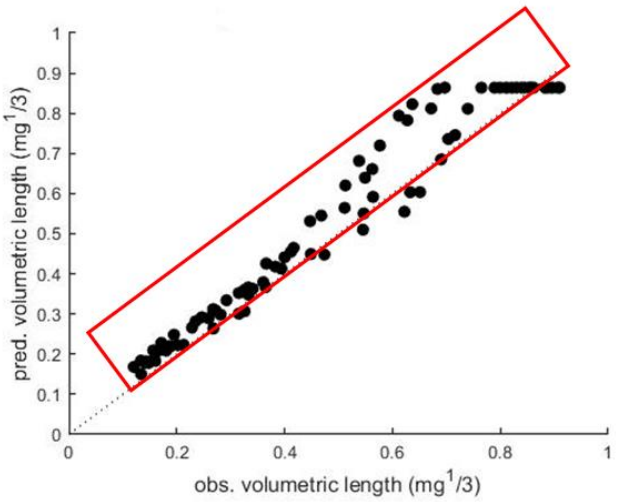


Figure 4.4. Predicted VS Observed effects of cypermethrin and prochloraz in mixture on *M. brassicae* growth. The red rectangle denotes the under estimation of the effects on growth predicted by the model compared to the true observed effects.

4.3.3. Development

The GLM and one-way ANOVA found that cypermethrin had a significant concentration related effect on development time as indicated by the effects of exposure for day of moult for some instars ($p<0.001$). Post hoc Tukey HSD test indicated that larvae exposed to 0.25 and 1.25 mg/kg of cypermethrin develop to each instar at a significantly ($p<0.001$) later time point than any other treatment (Table 4.4). This delay in maturation is particularly prominent between instars four, five and six, for which moulting occurs significantly later than for every other cypermethrin treatment (Table 4.4). A significant difference in the time between moults was also found in the larvae exposed to prochloraz (one-way ANOVA: $F(5)=35.6$ $p<0.001$). Slower development times were seen in the higher concentrations prochloraz exposure. This difference was, however, not significantly different from the control in instars 3 and 4 in the prochloraz 40 mg/kg treatment Tukey's post hoc test ($P>0.05$) (Table 4.5). Effects of prochloraz exposure were, however, seen for the later instars. Thus, moult times

for instars five and six were significantly later that those of control treatment for the two highest concentration prochloraz treatments (20 and 40 mg/kg).

Table 4.4. Average day of moult to each instar in *Mamestra brassicae* larvae exposed to cypermethrin. A different letter denotes significant differences between concentrations ($p<0.05$).

Cypermethrin Concentration (mg/kg)	Average Day of Moult to Instar:			
	3	4	5	6
Control	2.8 A	5.5 A	10.6 AB	15.5 A
0.01	3.5A	6.3 AB	10.8 AB	15 A
0.05	3.1 A	6.1 AB	10.3 A	15.8 A
0.25	3.7 AB	7.1 B	11.8 B	25 B
1.25	5.67 B	10.8 C	19.8 C	31.25 C

Table 4.5. Average day of moult to each instar in *Mamestra brassicae* larvae exposed to prochloraz. A different letter denotes significant differences between concentrations ($p<0.05$).

Prochloraz Concentration (mg/kg)	Average Day of Moult to Instar:			
	3	4	5	6
Control	2.8 A	5.5 A	10.6 AB	15.5 A
1	3.5 A	6.2 AB	9.7 A	15.7 A
5	2.9 A	5.9 AB	10.7 AB	15.5 A
10	2.7 A	5.5 A	12.3 B	17.5 AB
20	3.7 A	7.6 B	14.8 C	19.8 BC
40	3.2 A	5.6 A	15.6 C	22 C

Table 4.6. Average day to moult of larvae exposed to mixture of cypermethrin and prochloraz. A different letter denotes significant differences between concentrations ($p<0.05$).

Cypermethrin + Prochloraz Concentration (mg/kg)	Average Day of Moult to Instar:			
	3	4	5	6
Control	2.8 A	5.5 A	10.6 A	15.5 A
0.01 + 1	3.3 AB	6.2 AB	12.8 AB	20 B
0.05 + 1	3.7 AB	12.3 DE	16.1 C	20.8 BC
0.05 + 5	3.6 AB	10.5 CD	15.2 BC	20.9 BC
0.25 + 1	4.3 B	10.6 CD	17.2 C	23.5 BC
0.25 + 5	3.5 AB	8.8 BC	16.3 C	23.8 C
1.25 + 10	7.4 C	13.6 E	22.4 D	38.3 D

Mixture treatments showed significant difference in moult times for all instars ($p<0.001$). The 6th instar moult occurred significantly later in the 0.01 + 1 treatment group compared to the control. This difference was not seen in either single cypermethrin 0.01mg/kg or the prochloraz 1 mg/kg treatment, highlighting synergy at low concentrations for the effects of the two chemical on pupation time. The day of moult to 6th instar was also significantly

different from the controls in all mixtures treatment group (Table 4.6). This is in contrast to the single chemical treatments, where an effect on development was only seen in the two highest concentration exposures of both cypermethrin and prochloraz. Within the two 0.05 mg/kg cypermethrin mixture treatments, significant changes in days to moult occurred as early as instar 4 and continued to show significantly delayed development compared to control throughout each successive instar moult. The extent of this effect again differs from the single cypermethrin 0.05 mg/kg treatment for which there was no significant difference from the control from the 0.05 mg/kg exposure alone (Table 4.4). With the exception of the 0.25 + 5 mg/kg treatment at instar 3, all other treatments displayed a significant difference to control development in every instar. This mimics the slower development seen in cypermethrin 0.25 and 1.25 mg/kg single chemical treatments. As can be seen in Table 4.4, 4.5 and 4.6 increases in concentration extend the duration between moults, a delay that is lengthened with each successive instar.

4.3.4. Pupation

Day of pupation was recorded along with any incidences of arrested pupal ecdysis. Arrested pupal ecdysis is referred to as a pupal failure that would result in the pupae being unable to eclose. For example, larvae that begin to shed their old cuticle from the abdomen, but are unable to shed from the head and ventral thorax (Krishnan et al. 2021) (Figure 4.5). No larvae reached the pupa stage within 40 days in either 1.25 mg/kg cypermethrin treatment, as a single chemical or in a mixture combination. Hence, it is uncertain whether there could be effects on pupation at this concentration after further growth. Incidences of pupation failure in both chemical for all other treatments were calculated per chemical, excluding larvae that died before pupation. No significant difference in incidences of pupation failure was found for the different treatments of cypermethrin ($X^2(3)=1.4$ $p>0.05$), prochloraz ($X^2(5)=5.52$ $p>0.05$) or the mixture of cypermethrin and prochloraz ($X^2(15)=22.1$ $p>0.05$) respectively (Figure 4.6).

Figure 4.5. Larva that has failed to pupate correctly (left image), resulting in arrested pupae ecdysis. Here known as pupation failure. Note head capsule is not shed and abdomen is not completely encased. Normal pupation is shown in the right-hand image.

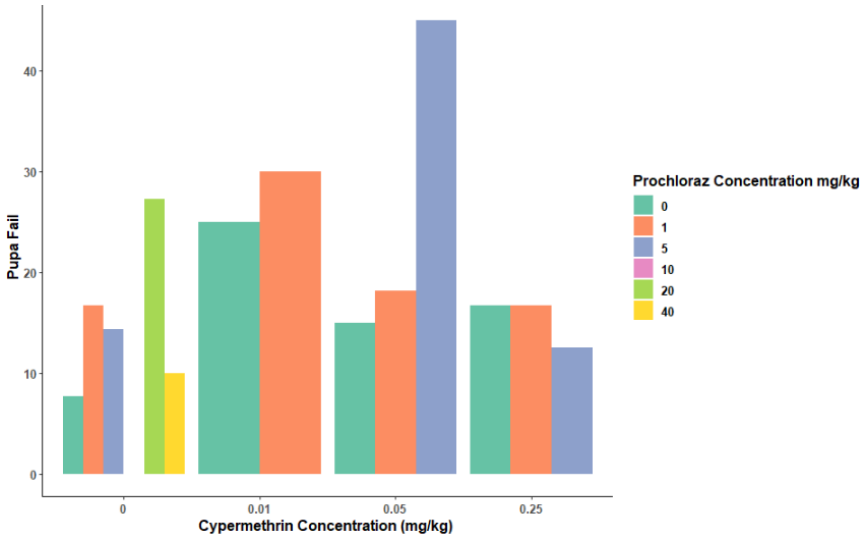


Figure 4.6. Percentage of pupation failure in each treatment group. Date of pupation was taken only from larvae that reached pupation, i.e. excluding any dead larvae and larvae at 1.25 mg/kg that did not reach pupation during the exposure period. There was no significant differences in incidences of pupal fails between any treatments.

One-way ANOVA indicated a statistically significant difference in day to pupation between for treatment groups of both single chemical and the mixture (cypermethrin $F(3)=52.4$ $p=<0.001$, prochloraz $F(5)=10.8$ $p=0.001$, mixture $F(5)=2.94$ $p<0.05$). Cypermethrin significantly increased pupation time in the 0.25 mg/kg exposure. Further all treatment

groups that contain prochloraz took significantly longer to pupate than those with cypermethrin alone ($p<0.05$). This increase appeared to be concentration dependent as significant effects on time to pupate were only identified by post hoc Tukey's test in the 5 mg/kg of prochloraz mixture treatments and not in the 1 mg/kg mixture treatments (Figure 4.7). The last larvae to pupate within the 40 day experimental period were those in the 0.05 + 5 mg/kg mixture. However, as prochloraz and cypermethrin significantly affected development and pupation times, this effect cannot be determined as synergistic as it cannot be ascertained if effects are the product of an interaction or the additive effects of both chemicals.

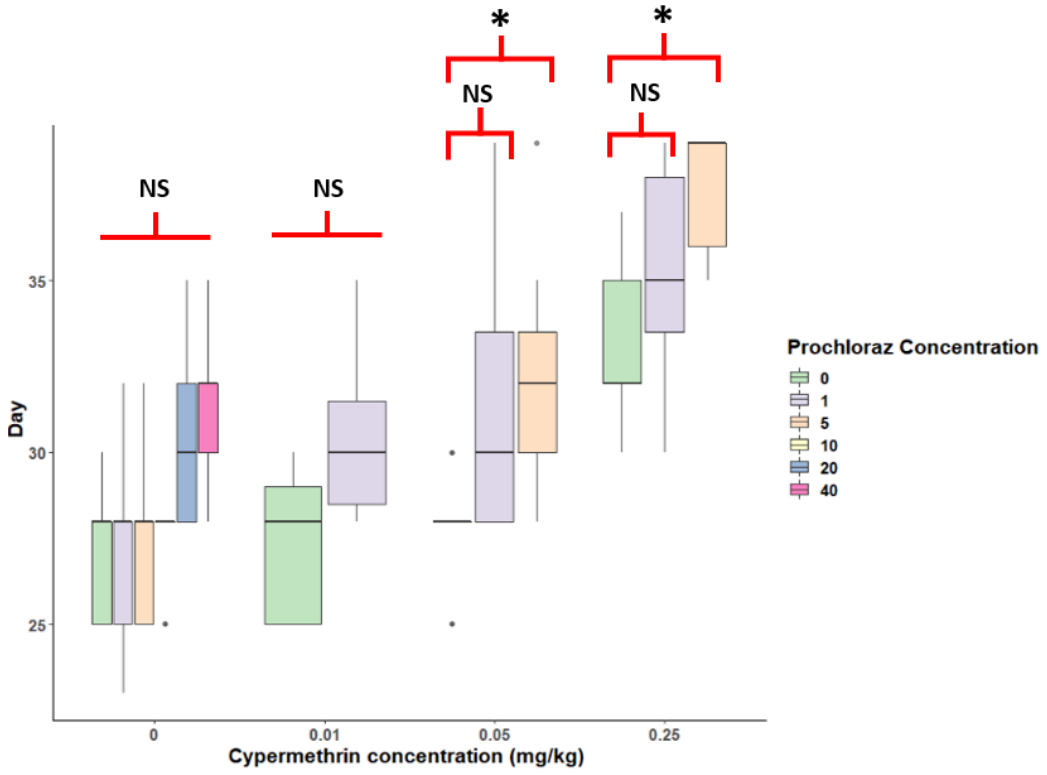


Figure 4.7. Box and Whisker plot of the mean (bold line) 75th percentile (upper and lower box limits) and 95% confidence intervals (vertical lines) of day to pupation for larvae exposed to treatments of cypermethrin, prochloraz and their mixture. Significant effects on day of pupation as a result of prochloraz exposure as determined by glm are showed as 0 '***' 0.001 '**' 0.01 '*' 0.05. Not significant results are denoted as NS.

4.4. Discussion

Energy apportionment models such as DEBTox can be utilised in ecotoxicology to determine sublethal and mortality effects of chemical exposure. Here, larval growth and mortality was modelled from 2nd to 6th instar in a range of concentrations of cypermethrin, prochloraz and a mixture of both. In contrast to Chapter 3, it was found that with prolonged exposure both prochloraz and cypermethrin had an effect on larvae survival which became more pronounced with increasing concentration. However, in accordance with Chapter 3, the model underpredicted survival and growth for the mixture of cypermethrin and prochloraz; indicating a synergistic interaction between this chemical pairing. Exposure to these two chemicals also impacted the time taken to moult between instars and reach pupation but no significant effect was recorded on arrested pupal ecdysis when exposed to these chemicals either alone, or as a binary mixture.

4.4.1. Survival

Exposure until pupation allowed cypermethrin and prochloraz to be assessed for effects on *M. brassicae* development and survival throughout the larvae stage after the 2nd instar. Exposure to earlier instars, and over extended exposure time, resulted in a different mortality response compared to the results in the previous chapters in which 4th instar larvae were exposed to both cypermethrin and prochloraz for only 96 h. Chapter 3 highlights that prochloraz had no effect on survival or weight gain on larvae exposed to up to 1000 mg/kg. Whereas here, there was mortality detected above background for larvae exposed to prochloraz at only 10 mg/kg. This near 100-fold difference in sensitivity for effects on mortality emphasise the effects that prolonged exposure can have on exposed species. In larvae exposed to cypermethrin, there is pronounced decrease in the survival of larvae in all treatments above 0.25 mg/kg. This level of sensitivity again contrasts with the results of Chapter 3 where the LC_{50} of cypermethrin exposed to 4th instar larvae is 11.6 mg/kg at 96 hours post exposure.

The greater responses in the current study may relate both to the instar exposed, a result of higher sensitivity of early instar, and the extended exposure time, allowing continued TK and TD dynamics leading to greater effects. Several studies have reported a decrease in sensitivity to toxicants in Lepidoptera as body size and instar increases (De Armas et al. 2020; Knight et al. 2001; Bouvier et al. 2002). One potential explanation for the size dependent

effect is the differential uptake rates at different instars, caused for example by cuticle variation. In addition, a phenomenon of dilution by growth may change the toxicokinetic of the pesticides; the increase in biomass reduced the internal concentration, and the growth is faster for bigger individuals (4^{th} instar) compared to small individuals (2th instar). The dilution by growth explained the difference in sensitivity in earthworm growth (Bart et al., 2020), and may applied here with caterpillars. Also, increase in lipid content in the body has also been found to decrease insecticide susceptibility , potentially due to altered uptake rates (Rubach et al. 2012; Prasad and Roy 2018). A further possible cause of the lower sensitivity of later instars may be related to changes in detoxification enzyme expression in different larval stage. For example, *GST* activity has been shown to positively correlate to instar succession underpinning a potential decrease in sensitivity (Bouvier et al. 2002). It is thought that this second hypothesis may better describe an observed decrease in toxicity, as susceptibility decreased with instar even when toxicants were injected through the cuticle and lipids of insects (Yu 1983; Yucel and Kayis 2019).

Larvae exposed to mixture treatments had higher mortality than those exposed to cypermethrin alone without the addition of prochloraz to the predicted additive effects of the two chemicals combined. The greater than additive toxicity indicates synergistic interactions between the two chemicals confirming the results from the short-term exposures conducted reported in Chapter 3. Synergism was found only in the lower cypermethrin concentration mixture, at the highest concentration mixture treatment of 1.25 + 10 mg/kg, there was no evidence of synergism. Similar results have been previously reported that have used TKTD models to analyse the joint impact of α-cypermethrin and prochloraz in which synergism was seen in low concentration mixtures but not at higher exposure levels (Cedergreen *et al.* 2017). The cypermethrin and prochloraz mixture tests in Chapter 3 also showed synergism in low to medium treatment groups and not at the highest mixture concentrations tested. In the 96 h assay, synergism was not seen in treatments above the LC_{50} value. The similarity of these results indicates that across these different exposure systems these two chemicals show synergism at intermediate, but not high, concentration and effect levels for cypermethrin.

4.4.2. Analyses of the growth data with DEB-TKTD modelling

In the absence of a chemical stressor, larvae use energy for metabolism and growth, or maturation. On exposure to a toxic stressor, there is the potential that a greater proportion of overall energy would be required to maintain metabolism through damage repair. This increase in cost to maintain somatic tissues, in turn reduces the amount of excess energy available for growth (Soetaert et al. 2007). In the exposures carried out here, impacts on growth were seen on larvae exposed to concentrations of cypermethrin 0.25 mg/kg and higher. This is seen in both the growth curve and development times to each instar. Larvae in the 0.25 mg/kg of cypermethrin treatment group further showed significantly different times to moult from instar 4 upwards, with maturation times between stages increasingly extended at high cypermethrin exposure levels. This is even further pronounced in the 1.25 mg/kg treatment where time taken to moult is approximately 50% longer than the control treatment in every instar. Here, the threshold energy budget (z_b) is very low (1.7^10^{-5} mg/kg) with cypermethrin exposure. This highlights that even exposure to low concentrations of cypermethrin could potentially disrupt usual growth rates and delay time taken to pupation indicating the potential to this insecticide to have significant effects on a range of life-cycle traits in target and non-target species.

The dominant rate constant (K_d) represents the combined effect of metabolism and excretion to remove the chemical from the body. For both chemicals, this rate is intermediate (as defined by Jager and Ashauer (2018)) in both cases (1.32 for cypermethrin d^{-1} and 0.21 d^{-1} for prochloraz, Table 4.3). The threshold for effect on energy budget is low for both chemicals, but especially so for cypermethrin (1.7 10^{-5} mg/kg) compared to prochloraz (0.002 mg/kg). This value provides a threshold for sublethal effects and the likelihood of these increased as the exposure concentration further exceeds this threshold. Thus, both chemical exposure at all concentrations will take up the chemical and as internal concentrations increase this exposure will disturb the larval energy budget, leading to sublethal effect at low concentrations.

The nature of the effects on growth and development seen in larvae seen with cypermethrin exposure passing the threshold dominant rate constant is determined by the pMoA of the chemical. For cypermethrin, the best fit pMoA to the observed effect data was an increase of the growth energy cost. This implies that the cost of production of somatic tissue increases

with exposure. In other words, larvae will have a greater expenditure on production of new biomass (Jager et al. 2010). A previous study has linked this pMoA with a strong effect on the ability to synthesis amino acids and proteins, due to inhibition of ribosomal activity- the site of protein production. This study identified disruption to the KEGG protein transport pathway, suggesting a decrease in protein may be responsible for increased tissue growth costs (Swain *et al.* 2010). An alternative, although potentially assimilatory explanation, could also be slow weight gain due to depletion of fats as a result of lipids converting to protein as a detoxification process to combat toxicants entering the body.

To fully understand the pMoA of cypermethrin, knowledge of the primary biochemical MoA may help to understand the mechanisms involved. Cypermethrin exerts its toxic effects on insects by binding to the voltage-gated sodium channel (VGSC), converting it from its active ion-conducting state to inactive and non-conducting (Davies *et al*. 2007). As a result, cells become depolarised and this leads to paralysis (Field *et al*. 2017). Detoxification enzymes such as *CYP450*s and esterases have been reported to be key to the metabolism of cypermethrin. Esterases hydrolyse cypermethrin molecules and *CYP450*s oxidise them to render them into an excretable form than can be conjugated by phase II enzymes (Nakamura *et al*. 2007; Baek *et al*. 2010; Muthusamy and Shivakumar 2015). This detoxification is a costly process. A study of trout by Bains *et al.* (2004) suggested that the cost of detoxification of xenobiotics could be substantial due to increased cell respiration rate. A further mechanism that could be linked to costs for growth.

Further effects could be identified on nerve and other cells that result in cell loss through apoptosis. Cell apoptosis may be mediated by the effects of chemical stressors (Ryter *et al*. 2007), and can therefore, be regarded as an important endpoint in toxicity exposure. Cypermethrin exposure has been seen to increase instances of cell arrest and decrease the viability of cells (Huang *et al.* 2016; Ji *et al*. 2021; Jin *et al.* 2011). While cell apoptosis is a process that occurs throughout the life of an organism (Elmore, 2007), the energy invested in the creation of new cells would be higher if a fraction of the cell cycle is not completed due to cypermethrin exposure.

Azoles fungicides, such as prochloraz, are known to disturb sublethal processes such as hormone synthesis and neonatal development in both vertebrate and invertebrate species through the inhibition of *CYP450* mediated pathways (Zarn *et al.* 2003). Azole fungicides can

delay time between moults of invertebrate species or even completely prevent this process from taking place (Hassold and Backhaus 2009). The growth data generated from prochloraz exposure indicated a decrease on larvae growth rates and development. Whereas in previous exposures of prochloraz alone where there was no effect on weight (see Chapter 3), in this exposure there is significant inhibition of growth from 20 mg/kg upwards that increases with concentration at the 5th and 6th instar moults. These combined effects of prochloraz exposure on both reproduction and growth could be consistent with changes in the physiological regulation of these processes, potentially through effects on hormone systems.

The nature of the effects observed mean that it is not feasible to reliably attribute a cause of death for the exposed larvae. However, it was noted that several of the larvae died during or just after moults in which the cuticle was not shed correctly. This phenomenon has been previously reported in crustacean species as a result of prochloraz inhibition of ecdysteroid synthesis, a process responsible for instigating moulting (Hassold and Backhaus 2009). The frequently observed effects on moulting observed here may indicate that prochloraz exposure also inhibits this pathway in lepidopterans. Such inhibition would account for the mortality seen in prochloraz exposure in addition to delayed maturation. As the pMoA of prochloraz could not be clearly identified, there is no clear information from which to determine which process is impacted by prochloraz. However, in lepidopterans it is the hormone prothoracicotropic hormone (PTTH) that stimulates moulting (Pérez-Hedo *et al*. 2010). There is no evidence of prochloraz mediated inhibition of this hormone, but this could be a viable hypothesis to investigate in future studies.

As seen in Chapter 3, combination treatments of cypermethrin and prochloraz interacted synergistically to affect larval survival and weight change beyond additive predictions. Consistent with these previous results, effects on mortality or growth were commonly synergistic in the lower concentration mixture, although additive in the high level exposures. This observation of different synergism extents at different exposure levels is consistent with the concept of dose level dependence for interactions which was first formalised by Jonker *et al.* (2004).

Due to the MoA of prochloraz which is based on inhibition of a *CYP450* involved in sterol synthesis, it is likely that the inhibition of endogenous *CYP450*s play a role in the synergistic

interactions between cypermethrin and prochloraz. Azole fungicides are commonly reported to increase the toxicity of pyrethroid insecticides by inhibiting *CYP450* isoforms which are essential for the metabolism of lipophilic toxicants such as cypermethrin (Hernández *et al*. 2017; Pilling *et a*l. 1995). Of several azoles tested, prochloraz is thought to be one of the more lipophilic of the azoles and so has one of the greatest potential for *CYP450* interaction (Hassold and Backhaus 2009). This is indicative of the increased binding potential of cypermethrin and could at least partially explain the effects of combination treatments beginning at concentrations as low as 0.05 mg/kg of cypermethrin.

4.4.3. Pupation

Pupation is the process of recycling larval tissues into structures required for the adult form. Larvae are transformed from legless organisms primarily prioritising feeding into flying adults, capable of reproduction. In terms of energy; as pupa, energy flow is one directional. Energy (food) is no longer taken in, but there is a significant amount of transformation occurring within the pupa which requires an energy source of metamorphosis (Nestel *et al.* 2003). Therefore, a supply of energy must be built up during the developmental larvae stages in the fat body (Merkey *et al*. 2011). For this reason, the larval stages of lepidopterans have a much higher metabolic rate than pupae, as they are continuously feeding to store fat and nutrients for pupation (Jiang *et al*. 2019).

Based on the delay in development through instars, pupation was significantly delayed in response to both single chemicals and the mixture combination. Further, no larvae in either of the 1.25 mg/kg treatments of cypermethrin, either as single chemical or as a mixture, pupated within the 40 day exposure. This developmental delay was lower than for cypermethrin in exposures with prochloraz only. Insecticide exposure has been previously reported to delay development to pupa and impact the amount of larvae that fail as pupa or emerge as adults with deformities (Krishnan *et al*. 2021; Dong *et al*. 2017). Therefore, this late pupation was consistent with these finding. However, in contrast to this previous work, sublethal doses of the pyrethroid, permethrin, have been seen to result in faster pupation times in the parasitic wasp *Orgilus lepidus,* indicating species specific differences in the effects of pyrethroids on developmental traits (Symington 2003).

In some insect species, a spike in glycogen levels has been reported immediately before pupation (Sak *et al*. 2009; Nestel *et al.* 2003). Sublethal exposure to cypermethrin can

deplete glycogen stores throughout the larval stages, causing potentially lower availability of glycogen, as a key energy storage molecule at pupation. This change in resource allocation to pupation could explain the results seen here, as larvae took longer to pupate with exposure to all cypermethrin treatments and more larvae failed to pupate in each cypermethrin treatment compared to the controls. In terms of energy, this could be because larvae expend additional energy throughout their development for detoxification and damage repair processes. Any such increase would mean that they lack the energy needed for pupation. Furthermore, glycogen is needed for chitin synthesis which is essential for the structure of the cuticle (Arrese and Soulages 2010; Qu *et al*. 2021). Therefore, if glycogen stores are depleted, a new cuticle cannot be formed against something that could be critical to body reorganisation to support adult emergence.

All mixture treatments, with the exception of the 0.25 mg/kg of cypermethrin increased the incidences of arrested pupal ecdysis more than for any single chemicals, though these effects were not significant. Arrested pupal ecdysis has been reported to affect lepidopterans exposed to insecticides (Krishnan *et al*. 2021), but this does not appear to be the case here as there is no significant difference was found between the incidences of arrested pupal ecdysis in the different treatment groups. It is possible that these incidences are stochastic, indicated in part because they are seen in the control treatment. It is recommended that to fully explore the relationship between pupa ecdysis and pesticide use, a larger experiment is created with treatments over a larger range of concentrations.

4.5. Conclusion

A major criticism of laboratory toxicity testing is the short-term nature of the exposure methods and analysis of a limited range of sublethal end points at only a single time-point. In order to assess the effects of chemical contact on an organism, energy apportionment models such as the Dynamic Energy Budget model (DEB) can be used to determine which process will be impacted most by chemical exposure; growth, metabolism, assimilation or reproduction, over extended exposure durations. Such data can be of value in risk assessment as understanding of the nature of the physiological processes that drive toxicity can ultimately support the development of AOPs that can be used to interpret and monitor chemical effects.

Here it has been demonstrated that a simplified version of the DEB-TKTD model can be applied to simulate chemical effects on a range of life-cycle traits relevant to the full larval development of the lepidopteran *M. brassicae*. The model successfully simulated growth and survival of larvae exposed to cypermethrin and prochloraz. Prediction of mixture effects were frequently underestimated, highlighting a synergistic effect between the chemical leading to greater toxicity than predicted by the additive model. The success of the overall experimental and modelling approach, support the further applications of the method and model used here for pesticide testing for other chemical combinations of interest. Furthermore, the model could be applied to the larval stages of many lepidopteran species as all are isomorphic.

Beginning exposures with 2nd instar larvae and exposing throughout the full developmental period gave different lethal and sublethal toxicity with both cypermethrin and prochloraz compared to the effects seen in short-term single instar exposures conducted in Chapter 3. Thus, whereas in Chapter 3, there were no effects on larvae exposed to prochloraz even at concentrations of 1000 mg/kg, here growth and survival impacts were seen at much lower, and more field relevant, exposure concentrations. The method enables the analysis of delays in development brought about by slow growth. These investigations of development highlighted that both chemicals alter time to moult to each instar. This effect was more pronounced with cypermethrin and mixture treatments highlighting the impact of synergism on maturation times. These synergistic effects were greater at lower concentrations, but not at higher, effect levels.

Using TKTD modelling showed that the combination of azole fungicides and pyrethroids caused synergistic effects when exposed in combination. Synergism effects were found for survival, growth and pupation time. Knowledge of the modes of action of the chemical point to potential effects of prochloraz on detoxification enzymes such as *CYP450*s and inhibition of their role in the xenobiotic metabolism of cypermethrin. The potential for prochloraz to inhibit cypermethrin metabolism through this mechanism has been identified as a common potential scenario leading to synergism. While the methods of both data collection and analysis shown in this chapter are successful in identifying incidences of synergism and what sublethal effects may occur, internal metabolomics or transcriptomics would be need to validate a cause of synergism in further studies.

CHAPTER 5 -DIFFERENTIAL SENSITIVITY OF CABBAGE MOTH ADULTS AND LARVAE

5.1. Introduction

In laboratory ecotoxicology testing, susceptibility of organisms to toxins is often tested on a single life stage. Although there are tests for mammalian species covering the whole life cycle and generational studies (Buschmann, 2013), there are few standard tests available for invertebrates meaning that there is often little or no consideration for differences in sensitivity at different stages for these species. These laboratory studies are intended to inform on both pest management and beneficial species protection practices. Therefore, information on differential sensitivity between life stages would be beneficial to ensure understanding of the effects that agrochemicals have on differentially sensitive stages of pest and non-target species life cycles. Conversely, understanding differential sensitivity between life stages is critical for assessing the risk agrochemicals present to non-target species.

Lepidopterans fill an unusual position in ecology, as they are destructive plant predators in their larval stages, often earning them the undesirable classification as a pest species. However, following metamorphosis, Lepidoptera emerge from their pupa to fill a different ecological niche as obligate nectar feeders. Moths, particularly in the *Noctuidae* family, are reported to pollinate a range of plant species by acting as pollen vectors due to their method of feeding (Hahn and Brühl 2016). Although many lepidopteran species feed as adults on their same larval host plant (Altermatt and Pearse 2011); the impact of moths on agricultural pollination is unclear (Hahn and Brühl 2016). This highlights how contrast in diet can directly influence the ecological position occupied by holometabolous species (those with two distinct life stages that feed).

Adult and larvae Lepidoptera display arguably the clearest example of metamorphosis in the insect class. Upon hatching, larvae must focus on feeding to fuel growth and maturation; however, on eclosion from the pupae, the primary objective of the adult is to reproduce (Ojeda-Avila *et al.* 2003). Therefore, adults fly to find mates and require a high-energy food source, nectar, to feed the metabolic costs of reproduction. Visual identification of adult and larvae moths

indicates their drastic change of external structure. However, surprisingly few studies have explored whether these morphological changes extend to differential sensitivity to agrochemicals.

It is suggested that some generalist herbivore species may possess a greater ability to detoxify plant chemical defences due to their exposure to a larger range of these chemical toxicants than specialist species (Zhou *et al.* 2013). However, it has also been argued that the co-evolution of specialist feeders and host plants provides decreased susceptibility to insects from plant protection chemicals (Olaya-Arenas *et al.* 2020). This contradiction means that it is unclear if a species that is both generalist and specialist at different stages of its life, such as the larvae and adult stages of *Mamestra brassicae,* have increased detoxification capacities for agrochemicals at different points in their life cycles and in turn how these differences may affect lifetime sensitivity to pesticide exposure.

In this chapter, a dietary bioassay was developed for *M. brassicae* adults that is able to provide data on sensitivity that can be compared to that generated previous for larval life stages. In the first stage of this study, the adult moths are exposed to three insecticides, chlorpyrifos, cypermethrin and imidacloprid and three fungicides, prochloraz, azoxystrobin and chlorothalonil. Pesticides were selected due to synergistic interactions recorded in other chapters and sublethal effects on weight in Chapter 3. Imidacloprid was selected for adult sensitivity testing as the low sensitivity displayed by larvae in Chapter 3 was a somewhat surprising result. Survival data was analysed with the GUTS-RED-SD model.

In the next phase, two mixtures that were found to interact synergistically on larvae in Chapter 3 (cypermethrin and prochloraz and cypermethrin and chlorothalonil) were tested again here on adults. Mixture data is assessed using the GUTS_mix package and incidences of differential interactions (e.g. synergy) between chemicals in adults and larvae are investigated. The overall hypothesis for this work is that differential sensitivity will occur between adults and larvae exposed to single insecticides, potentially resulting from different molecular biology (i.e. expression of xeno-metabolic genes) or exposure routes and/or uptake by different life stages. However, it is hypothesised this difference will not be large (no more than 10-fold). It is also hypothesised that fungicides will not impact mortality in adult moths as reflected by larval testing in Chapter 3. Finally, using the approach developed

in Chapter 3, incidences of synergy will be identified for *M. brassicae* adults and compared with those found for larval stages.

5.2. Methods

5.2.1. Test Organisms

To avoid any differential toxicity caused by sex, only male *M. brassicae* were used for the adult bioassays. Males and females were sexed as pupae (Figure 5.1) and separated into the culture or experimental flight cages. Moths were from the same culture as previous chapters and reared following the same methods. Separated males were fed orange-flavoured Gatorade on their emergence and stored in a controlled temperature chamber at 20°c at a 16:8 dark light pattern.

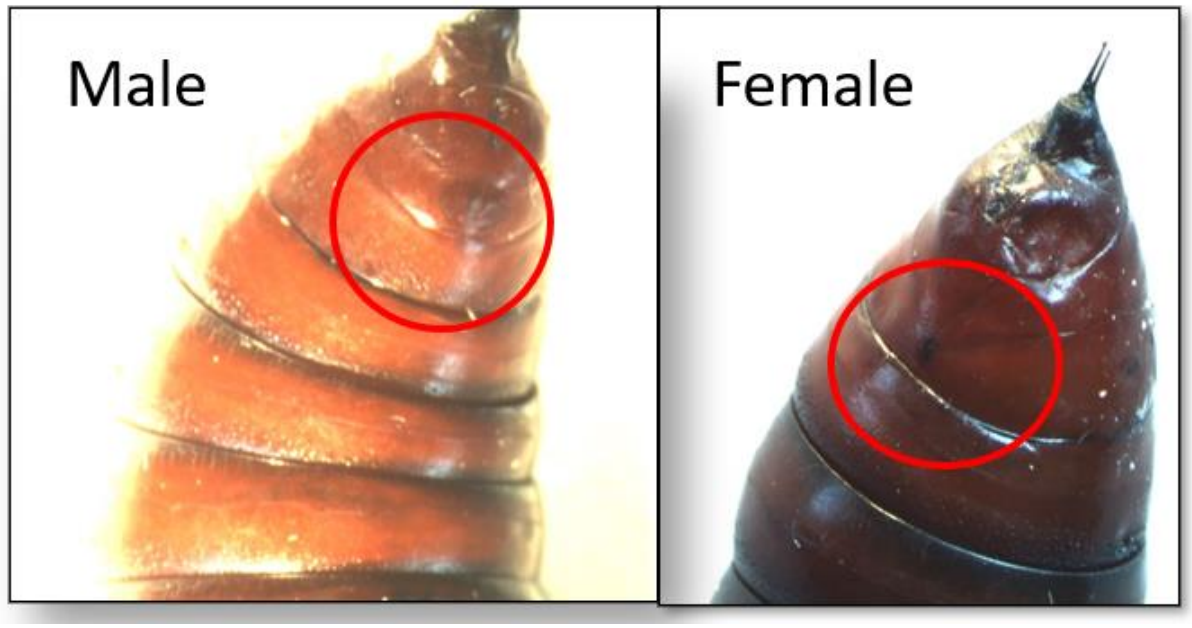


Figure 5.1. Male and female pupae front lower abdomen. Rings highlight the morphological differences.

5.2.2. Preparation of Test Chemicals

Single chemical adult bioassays were conducted for three insecticides, imidacloprid, chlorpyrifos, cypermethrin, and three fungicides, chlorothalonil, prochloraz and azoxystrobin. These bioassays used the same high purity analytical standards of each chemical (>99%, Sigma Aldrich, Poole, UK) as in Chapter 3 and were diluted in series in acetone following the same protocol as used previously. Each insecticide was tested at 7 different concentrations including an acetone control (Table 5.1). Chlorpyrifos was tested as

a model chemical to assess the feasibility and repeatability of this method of adult testing, in the same way it was selected in Chapter 2 for the larval bioassay. Fungicide tests included only two concentrations and a control, as Chapter 3 highlighted they had no effect on larvae survival. Had an effect been found, the full range of 7 concentrations and control would have been conducted as a follow-up to this initial screening phase study. Pesticides used in mixture tests were also diluted following this protocol but consisted of an acetone control, four concentrations of each single chemical and four or five of the combination of chemicals (Table 5.2).

Table 5.1. Pesticides tested and their concentration ranges in mg/L.

Chemical	Concentrations Tested (mg/L)
Chlorpyrifos	0, 1.09, 2.19, 4.34, 8.75, 17.5, 35, 70
Cypermethrin	0, 0.31, 0.77, 1.92, 4.8, 12, 30, 75
Imidacloprid	0, 4.41, 7.94, 14.23, 25.7, 46.3, 83.3, 150
Prochloraz	0, 333, 1000
Azoxystrobin	0, 250, 750
Chlorothalonil	0, 41.7, 125

Table 5.2. Chemical mixtures selected for adult *Mamestra brassicae* exposure and their concentrations

Pesticide Mixture	Concentration Chemical A (mg/L)	Concentration Chemical B (mg/L)	Combination Concentrations (mg/L)
Cypermethrin (A) + prochloraz (B)	0, 0.5, 2.5, 10, 50	0, 1, 10, 50, 200	0.5 + 1, 2.5 + 10, 10 + 10, 10 + 50
Cypermethrin (A) + chlorothalonil (B)	0, 0.5, 2.5, 10, 50	0, 1, 20, 50, 100	0.5 + 1, 2.5 + 20, 10 + 20, 10 + 50, 50 + 50

5.2.3. Test Procedure

Test cages were made of plastic pint-size cups, cut into three sections and assembled as per Figure 5.2. and 5.3. For single chemical tests, once diluted to the required concentrations, chemicals in acetone carrier were added to orange Gatorade solution in a 99: 1 (i.e. 1% acetone) ratio. Mixture tests were made up of 0.5% of chemical A + 0.5% of chemical B. For the single chemical treatments in the mixture study, the Gatorade was spiked with 0.5% pesticide and 0.5% acetone to ensure that there was an equal volume of acetone added to all treatments. The spiked solutions were then drawn into a 60ml leur-lock syringe without a rubber seal to prevent acetone damage to the seal. The syringe was then inserted into the cage through the hole seen in Figure 5.2 to allow the moth access to the dosed solutions.

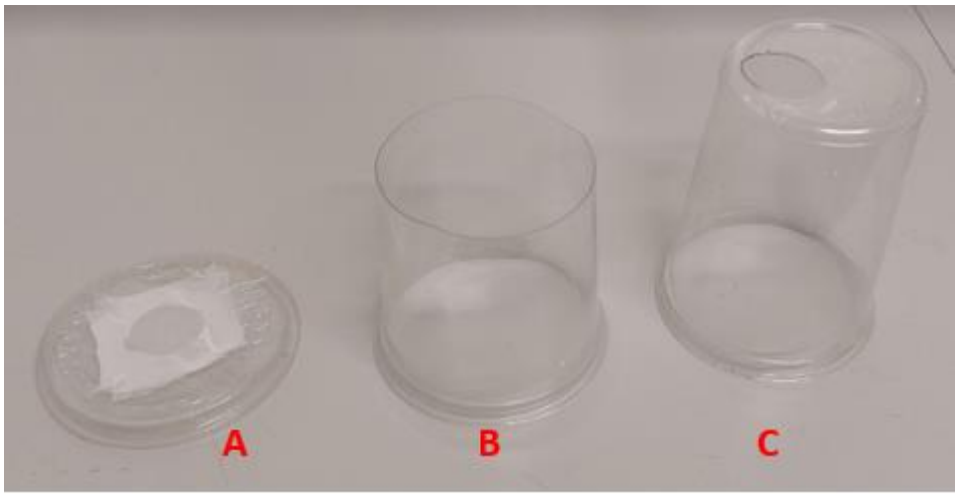


Figure 5.2. Sections of cups for assembly into adult Lepidoptera exposure pots. Note mesh insert into part A and hole for 60 ml syringe in part C. Part B makes up the stand of the exposure cage.

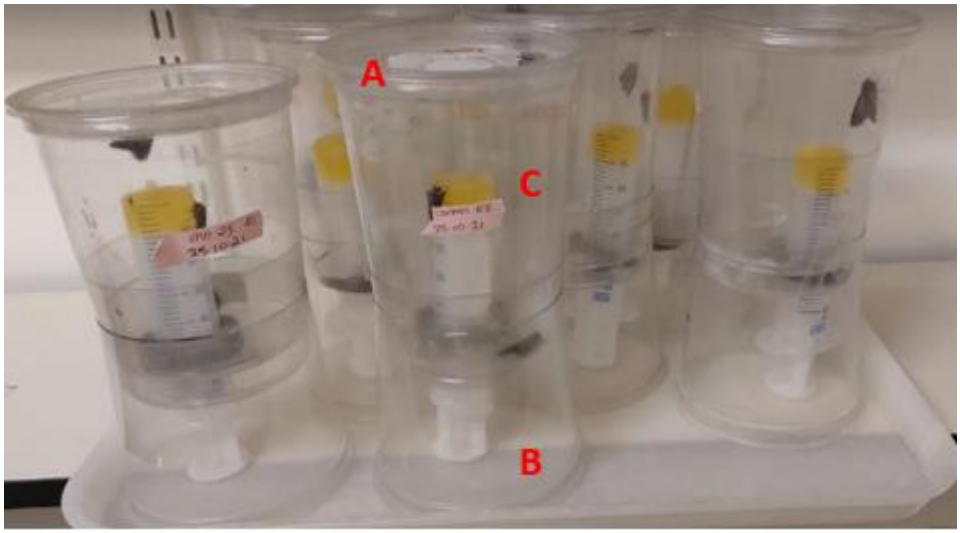


Figure 5.3. Fully assembled adult Lepidoptera exposure test pots. Syringe contains 10 ml of spiked Gatorade solution. Each cage contains three adult male *Mamestra brassicae.*

Tests were run as four replicates, each with 3 moths per cage giving a total of 12 moths per concentration. Survival was checked at 24, 48, 72, and 96 hours by stimulation with a paintbrush. Moths were recorded dead if there was no movement after stimulating behind the head and thorax region for 5 seconds. However, dead moths were often easy to visually identify as they were often found on the floor of the cage, proboscis facing upwards and forelegs crossed.

5.2.4. Data Analysis

As in preceding chapters, single chemical mortality data was analysed with the GUTS-RED-SD model, here, following the same approach. As all moths are genetically similar a stochastic death approach was chosen as individual tolerance was not appropriate given the limited genetic variation of the tested cohort. From the calibrated GUTS-RED-SD model, the LC_{50} values were calculated at each time point and simulated beyond 96 hours. Mixture bioassays were also analysed using the GUTS TKTD method set out in Chapter 3 using the GUTS_mix package *(*www.debtox.info/byom.html*)*. As both of the mixtures tested a fungicide and insecticide combination, the independent action model was chosen as the baseline models for the mixture data analysis. An under-prediction of mortality indicated synergism, an over-prediction antagonism and accurate prediction showed additivity.

5.3. Results

5.3.1. Single Chemical Data

The adult lepidopteran exposures were relatively quick to set up and monitor. The cages were easy to assemble, and the spiking process required less time than for equivalent sized larvae bioassays. An exposure test consisting of eight adult cages took less than an hour to assemble and around 10 minutes to check for survival. The fungicides chlorothalonil, prochloraz and azoxystrobin did not induce any mortalities and 100% survival was recorded in all treatments, including control and the highest treatments of all three tested chemicals. Therefore, they were not analysed with the GUTS-RED-SD model and no further concentration ranges were tested. The insecticides, chlorpyrifos, cypermethrin and imidacloprid, displayed some control mortality; 41.7%, 18.3% and 17.7% respectively. Control mortality of chlorpyrifos was far above the desired upper limit of 20% as set out in Chapter 2. Despite this, most of the chlorpyrifos treatments were simulated well by the GUTS-RED-SD model (Figure 5.4) which takes into account the mortality background, calibrated on the control treatment. However, chlorpyrifos was not continued further into mixture toxicity testing. As the chlorpyrifos test was conducted as an initial study for method development, it is likely that initial optimisation of the rearing processes and procedures for handling caused this high mortality in the control. For example, in this initial exposure, pupae were added to a rearing box and left to emerge, rather than being reared in a flight cage; limiting space on eclosion. This pre-treatment appears to have had an effect on mortality. This was rectified in all other chemical exposures which were reared in flight cages, meaning that the issues with control mortality did not occur subsequently in any other study.

The insecticides, chlorpyrifos and cypermethrin both caused 100% mortality at the highest tested concentrations, 70 and 75 mg/L respectively (Figure 5.4). Chlorpyrifos also saw full mortality in the 4.38 and 35 mg/L treatments. Imidacloprid did not cause full mortality in any treatment, but mortality was increased in all the 46.29 mg/L and higher concentration treatments.

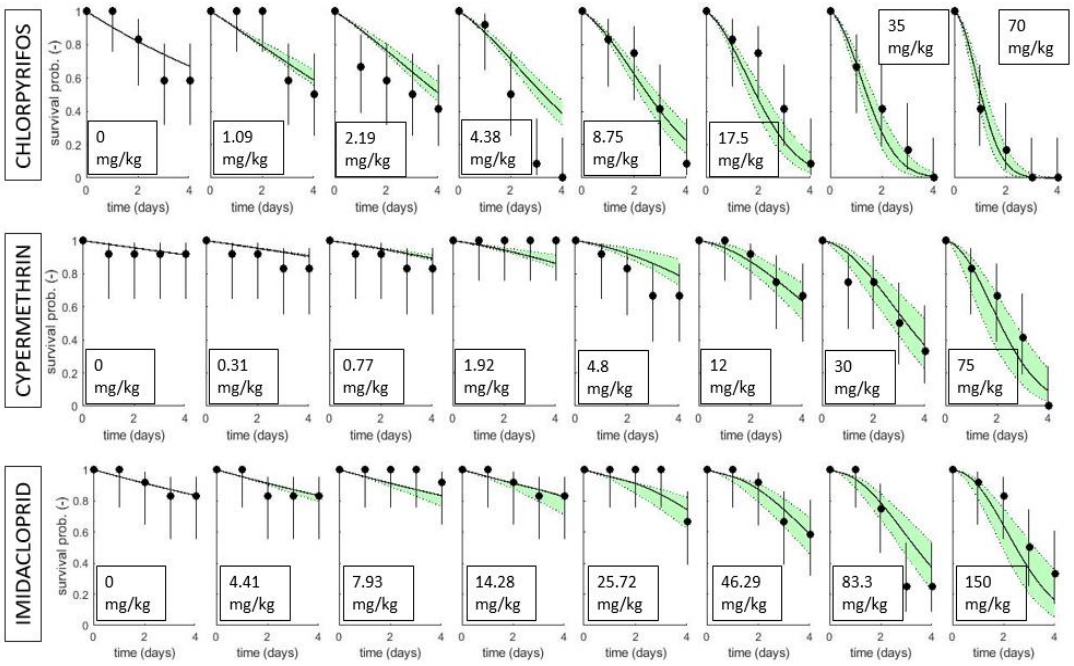


Figure 5.4. Observed and predicted survival of *Mamestra brassicae* larvae exposed to chlorpyrifos, cypermethrin and imidacloprid. Separate rows show different chemicals at each of the 8 concentrations tested. The points are observed data with error bars showing Wilson score confidence and solid black lines are the simulated survival using GUTS-RED-SD model, green areas show 95% confidence intervals.

Insecticide driven mortality was analysed with the GUTS-RED-SD model as in Chapter 2 and an LC_{50} value derived for 48 and 96 hours (Table 5.3). Here LC_{50}s are displayed alongside the values determined from larval testing. Chlorpyrifos was the most toxic insecticide to *Mamestra brassicae* adults (21.1 mg/L at 48h and 5.57 mg/L at 96h), followed by cypermethrin (40.3 mg/L at 48 h and 11.6 mg/L). As the exposure method differed for adult and larvae bioassays, differences in LC_{50} between adults and larvae were shown as a ratio to allow for comparison. Ratios of adult : larvae value were 11.1 : 33.8 for chlorpyrifos, 45.4 : 23.2 for cypermethrin and 148 : 1782 for imidacloprid at 96 hours. This comparison of 96 h LC50 values indicates the greatest difference in adult and larvae sensitivity is for imidacloprid for which the adults are 12-fold more sensitive than the larvae. Moths exposed to chlorpyrifos were 3-fold more sensitive as adults than larvae. Conversely, larvae displayed a near two-fold greater susceptibility to cypermethrin than adults.

Table 5.3. LC_{50} values (and confidence intervals)at 48h and 96h following the exposure of *Mamestra brassicae* to spiked gatorade solution derived with the GUTS-RED-SD model. Larvae data from chapter 2 and 3 three is also presented here.

Chemical	Adult 48 h LC50 (mg/L)	Adult 96 h LC50 (mg/L)	Larvae 48 h LC50 (mg/kg)	Larvae 96 h LC50 (mg/kg)
Chlorpyrifos	22.1 (15.9 - 32.4)	5.57 (4.02 - 8.16)	18.7 (18-19.6)	16.9 (16.4-17.4)
Cypermethrin	40.3 (26.4 - 66.4)	22.7 (14.9 - 37.50)	46.3 (36.7 – 59.3)	11.6 (9.22-14.9)
Imidacloprid	258 (162 - 475)	74 (52.1 - 123)	1980 (865 - 6060)	891 (406 - 1890)

Differences in sensitivity between larvae and adults were most pronounced with exposure to imidacloprid. As mortality in larvae did not exceed 50%, the GUTS-RED-SD model could not accurately calculate an LC_{50} of imidacloprid. In contrast in adults, much greater mortality was seen at lower exposure concentrations and, with the LC_{50} of 258 mg/L at 48h hours and 74 mg/L at 96 hours indicated approximately 8- and 12-times greater sensitivity at 48 h and 96 h respectively.

The GUTS-RED-SD parameter values showed slow kinetics for each of the insecticides as showed by the low value (<0.01) of the K_d parameter estimates in all three cases (Table 5.4). The median of threshold distribution (m_w) (i.e., NEC) were very low, but well characterised with high confidence intervals, and the killing rate (b_w) confidence intervals were even higher making their interpretation risky. In the larval testing in chapter 2, chlorpyrifos kinetics was not slow, as seen here (5 d^{-1}), and the m_w value was 15.6 mg/kg. However, it is likely that the high background mortality has impacted these values. For cypermethrin, the m_w parameter value was 0.003 mg/kg in larvae, this value is much higher in adults, indicative that a higher concentration is needed to induce a toxic effect. Imidacloprid values were similar in both larvae and adult testing, however, the low mortality in larval testing (Chapter 3) meant that parameter values for this chemical were not well characterised.

Table 5.4. Parameter values (including 95% Confidence Intervals) of the GUTS-RED-SD model estimated for three insecticide assays of *Mamestra brassicae* exposed to a spiked sugar based solution. The * denotes where lower CI cannot be calculated because the parameter CI has reached a boundary.

Chemical	Background mortality	Dominant rate constant	Median of threshold distribution	Killing rate
	h_b (d^{-1})	k_d (d^{-1})	m_w (mg L^{-1})	b_w (L mg^{-1} d^{-1})
Chlorpyrifos	0.1	0.007 (0.002*-0.44)	0.0003 (0.0003*-0.218)	2.34 (0.049-13.06)
Cypermethrin	0.022	0.005 (0.002*-1.62)	8.480e-05 (8.480e-05 - 2.653)	0.721 (0.007-3.62)
Imidacloprid	0.4547	0.002* (0.002* - 0.837)	0.068 (0.001* - 18.46)	0.0002 (0.0002*-0.09)

5.3.2 Mixture Data

Mixture exposures were set up to assess survival. However, as the experimental size was almost double that of the single chemical bioassays, many more pupae were needed which made these exposures much more time consuming in terms of both culturing and study set-up. Furthermore, the experiment required a large number of pupae to emerge at the same time, necessitating that some replicates were not carried out in the same week. Despite these challenges, both of the mixture experiments, cypermethrin + prochloraz and cypermethrin + chlorothalonil, had 100% control survival and use of the same stock solution, stored at 4◦C and wrapped in foil to prevent photo-degradation, which means that all replicates and treatments can be directly compared. Cypermethrin survival was well simulated by the GUTS-RED-SD model in both mixture experiments, which is indicative of a

robust experimental technique capable of achieving repeatable results with a high level of control survival.

Synergism in the mixtures was identified based on the under-prediction of the observed effect by the GUTS-Mix model as outlined in Chapter 3. Mixture data of cypermethrin + prochloraz (Figure 5.5) was identified as synergistic for at least one time point in every mixture treatment combination. Furthermore, the extent of this synergism became more pronounced with increasing prochloraz concentrations. For example, a 10 mg/L cypermethrin exposure resulted in partial mortality when exposed with 10 mg/L prochloraz, but full mortality was reached in a combination with 50 mg/L of the fungicide.

The mixture of cypermethrin and chlorothalonil was identified as additive as per the criteria outlined in Chapter 3 (Figure 5.6). Moths exposed to cypermethrin only, did not reach 100% mortality in any concentration treatment, however, deaths were monotonic and concentration-dependent. The mixture treatments were predicted well by the GUTS-RED-SD IA model until the 50 + 50 mg/L treatments of cypermethrin and chlorothalonil. This data suggests some extent of antagonism in this treatment, however, here the mixture is classed as additive as the scale of this interaction is small and further testing based on an expanded experimental design would be recommended to confirm an antagonistic interaction. This experiment also highlighted no change in mortality of adults exposed to 10 + 20 mg/L of cypermethrin and chlorothalonil and those exposed to 10 + 50 mg/L of cypermethrin and chlorothalonil. The reverse is true for cypermethrin and prochloraz in which mortality increases with prochloraz concentration.

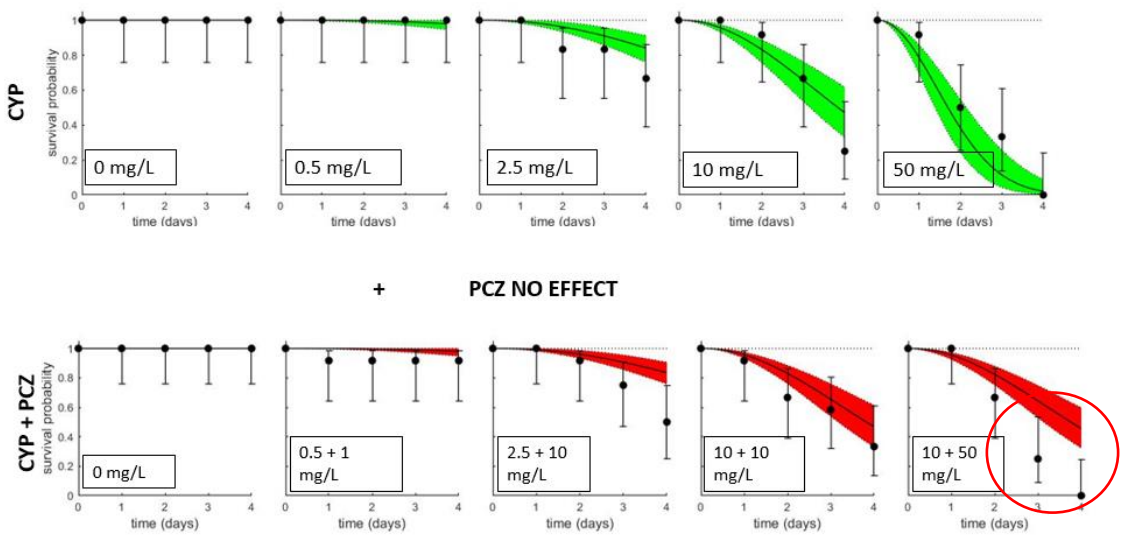


Figure 5.5. Mortality prediction for binary mixtures of cypermethrin and prochloraz modelled using the GUTS_RED_IA_SD model. Solid black lines show simulated survival, filled areas are 95% CI and black points observed survival data. Note in this exposure there is deviation from the survival prediction, therefore, it was identified as synergistic.

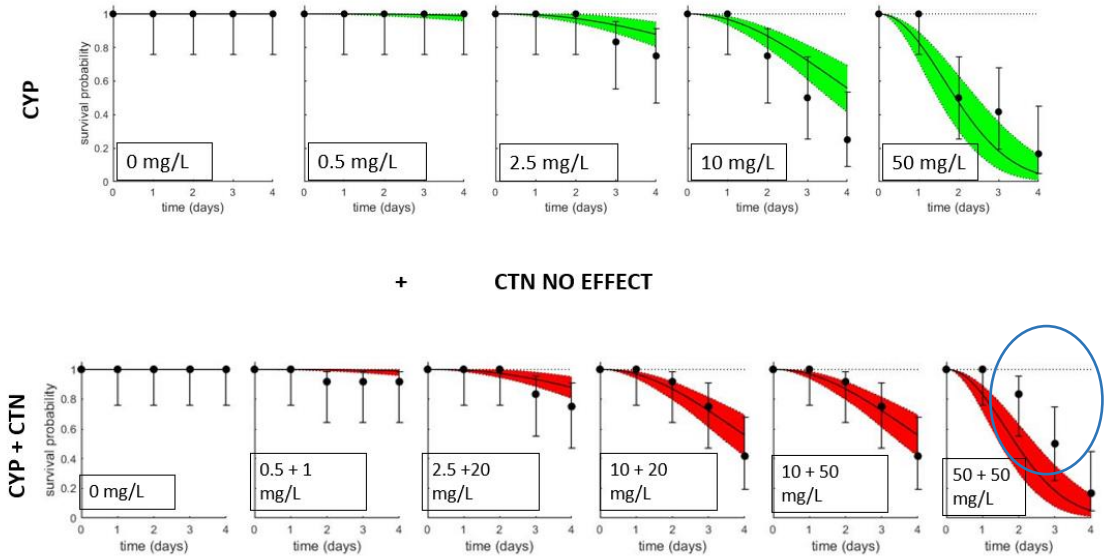


Figure 5.6. Mortality prediction for binary mixtures of cypermethrin and chlorothalonil modelled using the GUTS_RED_IA_SD model. Solid black lines show simulated survival, filled areas are 95% CI and black points observed survival data. Note in this exposure there is a slight over-estimation from the survival prediction but as this is only in the highest tested combination of chemicals, this mixture is classed as additive.

5.4. Discussion

Much of the literature detailing the efficacy of agrochemicals as larvicides and adult toxicity data were scarce (Usmani and Knowles 2001). Pest management strategies often rely on seasonal applications of agrochemicals. Therefore, if life-stages differ in sensitivity, then the timing of agrochemical application may have implications for the efficacy that an insecticide has the exposed individual. For example, where sensitivity to insecticides is greater in larvae compared to adults; pest management strategies should preferentially target the more sensitive life stage.

The single chemical bioassays conducted for *M. brassicae* adults mirrored the results of Chapter 3. Neither adult or larvae survival was impacted by fungicide exposure up to the maximum concentration tested in any study. However, the insecticides chlorpyrifos and cypermethrin were toxic to both larvae and adult life stages. The biggest contrast in life-stage sensitivity seen here is in imidacloprid exposure. The exposures performed here suggest there is a clear concentration dependent mortality effect on adults, giving an LC_{50} of 74 mg/L at 96 hours. This compares to a 96 hourLC_{50} for the larvae of 891 mg/kg.

Mixture testing was focused on fungicide-insecticide mixtures. Adult mixture testing again confirmed a synergistic interaction between cypermethrin and prochloraz as seen for the larvae in Chapter 3. The results of the mixture test of cypermethrin and chlorothalonil conducted for adults was not consistent with the results for larvae given in Chapter 3, previously, cypermethrin and chlorothalonil were found to interact synergistically in larvae whereas in adults no interaction was seen. In fact, the result of the 50 + 50 mg/L cypermethrin and chlorothalonil treatment appeared to identify an antagonistic interaction. However, this interaction was only seen in one treatment and further confirmatory testing would be needed to confirm any consistent deviation from the dominant observation of additivity.

The comparison of adults and larvae suggest differential sensitivity between the two stages of *M. brassicae* most notably for imidacloprid. There are a range of factors that could play a role in the increased sensitivity to of adults to this insecticide. Firstly, it should be noted that the exposure methods differ between adults and larvae and therefore, the results from the two assays are not directly comparable. Although both bioassays expose *M. brassicae* to

pesticides via spiking of feed, morphological differences in the life stages meant that the exposure method had to be adapted. For example, larvae have mouthparts capable of ingesting solid diet whereas adult moths have a proboscis and must ingest feed via a liquid medium (Powell 2009). This may mean that the wheat germ based agar diet and Gatorade solution differ in terms of bioavailability. Despite these potential differences, the adult and larval comparisons for cypermethrin and chlorpyrifos indicate that the life-stages are relatively similar in terms of their sensitivity. Imidacloprid was a stark contrast to this trend. Imidacloprid has a lower K_{ow} than either chlorpyrifos or cypermethrin, meaning that it may be expected to interact less strongly with any solid phase component such as wheat germ. As a result, it would be expected to have quite similar concentrations in the liquid phases of both diets. Hence, given the similarity for chlorpyrifos and cypermethrin, but difference for the more water-soluble imidacloprid it does not seem that dietary bioavailability is likely to fully explain the differences in sensitivity seen. In the adult bioassay, imidacloprid does not undergo the short period of warming to 50^{o}C that was required for the larvae diet. The absence of this heating step may limit the degradation that happened in the larval test. However, as discussed previously, the heat stability of imidacloprid should ensure this does not impact results (Zheng and Liu 1999). Furthermore, imidacloprid is relatively stable in both water and soil (Tisler *et al*. 2009) unless exposed to an alkaline solution (Zheng and Liu 1999; Liu *et al*. 2006). Hence, again this difference does not seem likely to explain the results, especially given the mildly acidic pH (pH 3) of the Gatorade solution (Reddy *et al*. 2016).

The larvae bioassays provide a dual exposure in which the agar diet provided both a contact and oral exposure. Although this bioassay was designed in this way to mimic field conditions in which a caterpillar would be in contact with a leaf it is feeding on, it was difficult to mirror this in the adult bioassay. It has been suggested that the main route of insecticide penetration is through the cuticle (Pedersen *et al*. 2020), however, the adult cabbage moths tested here had a much greater susceptibility to imidacloprid despite this lack of cuticle contact. Overall, it was difficult to extrapolate pesticide stability data to the conditions applied in the methods used in this thesis as the artificial diet (at least for larvae, potentially adult exposures) were novel. However, with further chemical analyses it would be possible to confirm that changes in bioavailability levels were responsible for the differential sensitivity seen in adults and larvae exposed to imidacloprid. While differences in bioavailability cannot be ruled out, there is no clear reason why the two methods should lead

to notably altered imidacloprid availability and this is an area recommended for further study.

Lipid content has been implicated in the sequestration of lipophilic insecticides (Prasad and Roy 2018; Roy et al. 2008. Variation of lipid levels may also explain the differential sensitivity seen between adult and larvae cabbage moths. Lipids provide several essential functions in terrestrial invertebrates. They are required for cell membrane structure and maintenance, are a rapidly mobilisable energy source and serve as waterproofing agents of the cuticle (Lease and Wolf 2011). It was suggested that juvenile arthropods have a higher lipid content than adults, to meet the metabolic demands of growth and maturation (Downer and Matthews 1976) and that lipid levels of larvae increase with instar (Scriber and Slansky 1981). Pedersen *et al.* (2020) calculated a weight-adjusted dose to compare the amount of active substance per mg of insect and found that adult grain beetles (*Tenebrio molitor*) were the most sensitive to α-cypermethrin as adults, that were expected to have the lowest lipid content. However, a study of the lepidopteran Tobacco hornworm (*Manduca sexta*) reported fat content of 10 to 20% in 5th instar larvae and 25-35% in adults, the day after eclosion from pupae (Ojeda-Avila et al. 2003). This difference may be species and diet-dependent, as it is recognised that artificial diets fed *ad libitum* result in higher lipid levels of caterpillars than leaf fed populations (Cookman *et al*. 1984). Elevated lipid levels in adult Lepidoptera has been implicated in fuelling flight, possibly for reproductive purposes, and is said to decrease rapidly, perhaps leading to lepidopteran death within 10 days (Gilbert and Schneiderman 1961). Thereby, highlighting the importance of selecting adults of a similar age for bioassay testing.

Lipid content can also vary by sex. Females are thought to require a greater lipid energy reserve to fuel oocyte production costs (Lease and Wolf 2011). While every effort was taken to remove this variable by testing only male adults, juveniles could not be sexed and therefore larvae testing was carried out on both sexes. This was not expected to impact the experiments of this chapter as the sexual dimorphism in the larval stages of Lepidoptera is not greatly pronounced. Gene expression analysis, a reasonable marker of sexual dimorphism, reveals relatively few differentially expressed genes between male and female larvae of the lepidopteran *Bicyclus anynana* (Ernst and Westerman 2021). However, it is important to highlight that imidacloprid is not as lipophilic as organophosphate insecticides, such as chlorpyrifos of pyrethroids, such as cypermethrin and so it is again unlikely to be the

sole cause of any differential sensitivity between life stages, given the similar sensitivities found for the two more lipophilic chemicals (Sun *et al.* 2016).

A final possibility is that physiological differences linked to the toxicokinetics and toxicodynamics of the larvae and adult life stage may account for the differential sensitivity to pesticides. The target for imidacloprid is the nicotinic acetylcholine receptor (nAChR). Differential expression of or binding affinity of the nAChR subunits between the two life-stages could be a cause of the observed differences in senstivity. The nAChR is a ligand gated ion channel made up of 5 transmembrane subunits arranged around a central ion-conducting pore (Casida 2018). Subunits can be arranged as homooligomeric and heterooligomeric (built of the same or different subunits) (Figure 5.7). This difference in structure may have implications for toxicity of pesticides. For example, the neonicotinoid family spynosyns only interacts with the α6 homooligomeric receptors (Lu *et al.* 2022).

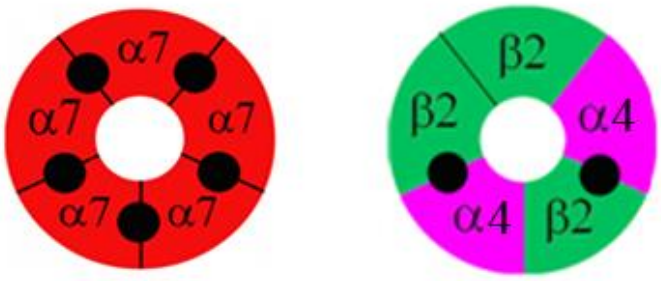


Figure 5.7. Schematic of two nAChR variants taken from Tsetlin et al. (2014). Probable binding sites of agonists are indicated by black circles.

The major target nAChR subunits of imidacloprid are α1/α2/β1/β2 (Lu *et al.* 2022). However, mutations of the α1 subunit were reported to cause resistance to imidacloprid in the brown planthopper (*Nilaverpa Lugens*), perhaps by alterations to the binding site (Liu *et al.* 2005). In *M. brassicae* is possible that variation between expression levels of these subunits in the larvae and adult stages could account for the differential sensitivity between life-stage found here. For example, target subunits α1/α2/β1/β2 may be expressed at a higher level in the adult than larvae, providing more binding sites for imidacloprid. Conversely, larval subunits may comprise of more homoligomeric receptors, which may have a lower binding affinity for imidacloprid.

Further physiological differences could also be associated with the expression of xeno-metabolic enzymes such as *CYP450*s, esterases and *ABC transporters*. It is widely acknowledged that the primary route of neonicotinoid resistance is over-expression of the phase I metabolic enzymes, *CYP450s*. This has been widely studied in the whitefly (*Bemisia tabaci*), as it was the first insect reported to develop neonicotinoid resistance in the field (Cahill *et al.* 1996). Studies reported that increased expression of CYP6CM1 was associated with resistance to imidacloprid in *B. tabaci* (Karunker *et al*. 2008; Nauen *et al*. 2013; Karunker *et al*. 2009; Yang *et al*. 2013). Furthermore, neonicotinoid resistance in this species can be age specific, with nymphs exhibiting greater sensitivity to neonicotinoids than adults (Nauen *et al.* 2008; Jones *et al*. 2011; Yang *et al.* 2013). This difference was attributed to an over-expression of CYP6CM1 in adults in comparison to juveniles. Although these results directly contradict the imidacloprid toxicity tests seen here, they do indicate that expression of metabolic enzymes can vary throughout the life of an organism and may therefore influence sensitivity.

Yang *et al.* (2013) suggested that, as well as CYP450s, Glutathione-S-Transferases (*GST*s) are also involved in the detoxification of neonicotinoids. This mechanism has only begun to receive attention in recent years and so is not as well understood as CYP450 detoxification. *GST*s play a major role in phase II metabolism (Jancova *et al.* 2010). Elevated *GST* expression has been reported in neonicotinoid resistant insect populations (Halappa and Patil 2016; Højland and Kristensen 2017; Zhou *et al*. 2022). However, it is unknown if the contribution of *GST*s is equal to, greater or less than that of CYP450s in metabolism of imidacloprid. Further, some studies suggest that although *GST*s are key in phase II metabolism processes, imidacloprid resistance is not affected by *GST* expression (Gao *et al*. 2020; Bakker *et al.* 2022; Herbert *et al.* 2020). Therefore, care should be taken when drawing conclusions on expression levels of this xeno-metabolic enzyme and imidacloprid sensitivity.

The impact of chlorothalonil on *GST* activity has been documented (Reyna *et al.* 2021). In the larvae, depletion of glutathione was identified as a likely cause of synergism. However, this pattern of interaction was not seen in the adult tests. Differential depletion and/or levels of glutathione in adults and larvae could explain the differing observations of synergy. Further investigations to determine glutathione depletion in adults and larvae following chlorothalonil exposure could reveal useful insights. However, speculatively, it is possible

that glutathione levels are higher in adults than larvae, therefore, requiring a higher chlorothalonil exposure to deplete.

The final phase of metabolism, phase III, is excretion. A superfamily of enzymes implicated in this process are the ABC transporters (Phang-Lyn and Llerena 2022). This superfamily is also often overlooked in neonicotinoid resistance studies. However, recent work is beginning to highlight the potential importance of this enzyme superfamily in neonicotinoid resistance,, as over-expression of ABC transporters results in lower sensitivity (Pan *et al.* 2020; Li *et al.* 2020; He *et al*. 2018). Chlorpyrifos sensitivity is also reportedly decreased in insects with greater expression of ABC transporters (Li *et al*. 2020).

Given the complexity of the different xeno-metabolic pathways and the remodelling of gene expression during metamorphosis, there are a range of potential causes of any difference in sensitivity and mixture effect response between juvenile and adult life-stage. Such changes may occur at the whole body level or even be tissue specific (Dussaubat *et al*. 2016). Thus, expression of key xenometabolic genes and resulting enzymes and transporters may differ in either Lepidoptera larvae or adult tissues because of the clear morphological structural differences between these stages. For this reason, further chemical and molecular analyses are required to identify the mechanisms of differential sensitivity and synergy in holometabolous species.

5.5. Conclusion

Lepidopterans occupy a distinct position in ecology as both pollinator and pest. Pest management strategies often focus on the control of larval stages. However, it is unknown how effects on this life stage for pest or beneficial species are related to effects on adults following insecticide applications. Although there is a general consensus that larvae susceptibility decreases proportionally with the size of larval, there are very few ecotoxicological studies detailing differential sensitivity between adult and larvae.

In this chapter, a bioassay was developed to test the sensitivity of an adult lepidopteran, *Mamestra brassicae*. In previous chapters, testing has focused on larvae and a wheat germ-based exposure method. However, as adults lack the mouth parts of juveniles, an isotonic drink feeding solution was created and spiked with insecticides and fungicides.

Comparing adult to larval mortality, differential sensitivity to imidacloprid was clearly demonstrated. Although it is difficult to directly compare results given the different designs of the assay, the fact that life-stage sensitivity was much greater for imidacloprid than for the other two insecticides indicates that any difference observed is not likely to be related solely to difference in bioavailability between the test methods. The finding of life-stage specific sensitivity for imidacloprid could have important consequences for pest management practices, highlighting that perhaps targeted management of adult lepidopterans may be a more efficient strategy than a focus on larval treatment alone.

Studies with binary mixture of an insecticide and two fungicides indicated consistent synergy in adults and larvae for the cypermethrin prochloraz mixture indicating a consistent mechanistic basis for this interaction in both life-stages. The synergy that was identified in the mixture of chlorothalonil and cypermethrin in the larval stages was, however, not found in the tested adults. This difference could again inform the way that agrochemicals are applied, as a tailored rotation of agrochemicals may provide a more cost-effective and ecologically-friendly solution, i.e by applications of a pyrethroid/organochlorine fungicide mixture in the larval season and a neonicotinoid to the adult.

While it is entirely possible that differential sensitivity seen between these life stages is in some way related to inter-experimental variability, it is also probable that metabolic enzyme

expression differs between life stages. Analyses of transcriptomic data would identify the extent relevant xeno-metabolic enzymes are differentially expressed between adult and larval stages. This data could be pivotal to protecting beneficial species by development of targeted pest management strategies designed to be most efficient throughout growth phases of organisms. Furthermore, analysis of gene expression could aid identification of mechanisms behind synergy in agrochemicals; essential for both crop protection and biodiversity conservation.

CHAPTER 6 – MECHANISMS OF DIFFERENTIAL SENSITIVITY AND SYNERGY

6.1 Introduction

Sensitivity to pesticides, including neonicotinoids, has been associated with both toxicodynamic and toxicokinetic changes, including altered receptor sequences and divergent xeno-metabolic capacities (Matsuda *et al.* 2020). The nAChR is made up of α and non-α subunits, arranged in a pentamer formation. Pentamers can consist of the same (homopentamer) or different subunits (heteropentamer) (Matsuda *et al.* 2005) however, heteropentamers require α and non-α subunits. Within these pentamer structures, each subunit is connected by a ligand binding domain (LBD) forming several interacting loops. Using the framework created by Short *et al* (2021) residues in loop sequences can be identified which in turn characterise the expected response of the nAChR to imidacloprid exposure. Thus, allowing a prediction of functionality of nAChR structures.

In order to mitigate the toxicity caused by xenobiotic compounds, insects possess a system for the detoxification of organic molecules based on three phases of xeno-metabolic processing and transport. This elaborate detoxification system can nullify the toxic effects of pesticide application by transforming, metabolising and excreting toxicants. During the modification stage (Phase I metabolism), the parent organic compound molecule is biotransformed into a more water-soluble metabolite (Phang-Lyn and Llerena 2022). The reaction process is often aided by esterases and enzymes of the *CYP450* enzyme families. The metabolites arising from Phase I metabolism are often further transformed by Phase II enzyme families such as glutathione S-transferases (*GST*s) performing conjugation reactions that further increase water solubility and that also prevent metabolites from diffusing across cell membranes (Pavlidi *et al.* 2018). Finally, these metabolites may be excreted from the cell into the excretory system via active transport by Phase III system proteins, such as *ABC* transporters (Merzendorfer 2014). Through the ability to detect and respond to chemical exposure, toxicants can, therefore, result in the upregulation of one or more xeno-metabolic enzymes critical to detoxification (Liang *et al*., 2015) and mutations that lead to constitutive overexpression are often found in resistant species (Højland and Kristensen 2017; Mansoor *et al.* 2019). Therefore, it is essential to understand the internal mechanisms of pesticide detoxification and their implications for resistance.

Probably the most important enzymes in Phase I metabolism are the xenobiotic metabolising members of the *CYP450*s. *CYP450*s are a superfamily of proteins found in all kingdoms of life (Kweon *et al.* 2020). In insects, *CYP450*s are generally divided into four clans, CYP2, CYP3, CYP4 and mitochondrial (Mito) clan. Each of these clans have roles in essential life functions. For example, the CYP 2 and the Mito clan are primarily involved in ecdysteroid and hormone synthesis and metabolism (Feyereisen 2006), whereas *CYP450*s from clans 3 and 4 are well documented to have links to xeno-metabolic detoxification and are well studied in regard to their functional role in pesticide resistance (Feyereisen 2006). In Lepidoptera, it has been suggested that this family has largely diversified in insects in response to the wide range of anthropogenic and plant chemicals encountered in the environment in comparison to bee species (Claudianos *et al.* 2006). This is likely due to the polyphageous diet of the larval stages which require detoxification capabilities for volatile metabolites produced by host plants in addition to xenobiotic exposures (Gouin *et al*. 2017). Based on the potential for organic molecule catabolism, *CYP450* expression has been found to detoxify a range of insecticides, including pyrethroids, neonicotinoids and organophosphates (Niwa *et al*. 2011; Liu *et al.* 2018a; Shi *et al.* 2021).

*GST*s are a detoxification enzyme super-family that are important for xenobiotic metabolism. In insects, *GST*s are grouped into six classes, delta, epsilon, omega, sigma, theta and zeta (Yamamoto *et al*. 2009) based on their location, function or genetic structure. Enzymes from this family are widely important in Phase II metabolism and can detoxify both exogenous and endogenous compounds via the conjugation of glutathione, a modification that also generates metabolites that are easier to excrete (Enayati *et al.* 2005; Skopelitou *et al*. 2012). For example, upregulation of delta and epsilon *GST*s is suggested to increase resistance to the organophosphate chlorpyrifos in the rice leaffolder (*C. medinalis*) (Liu *et al* 2015).

ABC transporters are a further superfamily of proteins that are important in Phase III metabolism. The proteins from this class are involved in the active transport of substrates across cellular membranes, although they have also been found to act as ion channels and receptors in humans (Hopfner 2016). The *ABC* transporter superfamily is grouped into eight classes, depending on the structure of their ATP-binding site (Merzendorfer 2014); ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, ABCG and ABCH. There is good evidence that *ABCs* are involved in pesticide elimination for cells. Overexpression of ABCB genes has been detected in pyrethroid-resistant *Aedes egyptii* and DDT-resistant *D. melanogaster* (Bariami *et al.* 2012;

Wu *et al.* 2019), suggesting this class has an important role in pesticide transport. Further, classes A, C, G, H, and F were upregulated in strains of P. *xylostella* resistant to chlorpyrifos (He *et al.* 2012); suggesting each different *ABC* transporter may have their own specific roles in detoxification for a given toxicant.

As genomic and transcriptomic tools become more accessible, insights into the metabolomic capacities of insects can be explored in greater detail. This enables the identification and characterisation of xeno-metabolic enzymes and their functions in a specific species. This data can also be used to investigate resistance and detoxification pathways at a species and even population level (Kouamo *et al*. 2021); informing both pest management and conservation practices. Although *CYP450*s, *GST*s and *ABCs* have been characterised for many arthropod species, they have not yet been studied in the lepidopteran *Mamestra brassicae*. *M. brassicae* is a member of the *Noctuidae* family, the second largest family of moths, containing over 11,000 species (Fritz 2011). As such, if the xeno-metabolic enzyme gene repertoires and expression in *M. brassicae* are well conserved throughout this family, the study of xeno-metabolic genes in this species could have wider implications for both conservation and pest management for many lepidopterans in relation to their responses to pesticide exposure.

Lepidopteran metabolomic testing has previously indicated that the response of xeno-metabolic enzymes can vary between the adult and larval stages. For example, Ottea *et al.* (2000) found that *CYP450* activity significantly differed between the adults and larvae, with adults displaying the highest enzymatic activity. Furthermore, studies of the fellow Noctuid, the cotton leafworm (*Spodoptera littoralis*) found that several *CYP450*s were only found in one life stage, either larvae or adult (Pottier *et al.* 2012). It was also reported that CYP6B7 was rarely found in adult *H. armigera* but expression increased throughout larval stages (Zhang *et al.* 2010). Conversely, in *D. melanogaster* CYP6t3 was only found in adults (Chung *et al*. 2009). As *CYP450*s from this family (family 6, clan 3) are known for their involvement in insecticide detoxification, differential expression between life stages may explain any differential sensitivity. This knowledge of the repertoire and expression of different components of the metabolic enzyme systems can support research to understand species and life-stage specific responses to chemical exposure within a predictive ecotoxicological framework.

In the previous chapter, it was highlighted that lepidopterans occupy an interesting position in ecotoxicology as the metamorphosis between larvae and adult present a clearly different phenotype from organisms that ultimately share the same genome. This provides the opportunity to compare differential sensitivity to pesticides between life stages. Therefore, *M. brassicae* adults were exposed to range of pesticides to assess survival and determine differences in sensitivity to adults and larvae. Results from this experiment indicated a clear difference in the sensitivity of adults compared to larvae for the neonicotinoid imidacloprid. This case of life-stage specific differential sensitivity provides a case study through which the role of internal mechanisms linked to processes such as biotransformation can be explored. In this chapter, toxicodynamic traits will be investigated by comparing the sequence and expression patterns of the nAChR subunit gene.

Then, the second part of the chapter aims to identify xeno-metabolic enzymes from the genome of *M. brassicae*, i.e. *CYP450*s, *GST*s and *ABC* transporters using *D. melanogaster* as a comparative species. These enzymes will then be compared to other insect species, for example, the honeybee *Apis melifera* and silkworm *Bombyx mori*. The expression patterns of these enzymes and transporters are then compared between two distinct life stages of *M. brassicae*, larvae and adult in order to identify those candidate components of the xenometabolic systems that show the greatest variation in activity between the adult and juvenile stages.

This study is, thus, multi-faceted in its aims as it intends to; i) compare sequence and expression patterns of the nAChR, ii) Identify *CYP450*s, *GST*s and *ABC* transporters present in this lepidopteran species, iii) compare genomes to that of another lepidopteran to highlight conserved gene families within this order, iv) investigate transcriptomic data to determine differential expression patterns between larvae and adult life stages. By drawing on survival data obtained in the first part of this chapter and linking these to xenometabolism status of each life-stage, it is intended to identify putative enzymes that may influence the differential sensitivity of lepidopteran life-stages to imidacloprid and potentially further pesticides.

6.2 Methods

6.2.1 Generation of *M. brassicae* Genome

The *M. brassicae* genome was prepared by the Wellcome Sanger Institute (Cambridge) using 4th instar larvae cultured at UKCEH (i.e., the culture used for previous chapters, NCBI Genbank Sample ID: SAMEA7524129). The assembled genome has been deposited in Genbank (NCBI) database (accession GCA_905163435.1). Detailed assembly details are available in Genbank. Briefly, the assembly is built using 35x coverage PacBio data, 10X Genomics Chromium data, and Arima Hi-C data. The assembly involved the following steps: initial PacBio assembly was generated with Hifiasm (Cheng *et al.* 2021), and retained haplotig separation with purge_dups (Guan *et al* 2020). The assembly was polished with the 10X Genomics Illumina data by aligning to the assembly with longranger align (https://support.10xgenomics.com), calling variants with freebayes (Garrison & Marth, 2012), and Hi-C scaffolding with SALSA2 (Ghurye *et al*. 2019). The mitochondrial genome was then assembled with MitoHifi (Uliano-Silva *et al.,* 2021). Lastly, the assembly was manually assessed using gEVAL (Chow *et al*., 2016).

6.2.2 Genome Annotation

Genome annotation was carried out at Cardiff University (Prof. P. Kille). In brief, predicted proteins generated from ensemble annotated transcripts were further annotated by identifying orthologues using NCBI blastp (v2.12) (Altschul *et al.* 1990) against the following Uniprot proteomes: *Homo sapiens* (UP000005640), *Mus musculus* (UP000000589), *Drosophila melanogaster* (UP000000803), *Caenorhabditis elegans* (UP000001940) and *Saccharomyces cerevisiae* (UP000002311). Orthologue matches were identified if the blast e-value, the number of expected hits of similar quality (score) that could be found just by chance, was less than 10^{-10}. Uniprot IDs of orthologous matches were used to retrieve further annotations including pfam, interpro, GO ontology and reactome links using the Uniprot retrieval.ID mapping tool (https://www.uniprot.org/id-mapping/).

6.2.3 Generation of Transcriptome

Tissues (nerve cord and whole body) were dissected from a single 4th instar larvae and adult males taken from the UKCEH culture. Tissue was homogenised in 600 µL TRIzol (ThermoFisher, UK), before RNA was purified with Direct-Zol RNA MiniPrep kit (Zymo Research, CA, USA). Samples were then DNAse I treated (New England BioLabs, Ipswich, MA, USA) and concentrated (RNA Clean & Concentrator-5, Zymo Research) before being quantified using the 'Broad-Range' RNA Qubit kit (Invitrogen). RNA quality was then assessed using agarose gel electrophoresis. RNASeq libraries were generated using the KAPA mRNA HyperPrep kit (Roche, CA, USA) before being quantified and quality-assessed with a D1000 chip on an Agilent Tapestation system. All samples were then pooled evenly at 1nM and sequenced (1x 75 bp paired-end) on 1/12th of an Illumina NextSeq 550 high capacity chip. The resulting raw reads were trimmed using Trimmomatic24 (v. 0.36) (Bolger *et al.* 2014). Transcript quantification was performed by first building a Star (v2.73a) (Dobin *et al*. 2013) index file using a softmasked version of the genome GCA_905163435.1 and the Ensembl feature file (GTF), that represent predicted gene features including all intron/exons boundaries and differential transcripts. Transcript counts were then derived by using Star (v2.73a), mapping each to the indexed genome in the unstranded mode. Uniquely mapped reads thus derived were used to generate feature counts which were subsequently normalised using RSEM (v1.3.3) (https://github.com/deweylab/RSEM(Li and Dewey 2011).

6.2.4 Characterisation of M. brassicae nAChRs

The predicted protein sequences of *M. brassicae* genes associated with the annotation term 'acetylcholine' and 'nicotinic' were collated. This list was then manually assessed and any non nAChR subunits were removed. The resulting twelve sequences were aligned with the MUSCLE (Edgar 2004) alignment tool using the default alignment settings (Geneious Prime). This alignment included the *D. melanogaster* nAChR alpha 2 gene (NCBI Genbank Accession NP733001.1), that was annotated across the ligand binding domain (LBD) with all functional regions critical to imidacloprid binding (Short *et al.* 2021). This alignment was used to identify anomalous nAChR subunits (e.g. pseudogenes) and to use the presence/absence of a specific interaction region (termed loop C) to categorise the subunits into alpha and beta forms. The resulting nAChR alpha and beta subunits were aligned using the MUSCLE alignment tool on Geneious Prime version 2022.2.1 with *Drosophila* subunits and a phylogenetic tree using the

Jukes-Cantor model was built. The *M. brassicae* subunits were named according to their relationship to the *D. melanogaster* orthologs. The putative *M. brassicae* genes were named using the format of species name initials, followed by alpha/beta designation and numbered according to the similarity to *Drosophila* genes. The expression patterns of the final *nAChR* gene list were compared using the normalised counts generated for the nerve cord tissue.

6.2.5 Characterisation of *CYP450*, *GST* and *ABC* transporter genes

6.2.5.1 CYP450s

The predicted protein sequences of any *M. brassicae* gene associated with the annotation term 'cytochrome P450' or 'P450' were collated. The gene list was manually assessed and any non *CYP450*s were removed (e.g. NAPDH cytochrome P450 reductase).

All sequences were aligned alongside *Spodoptera litura* CYP6B7 gene (XP_022817035), which represented a well annotated *CYP450* gene, to identify functional domains and help eliminate pseudogenes. A common region spanning the functional domains (from residues 125 to 459, using residue numbers equivalent to that found in the *S. litura* CYP6B7 gene was then used for future alignments.

The obtained *M. brassicae CYP450* genes were putatively named using the format of species name initials_enzyme superfamily_flybase annotation and number of duplicates (Figure 6.1). For example, if a *D. melanogaster* gene had ten *M. brassicae* duplicates, they would be numbered from one to ten in no particular order. *CYP450* amino acid sequences were downloaded for the silkworm (*Bombyx mori*) and the honeybee (*Apis melifera*) from the Cytochrome P450 homepage website (http://drnelson.uthsc.edu/CytochromeP450.html). *D.*

*melanogaster CYP450*s were harvested using the *CYP450* database on Flybase (Larkin *et al.* 2021).

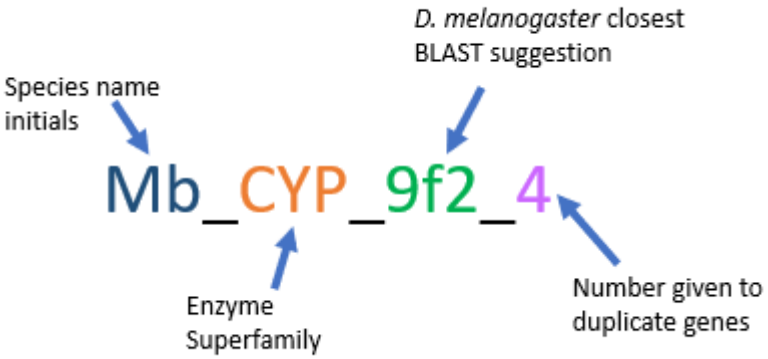


Figure 6.1. Schematic of the putative naming system for *M. brassicae CYP450* genes.

A number of conserved functional domains were identified (Figure 6.2). These included five domains that are always conserved in insect *CYP450*s (Rupasinghe *et al.* 2006). These include the WxxxR motif, the GxE/DTT/S motif, the ExLR motif, the PxxFxPE/DRF (PERF) motif, and the PFxxGxRxCxG/A motif (Feyereisen 2012) (Figure 6.2).

However, when the *M. brassicae CYP450*s were aligned in comparison to an annotated *CYP450* from *Spodoptera litura* (cytochrome P450 6B7-like- XP_022817035) (NCBI 2017) (Figure 6.2), it was noted that some sequences were missing one or more of these conserved motifs (Figure 6.3). Therefore, it was decided to classify these as pseudogenes, a common feature of *CYP450* families (Nelson *et al.* 2004) and remove them from further analysis (Figure 6.3). Sequences downloaded from the Cytochrome P450 homepage (*A. mellifera* and *B. mori*) were also discarded if they failed to display all of the five conserved regions described above.

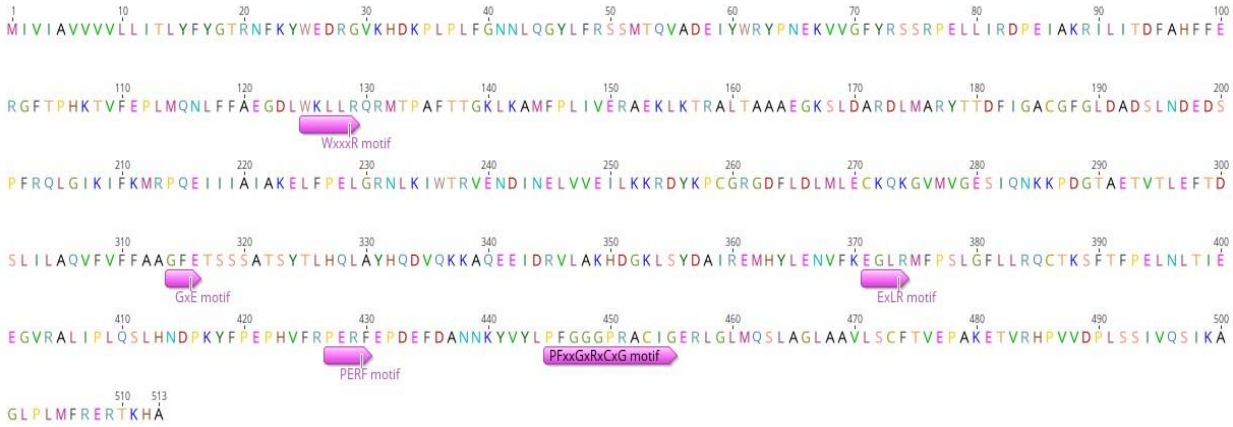


Figure 6.2. *Mb_ CYP314a1* conserved domains annotated as per *Spodoptera litura CYP6B7 like* sequence. Purple blocks highlight heme binding sites and conserved domains.

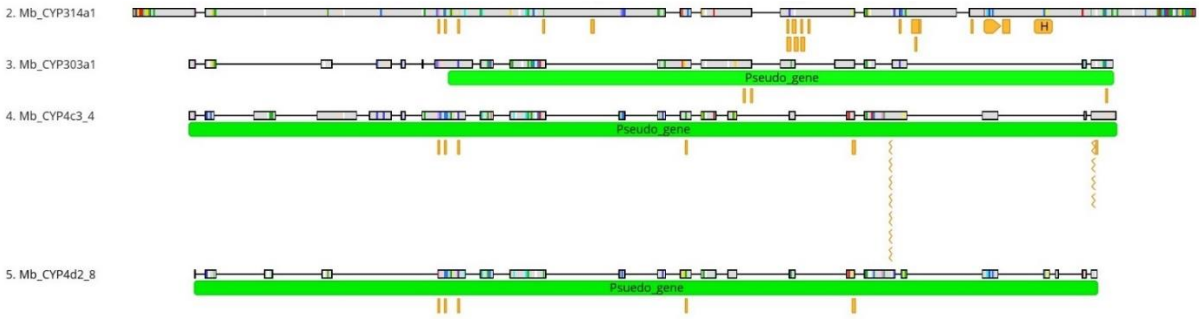


Figure 6.3. Example of pseudogene *CYP450*s. Yellow bars indicate conserved domains. The sequences Mb_CYP303a1, Mb_CYP4c3_4 and Mb_CYP4d2_8, classified as pseudogenes (see Pseudo_gene green bar) due to missing functional domains (yellow boxes). For example, Mb_CYP4d2_8 is missing the PERF and final heme binding domain. The *CYP450* Mb_CYP314a1 represents a *CYP450* containing all functional domains.

Alignments of *CYP450*s belonging to a single species were aligned using the MUSCLE alignment in the Geneious Prime software using default alignment settings (Edgar 2004). Sequences including more than one species were aligned using the clustal omega, with default alignment settings, on the Geneious prime 2022.2.1 software. A common region was extracted from all aligned sequences that encompassed all five functional domains.

Phylogenetic trees were created using the extracted alignments with the Geneious Tree Builder, using the Geneious Prime software version 2022.2.1 . In total, 97 *CYP450*s were analysed from the *M. brassicae* genome, 87 *D. melanogaster,* 80 *B. mori* and 46 *A. melifera*. Trees were constructed using the Maximum Likelihood performed using the Jukes-Cantor genetic distance model with the UPGMA tree build method. A bootstrap consensus was inferred with 100 replicates.

6.2.5.2 GSTs

The predicted protein sequences of any *M. brassicae* gene associated with the annotation term '*GST*' or 'glutathione' were collated. The gene list was manually assessed and any non *GST*s were removed.

All sequences were aligned alongside *Spodoptera litura GST* delta 4 (AIH07597), which represented a well annotated *GST* gene, to identify functional domains. A common region spanning the functional domains (from residues 11 to 206, using residue numbers equivalent to that found in the *S. litura GST* delta 4 gene was then used for future alignments.

The obtained *M. brassicae GST* genes were putatively named using the format of species name as for *CYP450*s: initials_enzyme superfamily_flybase annotation and number of duplicates (Figure 6.4). Duplicates were numbered in no particular order. *D. melanogaster GST*s were harvested using the *CYP450* database on Flybase (Larkin *et al.* 2021). *A. mellifera* and *B. mori GST*s were collected using an NCBI search of the search terms '*GST*' and 'glutathione' (NCBI 2017).

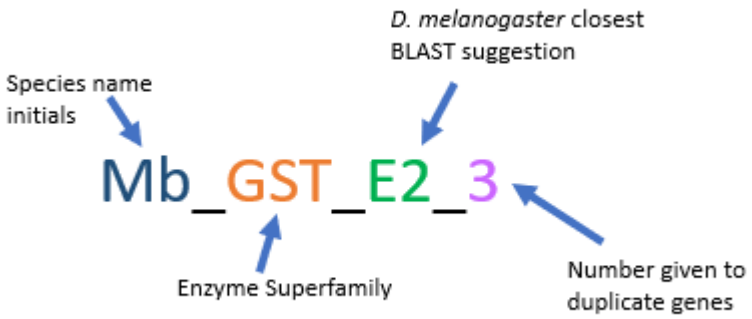


Figure 6.4. Schematic of the putative naming system for *M. brassicae GST* genes.

Alignments of *GST*S belonging to a single species were aligned using the MUSCLE alignment in the Geneious Prime software using default alignment settings (Edgar 2004). Sequences including more than one species were aligned using the clustal omega, with default alignment settings, on the Geneious prime 2022.2.1 software. A common region was extracted from all aligned sequences that encompassed all functional domains.

Phylogenetic trees were created using the extracted alignments with the Geneious Tree Builder, using the Geneious Prime software version 2022.2.1 . In total, 48 *GST*s were analysed from the *M. brassicae* genome, 40 *D. melanogaster,* 17 *B.mori* and 5 *A. melifera*. Trees were constructed using the Maximum Likelihood performed using the Jukes-Cantor genetic distance model with the UPGMA tree build method. A bootstrap consensus was inferred with 100 replicates.

6.2.5.3 ABC Transporters

A lack of sufficient annotation across the *ABC* transporter genes in *D. melanogaster* meant that the putative *M. brassicae ABC* transporters could not be retrieved using the *D. melanogaster* annotation alone. Instead, the *ABC* transporters characterised for the lepidopteran *B. mori* (Dermauw *et al.* 2014) were used to identify the *M. brassicae* genes. Specifically, each *B. mori ABC* gene from each of the *ABC* gene families (A-H) was used to perform a BLAST analysis against a database of all predicted *M. brassicae* proteins. From each BLAST search, the five hits with the lowest expectation value were collated and all duplicate accessions were removed. This set of *M. brassicae* genes were then classified into

one of the eight *ABC* families according to their functional domain organisation, sequence similarity to previously categorised *B. mori ABC* transporter genes (Dermauw *et al*., 2014) and the top BLAST returned when each sequence was used to performed a BLAST analysis using the UNIPROT database (Release 2022_03). The putative *M. brassicae ABC* transporters within each gene family were then aligned to identify protein isoforms generated from the same gene. In cases were multiple isoforms were present, the longest sequence was retained to represent the gene. The putative *M. brassicae* genes were named using the format of species name initials, followed by *ABC* transporter family (i.e. ABCA-ABCH) and an arbitrary number designation.

6.2.6 Transcript Expression

Expression heat maps were created from transcriptomic data, counts of less than 10 in both adult and larvae stages were removed from analysis. Expression in whole body tissue of adult and larvae was normalised and this was re-scaled again using the 'scales' R package to represent a value between 0 and 1.

Fold change differences in adults and larvae were calculated as larvae expression/adult expression. Counts equal to 0 were given a value of one to create a minimum representation. This analysis was designed to present a qualitative view of the transcriptomic data. In accordance with the findings of (Green Etxabe *et al.* 2021; Short *et al.* 2021) any differential expression with a less than 10-fold upregulation in either life stage is not considered notably different in this study. Fold change differences were plotted in Microsoft Excel (Office 365). As the Mito clan and clan 2 *CYP450*s are not commonly implicated in insecticide detoxification their transcriptomic data was not analysed.

6.3. Results

6.3.1 Genome Analysis

The genome was submitted to Genbank by the Wellcome Trust using the sequence ID GenBank GCA_905163435.1. The genome was sequenced from *M. brassicae* kept in culture at the UK Centre for Ecology and Hydrology. Genome assembly information can be found in Table 6.1.

Table 6.1. Sequence statistics of submission GenBank GCA_905163435.1 (*M. brassicae*).

Genome size	576.2 Mb
Number of chromosomes	31
Number of scaffolds	42
Scaffold N50	19.4 Mb
Scaffold L50	14
Number of contigs	69
Contig N50	17.8 Mb
Contig L50	15
GC percent	38
Assembly level	Chromosome

6.3.2 nAChR Sequence and Expression

A total of 10 functional nAChR subunits were identified in the *M. brassicae* genome, seven α and three β, mirroring those of *D. melanogaster* (Korona *et al*. 2022). Although twelve subunits were initially identified, two subunits were removed from the *M. brassicae* analysis; one appeared to be a nAChR pseudogene, as it lacked the majority of its LBD. Another appeared to be a nAChR gamma-like subunit, which presented no expression in the nerve cord and lacked key residues necessary for imidacloprid binding.

Of the seven α subunits identified, in both adults and larvae, the greatest expression was seen in *Mb_nAChR_alpha_6_like (*Figure 6.5). Similar expression patterns were found in all of the α and β subunits (Figure 6.5 and Figure 6.6). Three of the α subunits showed a >5% difference in expression proportion between larvae and adults but no changes >1% were found in β subunits so they were excluded from further analysis.

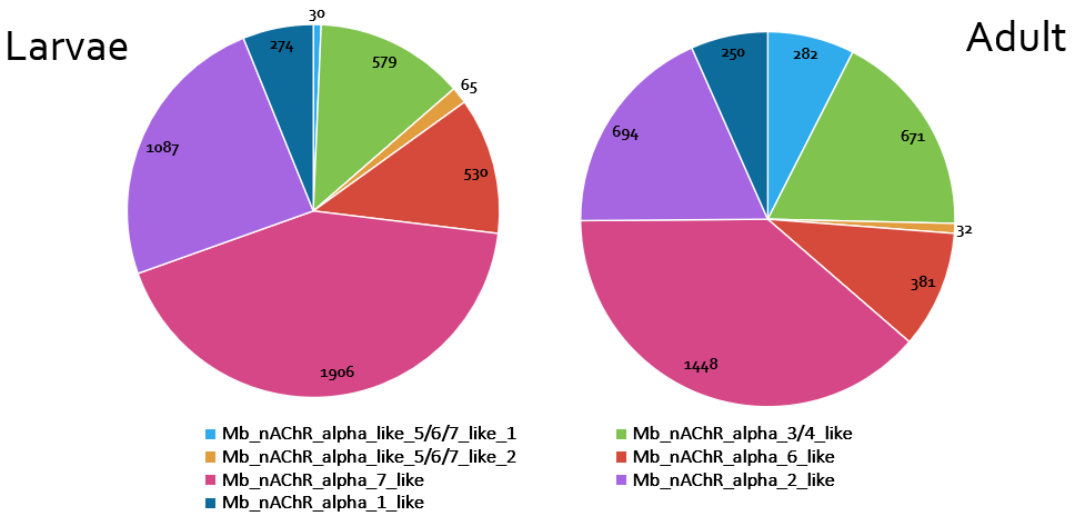


Figure 6.5. Comparison of adult and larvae nerve cord α nAChR subunit expression proportion.

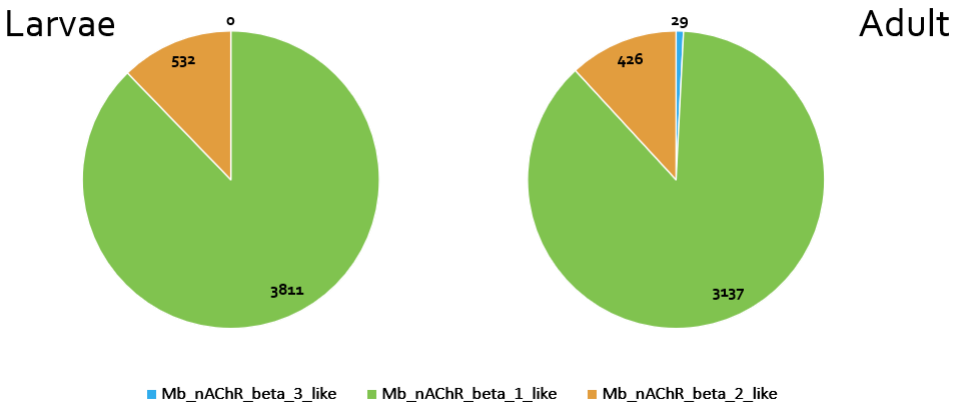


Figure 6.6. Comparison of adult and larvae nerve cord β nAChR subunit expression proportions.

Transcriptomic analysis of the α nAChR subunits (Figure 6.7) highlighted high expression in the gene *Mb_nAChR_alpha_7_like*. The genes *Mb_nAChR_alpha_2_like,* and *Mb_nAChR_alpha_6_like* were also upregulated in the larval stages. *Mb_nAChR_alpha_5/6/7_like_1* was notably upregulated in the adult stage.

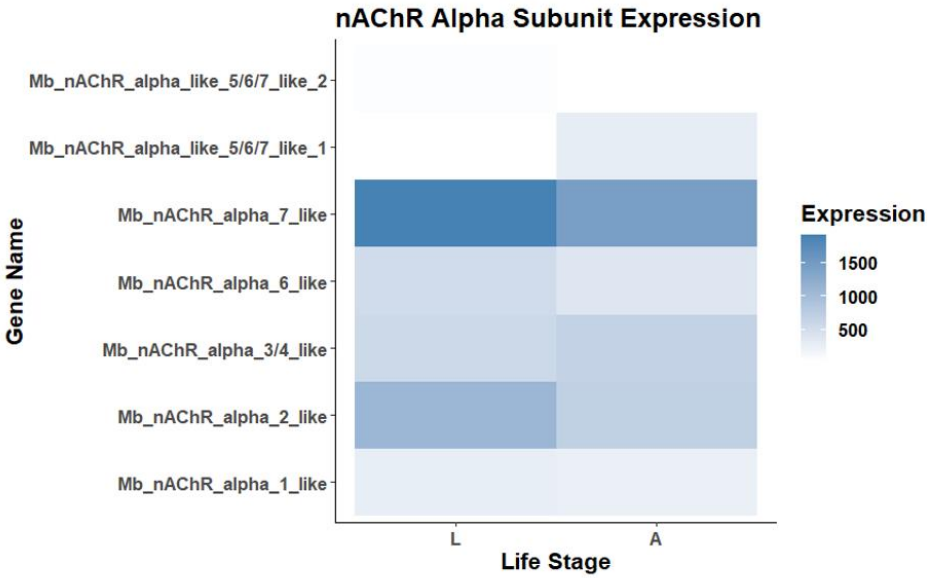


Figure 6.7. A heatmap of larvae and adult α nAChR subunit expression.

In concordance with the criteria set out by Short *et al*. (2021) for comparing binding affinity scores, residues at sites reported to influence binding affinity were compared between adults and larvae. Evidence for only one change in residue was found that was likely to affect binding affinity but this was found to benefit the adult stage.

6.3.3 CYP450 Genomic Complement

The *M. brassicae* genome expressed a total of 121 *CYP450*s. A total of 24 pseudogenes were removed from alignment, resulting in 97 'functional' *CYP450*s remaining. These sequences were all compared (aligned using phylogenetic analysis) to the *CYP450*s present in the *D. melanogaster* genome (Figure 6.8). This analysis indicated that many were duplicates of an existing *D. melanogaster CYP450* genes. For example, there were 28 *M. brassicae* genes orthologous to the *D. melanogaster CYP-4c3*. All of these genes were located at different positions on the *M. brassicae* genome, thus, strongly indicating that duplicated *CYP450*s are unique genes rather than isoforms of a single gene.

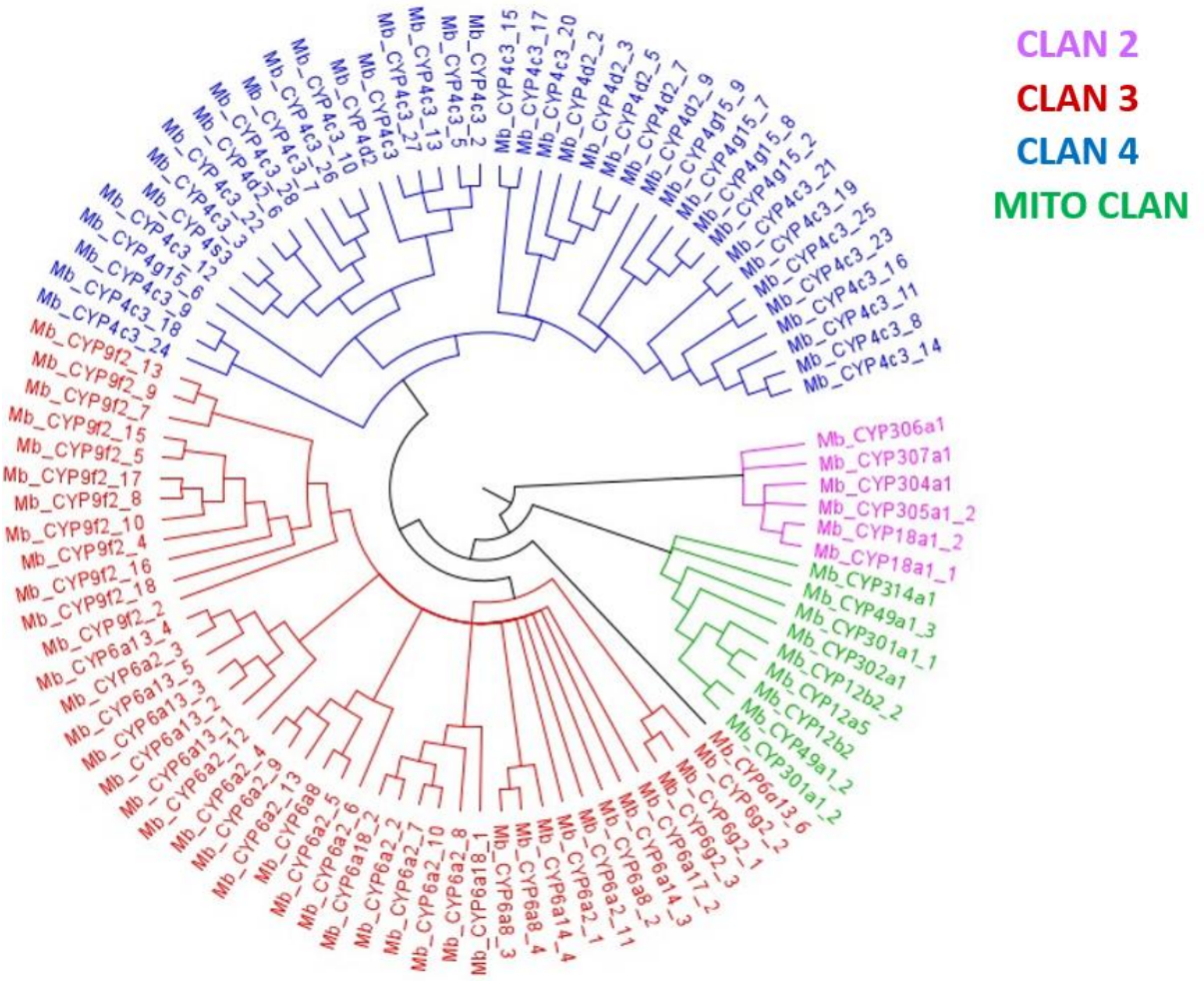


Figure 6.8. Phylogenetic tree of *CYP450*s of *M. brassicae* using Jukes-Cantor model. Clan 2 *CYP450s* are shown in purple, clan 3 in red, clan 4 in blue and the Mito clan in green. All *CYP450* genes fall into one of these clans with the exception of *Mb_CYP_6a13_6*, does not seem to be closely related clan 3 but given its annotation using the *D. melanogaster* sequences is expected to belong to this clan.

Of the 97 remaining *CYP450s*, 6 are clan 2 (6.19%), 43 are clan 3 (44.3%), 39 are clan 4 (40.2%) and 9 are classed as Mito clan (9.27%). *M. brassicae CYP450s* in the Mito clan and clan 2 fall into single clades and present less sequence divergence than genes in clan 3 and 4. Clan 4 CYPS form 3 distinct clades. The largest and most diverse clan is clan 3. Here two main families are found, CYP9s and CYP6s; CYP9s account for 30.7% of clan 3 and 69.3% are CYP6s. Of the CYP6s, five of these (*Mb_CYP_6a17_2, Mb_CYP6a14_3, Mb_CYP6a8_2, Mb_CYP6a2_11, Mb_CYP6a2_1*) appear to be evolutionary diverse and have long branches. Furthermore, *Mb_CYP_6a13_6* is not contained within either the CYP6 or CYP9 cluster.

The 39 clan 4 genes are split across four distinct *CYP450* gene types; *CYP4c3, CYP4g15, CYP4d2* and *CYP4s3*. There are 26 variants of *CYP4c3*, 5 of *CYP4g15s*, 7 of *CYP4d2s* and 1 *CYP4s3*. The genes annotating as *CYP4c3s* are distributed among each of the major 4 clades, with a distinct branches consisting of *Mb_CYP4c3_9, Mb_CYP4c3_18* and *Mb_CYP4c3_24* genes.

M. brassicae CYP450 genes were annotated using the *D. melanogaster* genome, as it is the highest quality and most extensively annotated of any current available insect genome (Jana *et al*. 2016). Thus, although the consensus between these genomes was unknown, the extensive knowledge that is available for *D. melanogaster* makes it easier to interpret the outcomes of any analysis. A phylogenetic tree using the Jukes-Cantor model was created of the *M. brassicae* and *D. melanogaster CYP450*s (Figure 5). This consensus tree highlighted the evolutionary conserved nature of clan 2 and Mito clan *CYP450*s, as both species contain similar numbers of genes with evidence of clear orthologous relationships within these clans, evidenced by the fact that each of the *M. brassicae CYP450*s fit alongside their corresponding *D. melanogaster CYP450*.

Among the clan 2 enzymes, are three of the so called 'Halloween' *CYP450*s of *D. melanogaster*; Spook (*spo*), Spookier (*spok*) and Phantom (*phm*). These genes are labelled on the phylogenetic tree using these names in concordance with *D. melanogaster* nomenclature. However, in *M. brassicae,* only *spo* and *phm* genes are present and are labelled by their synonymous *CYP450* names, *Mb_CYP_306a1* and *Mb_CYP_307a1* respectively. These two *CYP450*s are well conserved between *M. brassicae* and *D. melanogaster,* as is generally true of the *CYP450*s in this clan.

The Mito clan is also well conserved in *M. brassicae* compared to *D. melanogaster.* The Mito clan *CYP450*s of *M. brassicae* consist of only one clade. In this clan are two more 'Halloween' *CYP450*s; Disembodied (*dib*) and Shade (*shd*). In the *M. brassicae* genome they are putatively named *Mb_CYP_302a1* and *Mb_CYP_14a1* respectively. Both of these genes are well conserved with those of *D. melanogaster* and are found on the same branch. However, in this clan it was noted that genes are clustered by species. For example, all of the *D. melanogaster* CYP12s are in a separate clade to the *M. brassicae* CYP12s. The CYP49a genes of both species occupy the same clade, with the exception of *Mb_CYP49a1_3,* which is evolutionarily divergent to all other *CYP450*s within this clan.

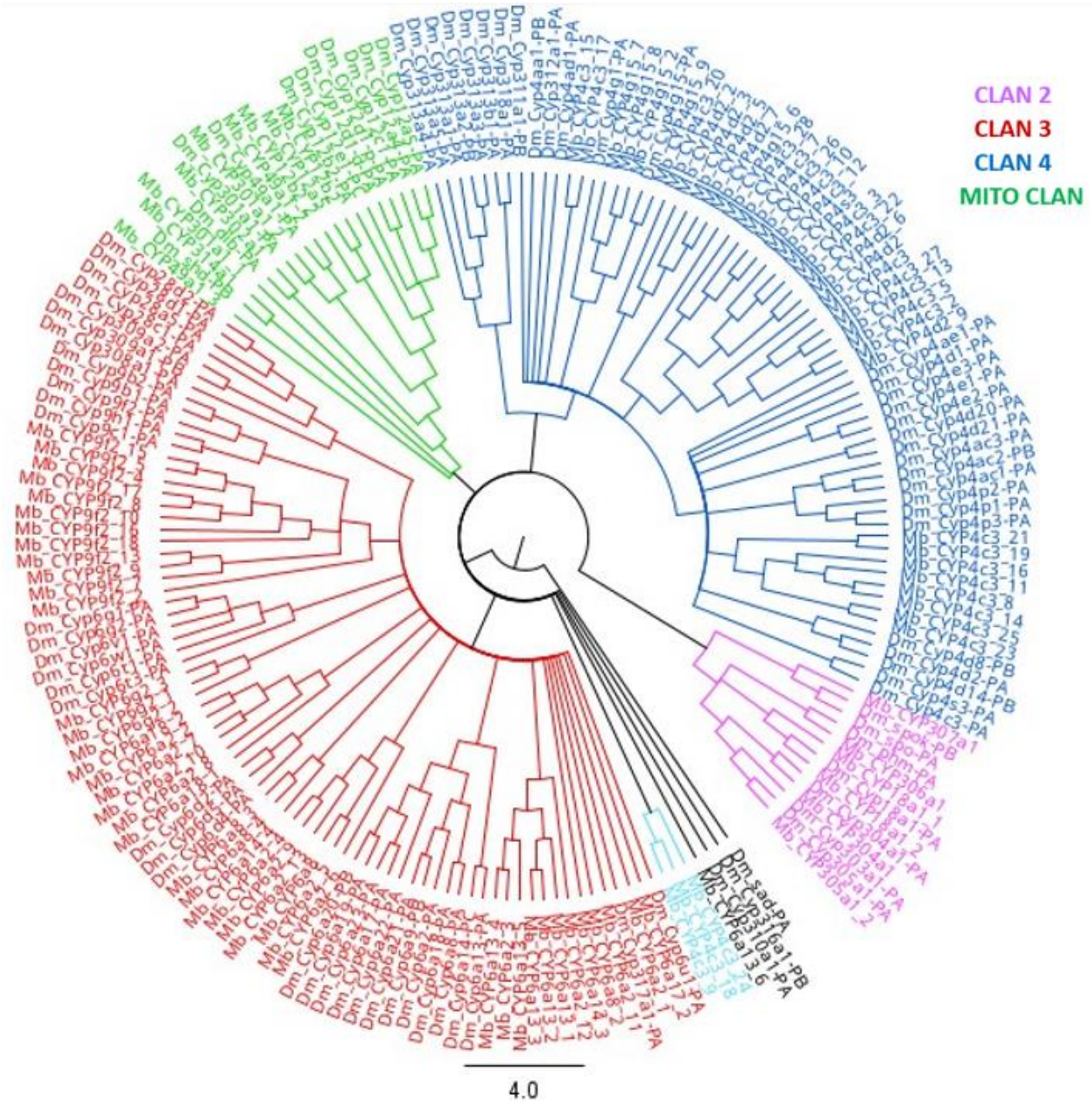


Figure 6.9. Jukes-Cantor phylogenetic tree of *CYP450*s in *M. brassicae* (Mb) and D. melanogaster (Dm) genome. Clan 2 *CYP450*s are displayed in purple, clan 3 in red, clan 4 in blue and Mito clan in green. Three *CYP450*s are highlighted in light blue as they hypothetically should be placed in clan 4 but have not placed within this clan using this phylogenetic method. The small number of *CYP450*s in black do not fit into any of the four clans.

Clan 3 is the largest clan in both species. In the consensus tree, there are four *CYP28* genes of *D. melanogaster*. However, there are no *CYP28s* identified in the *M. brassicae* genome. These *D. melanogaster CYP450*s are in a cluster also including *CYP308s* and *CYP309s* which again are not present in *M. brassicae*. The other two clades represented in this clan are the CYP6s and CYP9s. The *M. brassicae* genome has greater number of CYP9 representatives (12 in *M. brassicae* and 5 in *D. melanogaster*) and these are evolutionarily distinct with each species forming separate clusters (Figure 6.9).

The CYP6s in clan 3 are a diverse family of *CYP450*s. This family is separated into several clades consistently clustered by species. There are no CYP6g1 genes in *M. brassicae* but there are three CYP6g2s in a separate cluster to the *D. melanogaster* CYP6gs. The cluster containing the *D. melanogaster* CYP6g genes, also contains CYP6v1, CYP6t1 and CYP6t3, all of which are absent in *M. brassicae*. A further eight *CYP450*s appear highly divergent, as represented by their long branches. Of these, six belong to *M. brassicae* (*Mb_CYP6a2_12, Mb_CYP6a14_3, Mb_CYP6a8_2, Mb_CYP6a2_11, Mb_CYP6a2_1, Mb_CYP6a17_2*) and two *D. melanogaster* (*Dm_CYP317a1, Dm_CYP6u1*). As seen in the *single M. brassicae* phylogenetic tree (Figure 4), the gene annotating as *Mb_6a13_6* again falls outside of the clan 3 clade (Figure 5).

Clan 4 again identifies the genetic distance between *M. brassicae* and *D. melanogaster CYP450*s and highlights that clades are consistently species-specific. One clade contains *CYP450*s *311a1, 318a1, 13b1, 13a3, 13a2, 13a1, 13a5* and *13a4,* which are present in *D. melanogaster* but not *M. brassicae*. The *Mb_CYP4c3s* were the largest group of duplicates in the *M. brassicae* genome and here are found across two of the major *CYP450* clan 4 clades, having seemingly not evolved from a single common ancestor *CYP4c3* gene. A further difference between the two species is seen in the *CYP4s3*, with the genes in this family being found in different clades within the tree. However, *Mb_CYP4s2* is closely linked to *Mb_CYP4c3_3*, a pattern mirrored by *Dm_CYP4c3* and *Dm_CYP4s* but occupying different major clades.

The *CYP450*s found in the *M. brassicae* genome were next arranged into a phylogenetic tree alongside those *CYP450*s found in *A. mellifera* and *B. mori* (Figure 6.10). Once again, the *CYP450*s that are within clans 2 and Mito clan were well conserved and contained only one clade. In clan 2, the Halloween CYPs *Spo* and *Phm* (*CYP307a1 and CYP306a1*) were closely related in *M. brassicae* and *B. mori*. Not surprisingly, the branch length reveal that

orthologous moth genes were more closely related than either moth *CYP450* gene was to *A. mellifera.*

Within the Mito clan, *CYP314a1* was well conserved between all three of the species, however, this gene in *B. mori* shared a closer relationship with that in *M. brassicae* than that in *A. mellifera*. The *CYP301* family was also well conserved between the three species. The exception to this was *Mb_CYP30a1_1* which did not fit within this cluster and instead was clustered with *Am_CYP334a1* and *Bm_CYP339a1*. Two *CYP450*s in this clan were not closely related to any other, *Mb_CYP49a1_3* and *Bm_315A*. The other *CYP49a1s* (*Mb_CYP49a_2* and *Bm_49a1*) were found in a cluster with the CYP301 family.

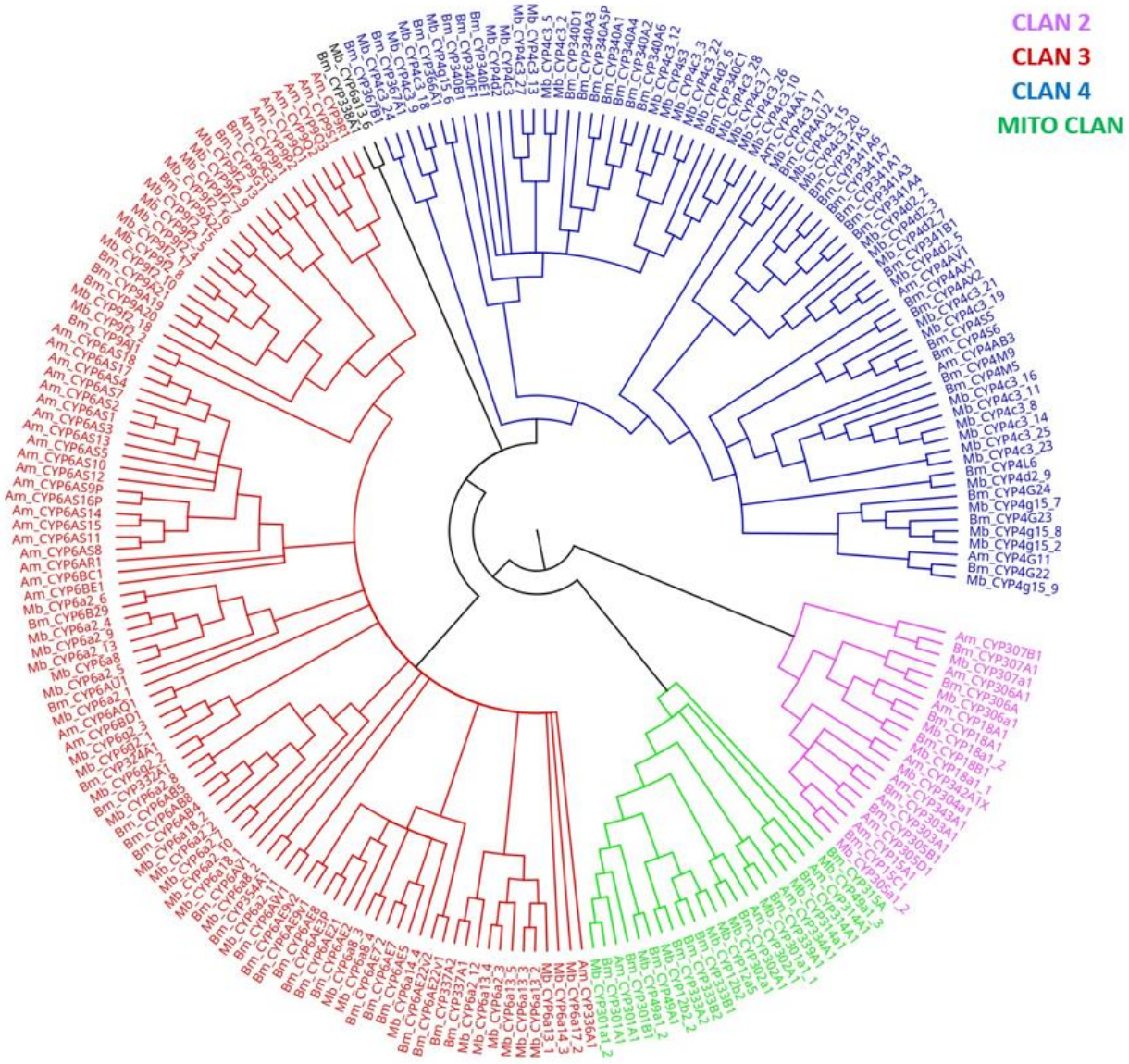


Figure 6.10. Jukes-Cantor phylogenetic tree of *CYP450*s in *M. brassicae* (Mb) *Apis mellifera* (Am) and *B. mori* (Bm) genome. Clan 2 *CYP450* genes are displayed in purple, clan 3 in red, clan 4 in blue and Mito clan in green. Two *CYP450*s in black do not fit into any of the clans using this phylogenetic approach.

Reflecting broader trends, clan 3 is large in all of the species analysed. In these analyses all of the CYP9 family within clan 3 were contained in a single major clade. Within this, all *A. mellifera* CYP9s were found in a single clade, whereas *B. mori* and *M. brassicae* were dispersed reflecting their closer evolutionary relationship. For the CYP6 family, this pattern

of separation of *A. mellifera CYP450*s from those of the other two species is largely repeated, with all but three *A. mellifera* CYP6s in a single clade. A further two CYP6s (*Am_CYP6aQ* and *Am_CYP6BD1*), form a separate clade and *Am_CYP336A1* is distinct from all other clan 3 *CYP450*s. The *B. mori* and *M. brassicae CYP450*s from the CYP6 family are found interspersed within the remaining CYP6 clades. This relationship simply suggests a closer evolutionary relationship for these two species than for *A. mellifera*. Two further *M. brassicae CYP450*s (*Mb_CYP6a17_2* and *Mb_CYP6a14_3*) did not fit into any clade in clan 3, suggesting a high level of sequence divergence.

Clan 4 was smaller than clan 3 in all three species. In *A. mellifera* there are a total of 4 clan 4 CYPs (*M. brassicae* have 39). However, unlike the evolutionary patterns seen in other clans, these *A. mellifera* genes are found dispersed among the major clan 4 clades that also include *B. mori* and *M. brassicae CYP450*s. This suggests that although *A. mellifera* have relatively few clan 4 *CYP450*s they likely possess a considerable functional diversity. Each of the major clades within this clan contained at least one *CYP450* from *B. mori* and *M. brassicae*.

6.3.4 CYP450 Differential Expression

6.3.4.1 Clan 3

Clan three is the largest *CYP450* family in *M. brassicae* and consists of two major families, CYP6s and CYP9s. Two *CYP450*s in this clan did not have a normalised expression count of above 10 in either life stage and were discarded from further analysis. In larvae, the clan 3 *CYP450*s were generally not highly expressed. Of the 43 *CYP450*s in family 6, 13 were upregulated with a fold difference of >10 in larvae, seven in larvae and six in adults. (Figure 9) . The gene *Mb_CYP6a2_2* was not expressed in adults and *Mb_CYP6a13_1* was not expressed in larvae. Two genes in this family were highly upregulated in larvae, *Mb_CYP6a2_2* and *Mb_CYP6a17_2* presenting ~3000-fold greater expression in larvae to adults. A total of 12 *CYP450*s were identified in family 9 in clan 3. Nine of these *CYP450*s were upregulated in adults and three in larvae (Figure 6.13), four of these with a >10-fold difference. The *CYP450*s with greatest upregulation in adults from family CYP9 were *Mb_CYP_9f2_10*, *Mb_CYP9f2_16*, *Mb_CYP9f2_15*, *Mb_CYP9f2_17*. In this family one, *Mb_CYP9f2_2* was expressed highly in larvae, but not at all in adults (Figure 6.14), leading to the large ~3000 -fold expression difference.

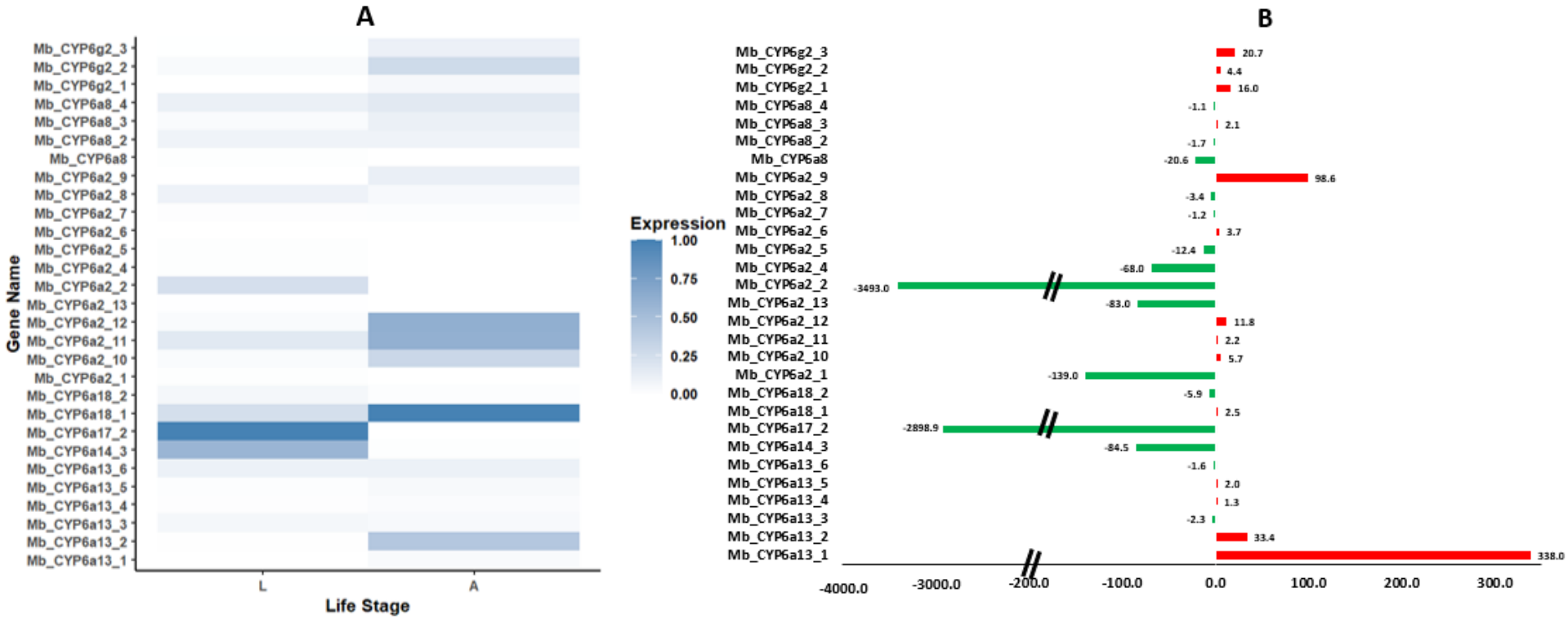
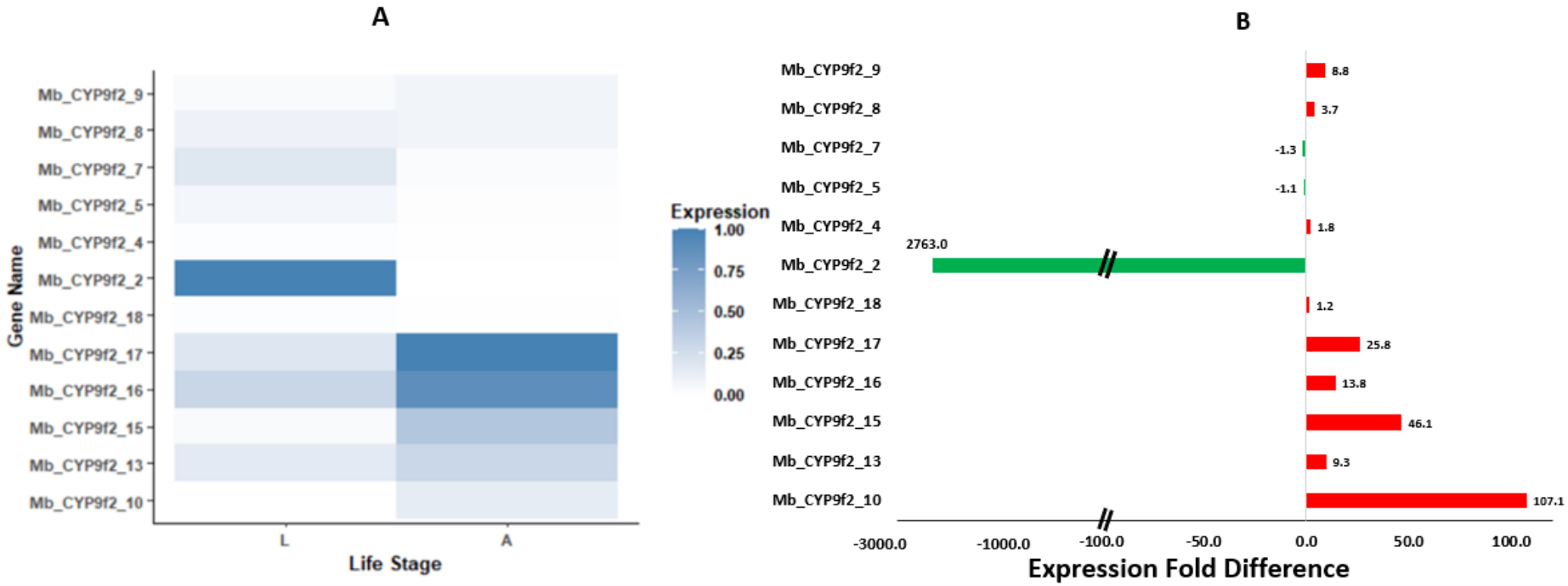


Figure 6.13. Normalised count of expression of *CYP450*s in family 6 (Clan 3) of *M. brassicae* adults and larvae (A). Plot B shows fold differences in expression levels of family 6 genes. Negative values (shown in green) indicate upregulation in larvae where positive values (red) indicate upregulation in adults.



6.14. Normalised count of expression of *CYP450*s in family 9 (Clan 3) of *M. brassicae* adults and larvae (A). Plot B shows fold differences in expression levels of family 9 genes. Negative values (shown in green) indicate upregulation in larvae where positive values (red) indicate upregulation in adults.

6.3.4.2. Clan 4

Clan 4 *CYP450* expression is dominated by that of *Mb_CYP4g15_9* (Figure 11). Of the 39 clan 4 *CYP450*s, nine had an expression count of less than 10 in both adults and larvae and were excluded from further analysis. A total of 24 clan 4 *CYP450*s were upregulated in adults, two of these with over 100-fold difference (*Mb_CYP4c3_22* and *Mb_CYP4c3_23*) and nine >10-fold difference. In larvae, four *CYP450*s were upregulated >10-fold including two with a >700-fold difference. Two of these *CYP450*s (*Mb_CYP4d2_3* and *Mb_CYP4c3_2*) were not expressed in adults.

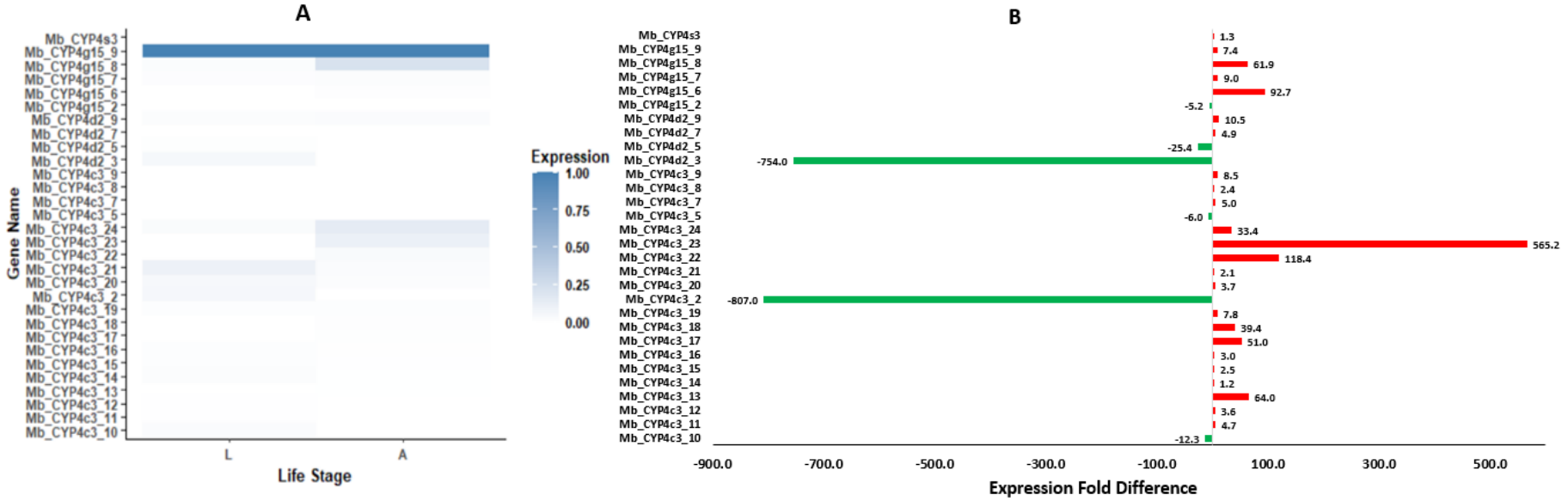


Figure 6.15. Normalised count of expression of *CYP450*s in Clan 4 of *M. brassicae* adults and larvae (A). Plot B shows fold differences in expression levels of clan 4 genes. Negative values (shown in green) indicate upregulation in larvae where positive values (red) indicate upregulation in adults.

6.3.5 *GST* Genomic Complement

In insects, cytosolic *GST*s are classified into one of six classes; delta, epsilon, omega, sigma, theta or zeta. A total of 48 *GST*s were identified from the *M. brassicae* genome based on mapping to the *D. melanogaster* genome (Figure 6.16). The largest class of *GST*s found in *M. brassicae* was epsilon, which contained 26 genes (55.3% of total *GST*s). The second largest was sigma, which contained 13 *GST*s (27.7% of total *GST*s). The delta class was found to be closely related to the epsilon class and was comprised of four *GST*s (8.5% of total *GST*s). The *M. brassicae* genome also included two omega, two zeta and one theta *GST*.

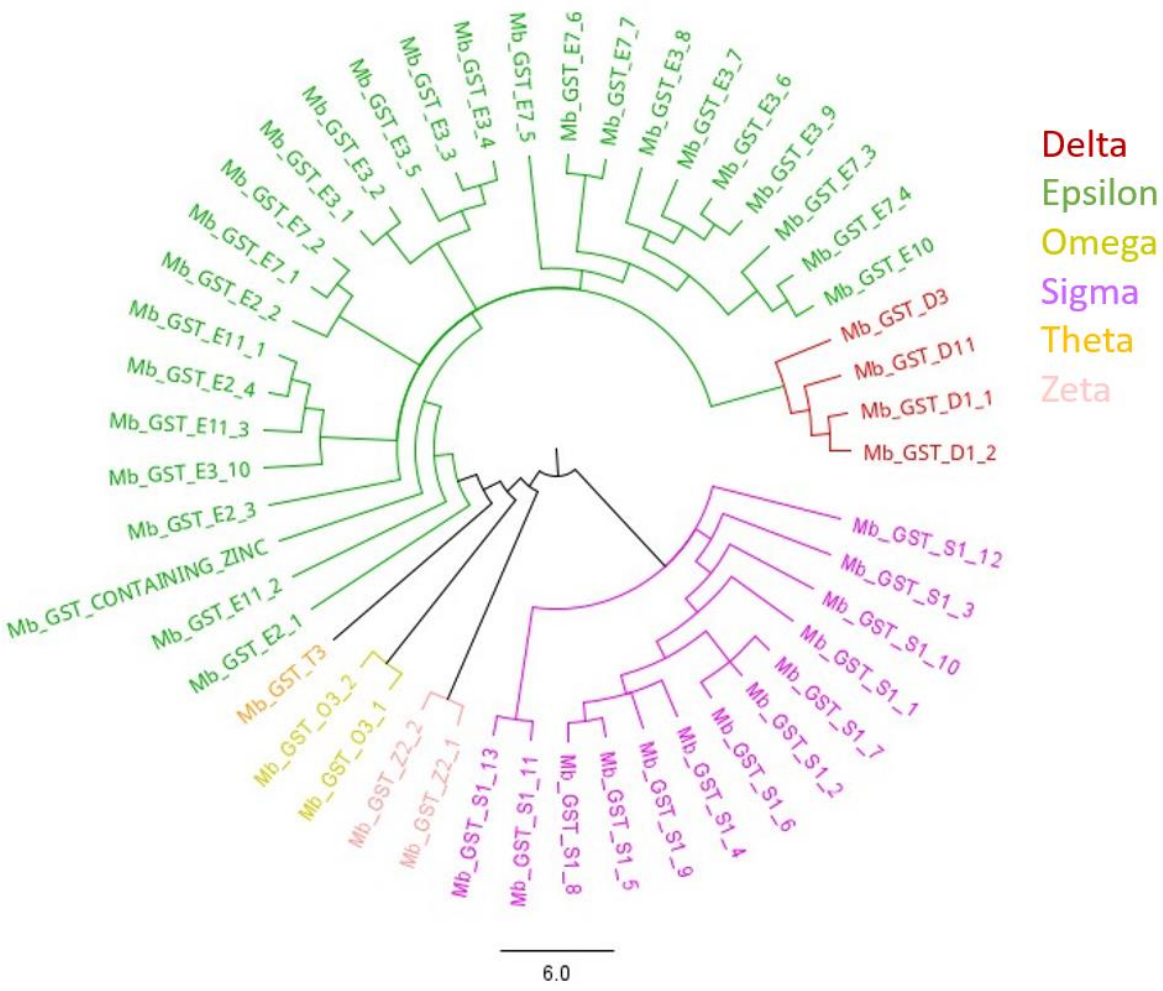


Figure 6.16. *GST* complement of *M. brassicae* genome as mapped to D. melanogaster. *GST*s from the class epsilon are represented in green, omega in olive, sigma in purple, theta in orange and zeta in pink.

*GST*s from the *M. brassicae* genome were then analysed in a phylogenetic tree alongside those from the *D. melanogaster* genome (Figure 6.17). The epsilon class of *GST*s has either expanded in *M. brassicae*, or there has been dramatic gene loss in *D. melanogaster (D. melanogaster has 14, M. brassicae 26*). Within this class, *D. melanogaster* and *M. brassicae GST* genes are largely found in separate clades. The single exception to this is *Mb_GST_containing_zinc* and *Dm_gfzf*, which form a single clade, indicating sequence conservation of these two genes. The delta class was once again found within the same evolutionary clade as epsilon; however, there were a greater number of genes in *D. melanogaster*, suggesting gene expansion or loss in one of these lineages. Despite this divergence, *GST_D11* was well conserved in both species. *Mb_GST_D3* was distinct from all other *GST*s in this class.

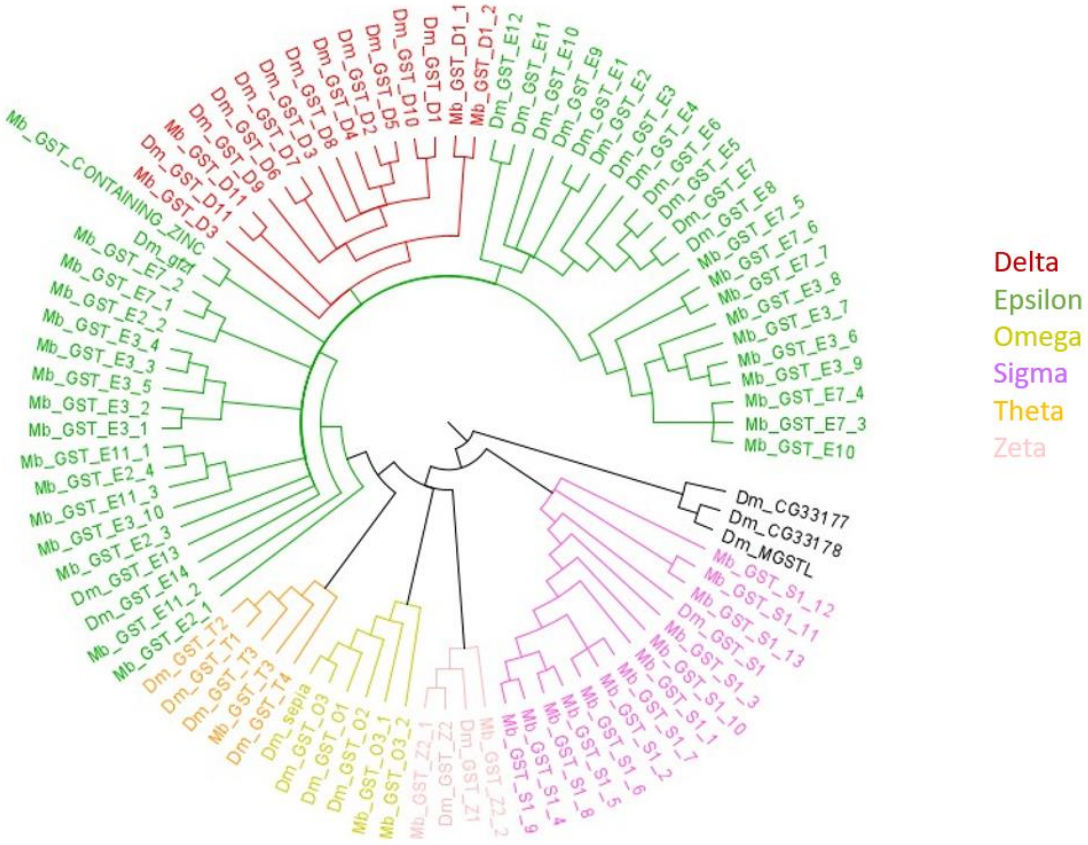


Figure 6.17. Jukes-Cantor phylogenetic tree of *GST* complement in *M. brassicae* genome and D. melanogaster. *GST*s from the class epsilon are represented in green, omega in olive, sigma in purple, theta in orange and zeta in pink. Black represents the class of microsomal *GST*s.

There were a greater number of theta *GST*s in *D. melanogaster* relative to *M. brassicae*, although even in the fruit fly this is still the second smallest class of cytosolic *GST*s. In *M. brassicae,* there was only one gene in the theta class, whereas *D. melanogaster* have four. In *D. melanogaster*, this class consists of one clade. This is also applicable to classes omega and zeta. The sigma class by contrast, is much larger in *M. brassicae* than *D. melanogaster* (13 to 1). Here, *Mb_GSTS1_12* is the most distantly related to the rest of the class. Finally, the microsomal *GST*s are a distinct evolutionary class and are only seen in the *D. melanogaster* genome.

The genomic complement of *GST*s in *M. brassicae* was further compared to those of *B. mori* and *A. mellifera* (Figure 6.18). There were many more *GST*s in the epsilon class of *M. brassicae* compared to *B. mori* (7 *B. mori* and 26 *M. brassicae*) these were found in all major clades within the epsilon class. The unclassified *B. mori GST* (*Bm_GST_U1*) was identified as sharing a close relationship with the zinc containing *GST* of *M. brassicae* within the epsilon class.

The delta class was contained within the epsilon class, with all genes found within a single clade. This class contained one *A. mellifera* gene, four *M. brassicae* genes and three genes from *B. mori*. The zeta and omega class did not contain any genes from *A. mellifera*, but were closely related in both lepidopterans, *M. brassicae* and *B. mori*. The theta class was also well conserved and contained a single gene from all three species.

The sigma class of *GST*s was much more diverse in *M. brassicae* compared to both *A. mellifera* and *B. mori*. Clades within this class also often consisted only of genes from a single one of the three assessed species. Both *A. mellifera* and *B. mori* genes were located on separate clades from the *M. brassicae* genes. One microsomal *GST* was identified within these three species, this being found in the *A. mellifera* genome.

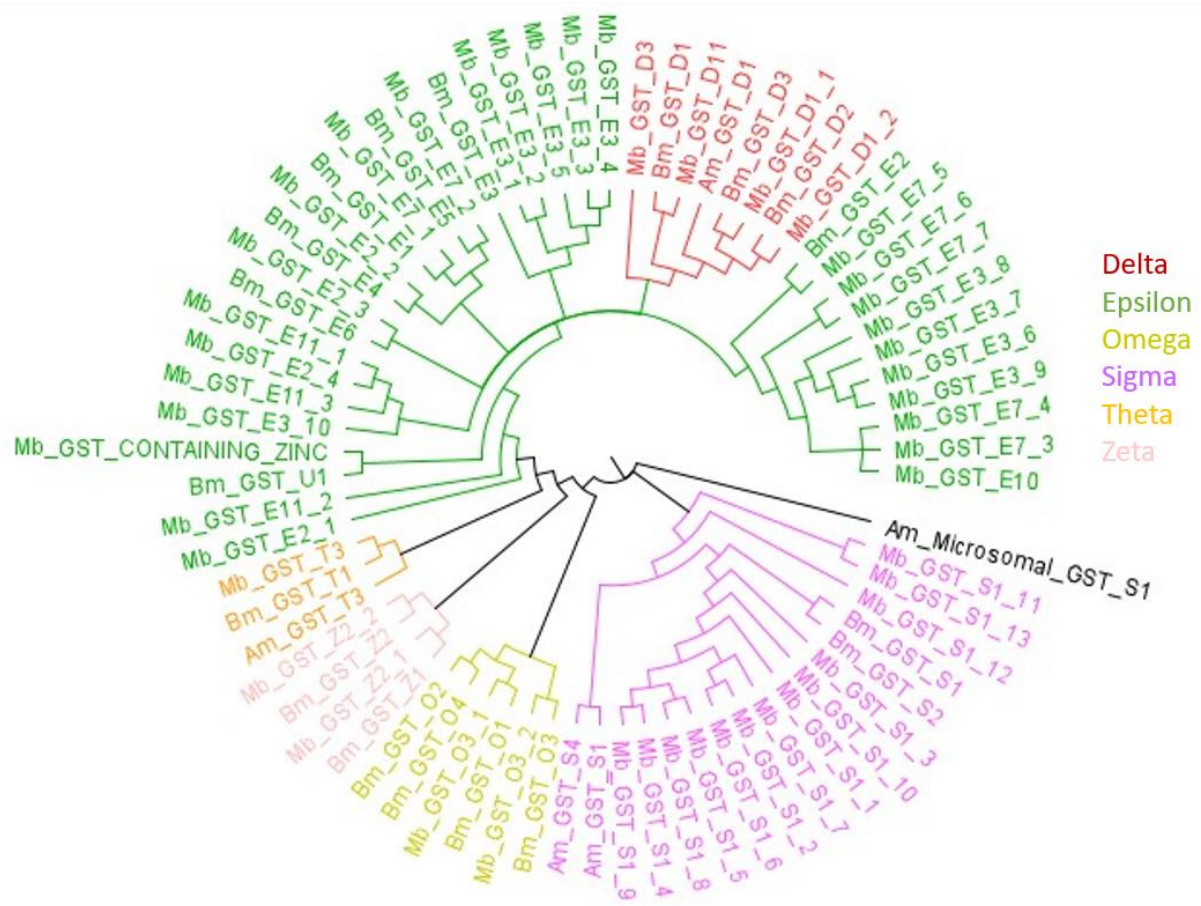


Figure 6.18. Jukes-Cantor phylogenetic tree of *GST* complement in *M. brassicae* genome and D. melanogaster. *GST*s from the class epsilon are represented in green, omega in olive, sigma in purple, theta in orange and zeta in pink. Black represents the class of microsomal *GST*s.

6.3.6 *GST* Differential Expression

For the analysis of differential expression, all *GST*s with a normalised expression count of less than 10 in both adult and larval stages were removed. No removed *GST*s were from either the groups delta, omega, theta or zeta but two were removed from the epsilon group. As the four smallest groups of *GST*s, expression of the delta, omega, theta and zeta genes were analysed together within a single assessment (Figure 6.19). In the zeta group, *Mb_GST_Z2_2* was expressed highly in both life stages and shows little differential expression between adult and larvae (Figure 6.19). *Mb_GST_Z2_1* was not expressed at all in adults. Likewise, the theta group gene Mb_*GST*_T3 was expressed highly in both larvae and adults but showed little differential expression. However, four of the *GST*s, one zeta, one omega, and two delta genes, showed clear upregulation (>100-fold) in the larval transcriptome relative to adult (*Mb_GST_Z2_1, Mb_GST_O3, Mb_GST_D1_2* and *Mb_GST_D1_1*) (Figure 6.19B).

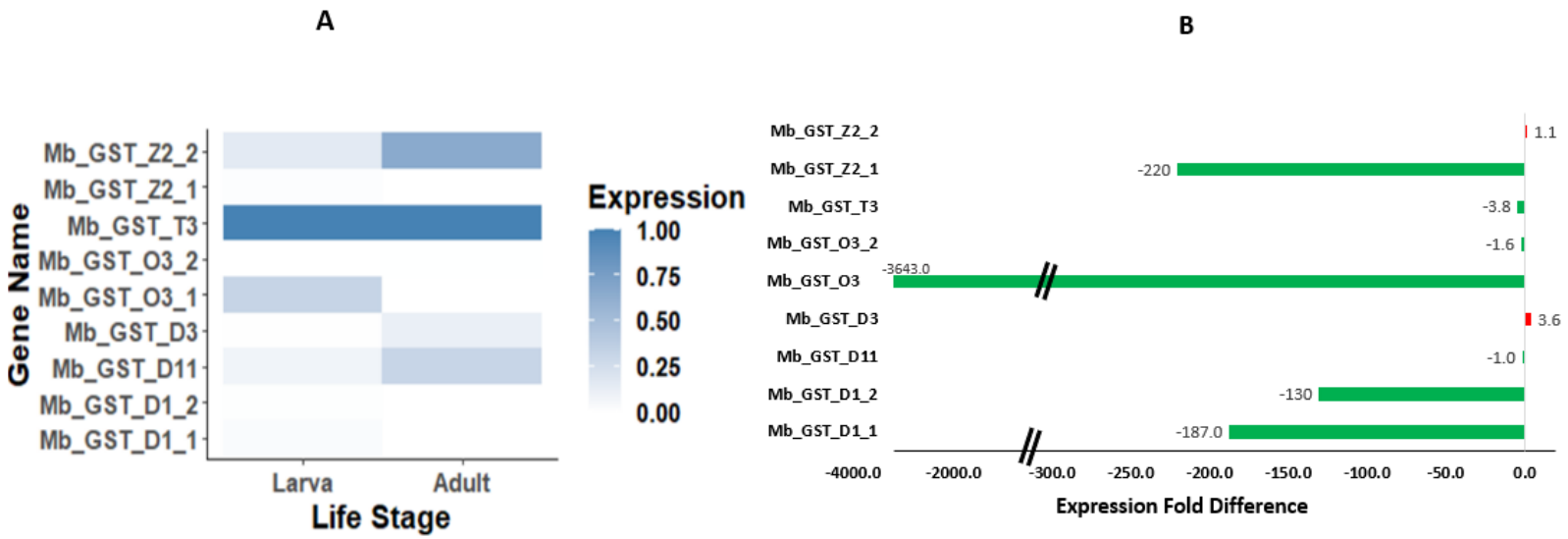


Figure 6.19. Normalised count of expression of *GST* in Class Delta, Omega, Theta and Zeta of *M. brassicae* adults and larvae (A). Plot B fold differences in expression levels of these *GST*s. Negative values (shown in green) indicate upregulation in larvae where positive values (red) indicate upregulation in adults.

Epsilon was the largest *GST* group in the *M. brassicae* genome. Two genes from this group were removed from transcriptomic analysis as they had a normalised expression count of less than 10 in both adult and larval stages. Of the remainder, there were five subgroups. The largest of these was E3. In this subgroup, six *GST*s were upregulated in larvae (*Mb_GST_E3_ 8, Mb_GST_E3_7, Mb_GST_E3_6, Mb_GST_E3_5, Mb_GST_E3_10, Mb_GST_E3_2*) and three in the adult transcriptome (*Mb_GST_E3_3, Mb_GST_E3_4* and *Mb_GST_E3_1*) (only one of these over the 10-fold difference threshold validation criteria) (Figure 6.20). *Mb_GST_E3_9* was very highly expressed in both life stages and *Mb_GST_E3_7*, *Mb_GST_E3_4* and *Mb_GST_E3_2* were not expressed in highly expressed in either life stage. Six genes were upregulated in larvae with a greater than 10-fold difference, two of which show greater than 100-fold difference

(*Mb_GST_E3_5* and *Mb_GST_E7_2*). The transcript of the gene *Mb_GST_containing_zinc* was analysed alongside *GST*s in the epsilon group, as it has strong phylogenetic similarity. This gene was, however, expressed at very low levels in both larvae and adult *M. brassicae*.

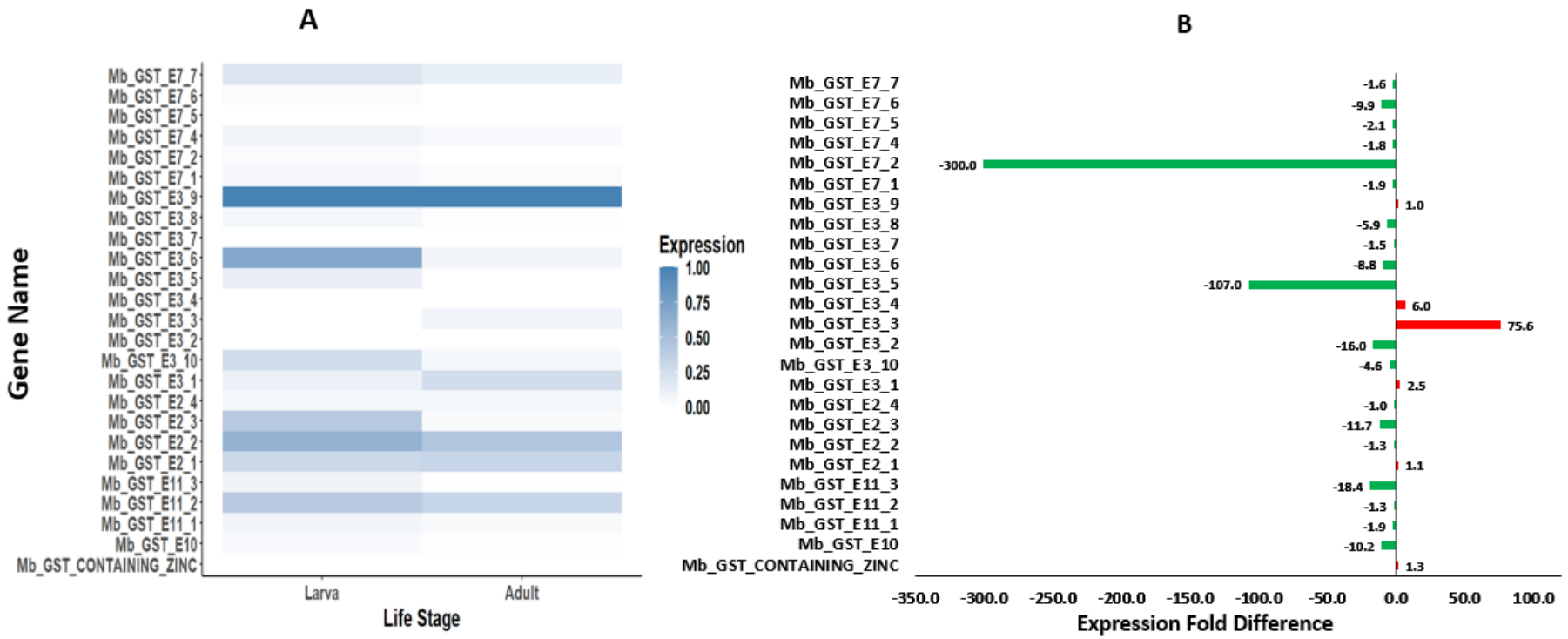


Figure 6.20. Differential expression of *GST*s from the epsilon class in the *M. brassicae* transcriptome (A). Plot B shows fold differences in expression levels of the Epsilon class. Negative values (shown in green) indicate upregulation in larvae where positive values (red) indicate upregulation in adults. *GST* containing zinc was also placed within this group due to its placement on the phylogenetic tree of *GST*s.

The sigma class of *GST*s of *M. brassicae* were widely diverged from those of *D. melanogaster*. Within this class, one sigma *GST* was upregulated greater than 10-fold in adults (*Mb_GST_S1_9)* and four in larvae (*Mb_GST_S1_7, Mb_GST_S1_6* , *Mb_GST_S1_3* and *Mb_GST_S1_2)* (Figure 6.21 B).

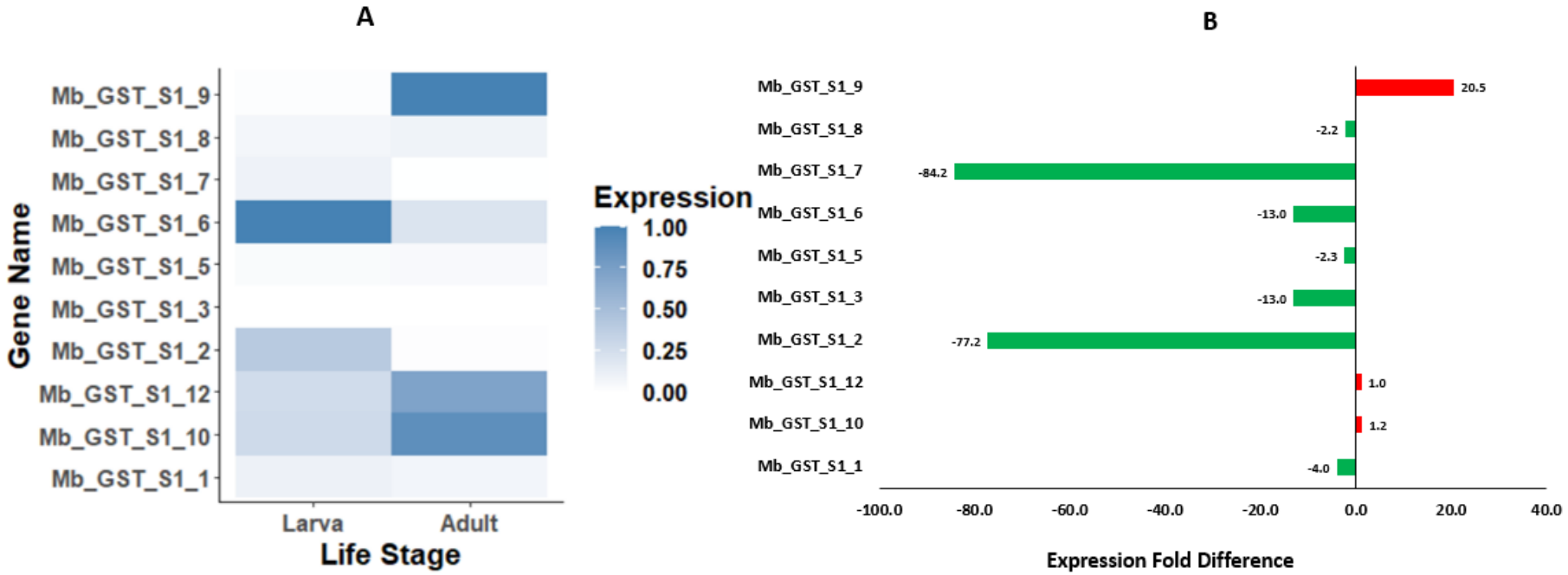


Figure 6.21. Differential expression of *GST*s from the sigma class in the *M. brassicae* transcriptome (left plot). The plot on the right shows fold differences in expression levels of the sigma class. Negative values (shown in green) indicate upregulation in larvae where positive values (red) indicate upregulation in adults.

6.3.7 *ABC* Transporter Genomic Complement

Unlike the *CYP450* and *GST* genes analysed in this chapter, the *ABC* transporter complement of *M. brassicae* could not be analysed together as a whole group due to their high degree of difference at the sequence level. Instead, it was opted to align and analyse the phylogeny of each *ABC* class separately. In total, eight different classes, named ABCA to ABCH, giving a total of 50 *ABC* transporters were identified.

6.3.7.1. ABCA Class

Of the 50 *ABC* transporters identified in *M. brassicae*, six (12%) belonged to the ABCA class (Figure 6.22). The *D. melanogaster* genome also contained six ABCA class *ABC* transporters, whereas the *B. mori* genome contained nine and the *A. mellifera* genome three (Dermauw and Van Leeuwen 2014). All of the *M. brassicae* ABCA class genes share the closest phylogenetic relationship with the *B. mori* ABCA genes consistent with the closer phylogenetic relationship of these two species.

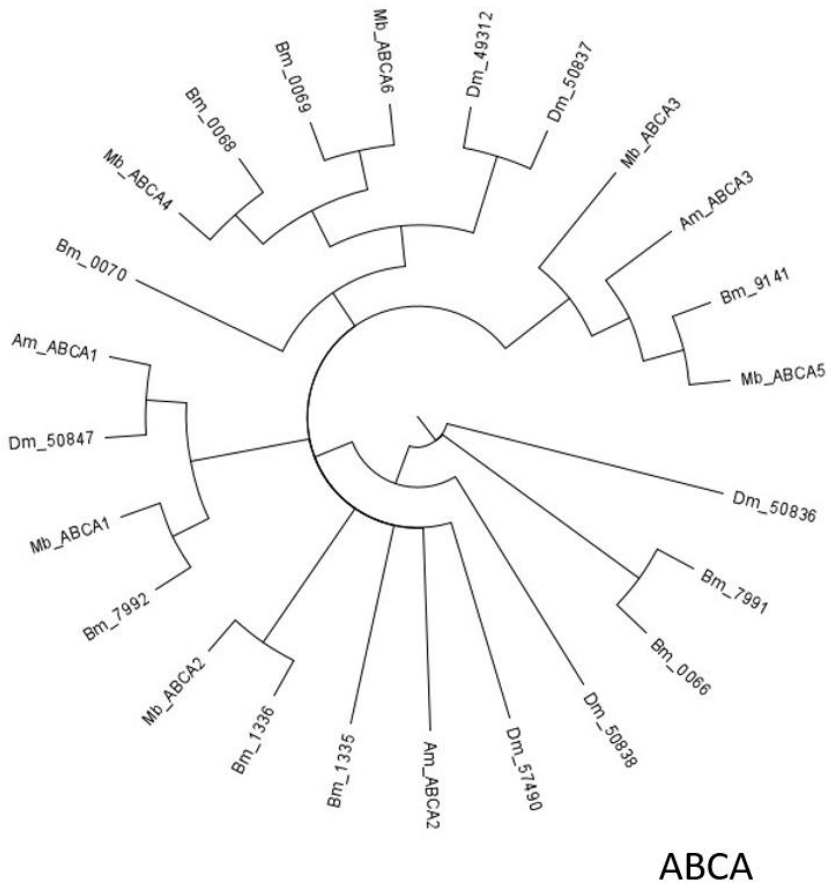


Figure 6.22. Jukes Cantor phylogenetic tree of ABCA class transporters expressed in the *M. brassicae* genome. *A. mellifera, D. melanogaster* and *B. mori* ABCA class transporters are also included for comparison.

6.3.7.2. ABCB Class

The ABCB class of *ABC* transporters are found in two distinct forms, full and half transporter. In the *M. brassicae* genome, five ABCB class transporters were full and four were half transporters. The *B. mori* genome also contains five full transporters, whereas the *D. melanogaster* and *A. mellifera* genomes contain four and one full transporters respectively (Figure 6.23). The *B. mori* genome contained the same number of half transporters as *M. brassicae. A. mellifera* expressed three half transporters, as did *D. melanogaster*.

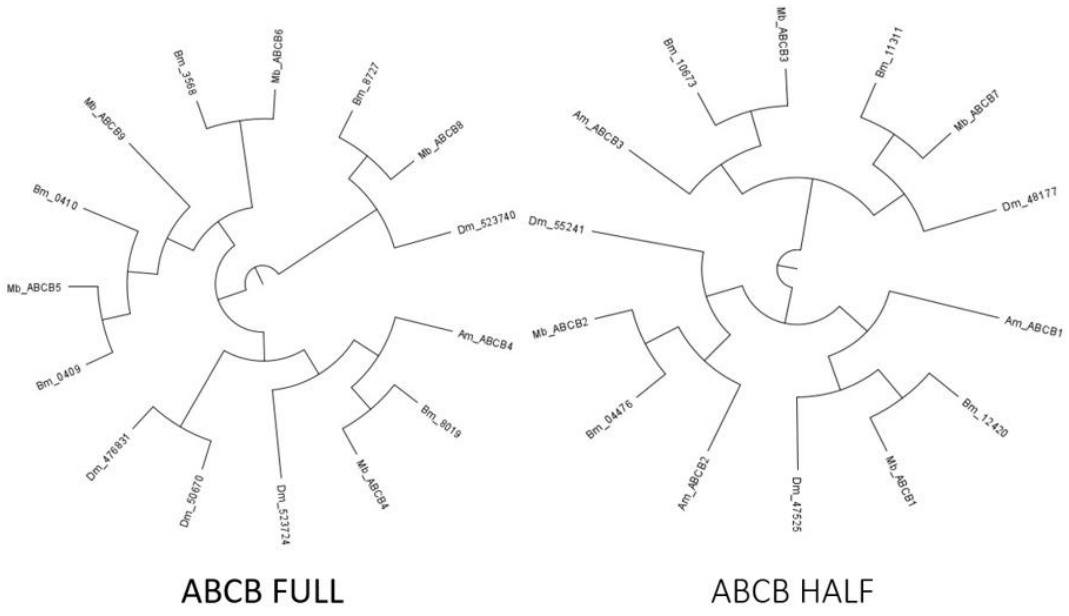


Figure 6.23. Jukes Cantor phylogenetic tree of ABCB class transporters expressed in the *M. brassicae* genome. This class of transporter can comprise of a half or full ABC transporter, here full transporters are displayed in the left hand plot and half transporters on the right. *A. mellifera, D. melanogaster* and *B. mori* ABCB class transporters are also included for comparison.

6.3.7.3. ABCC Class

The ABCC class was one of the largest *ABC* transporter groups, with twelve genes (24% of total complement) (Figure 6.24). Twelve ABCC class transporters are also present in the *B. mori* genome, while in the D. *melanogaster* genome, fourteen ABCC class transporters were identified, including two species-specific clades. The *A. mellifera* genome contains seven ABCC class transporters, consistently sharing a closer phylogenetic relationship with the ABCCs of *D. melanogaster*.

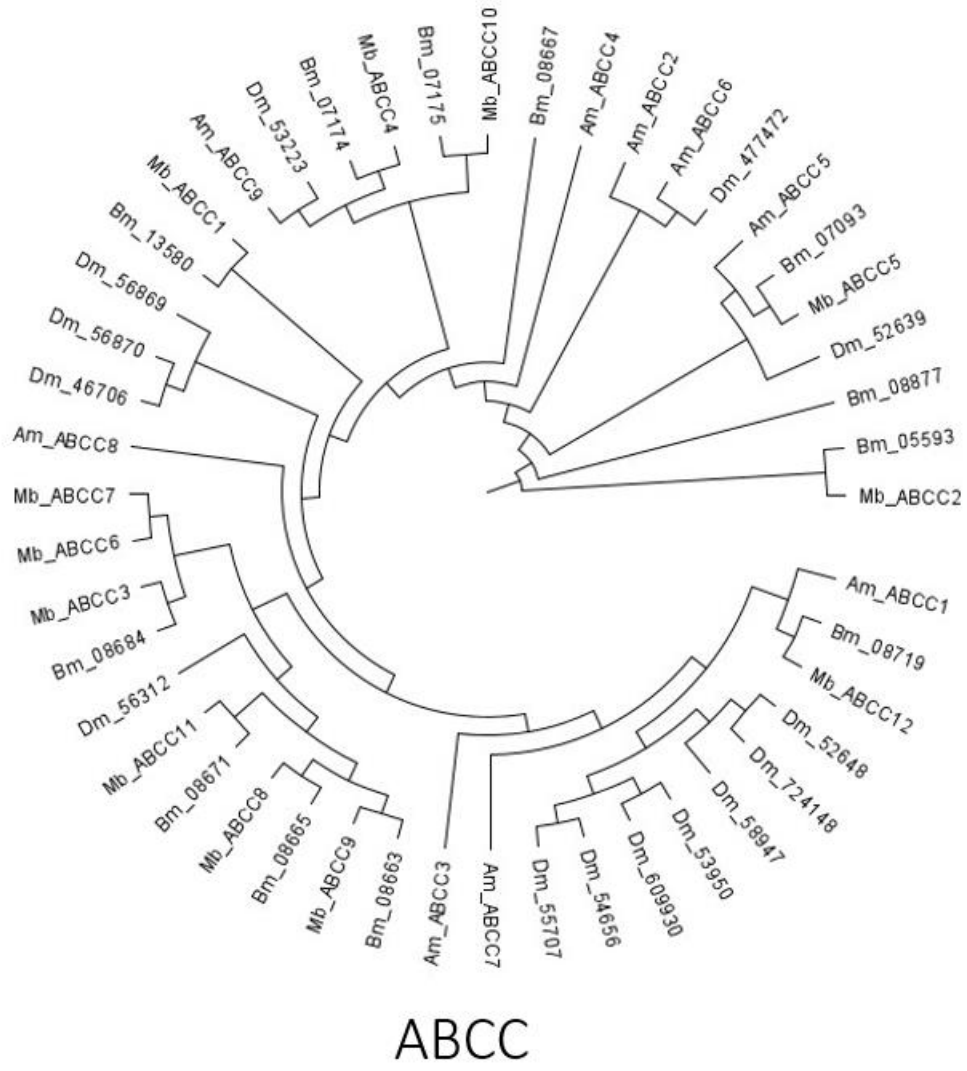


Figure 3.24. Jukes Cantor phylogenetic tree of ABCC class transporters expressed in the *M. brassicae* genome. *A. mellifera, D. melanogaster* and *B. mori* ABCC class transporters are also included for comparison.

6.3.7.4. ABCD and ABCE Class

The ABCD and ABCE class are two of the smallest classes of *ABC* transporters in *M. brassicae* (Figure 6.25). Each of the four species studied possess two ABCD class and one ABCE class transporter. In the phylogenetic tree of ABCD transporters, *M. brassicae* and *B. mori* share the closest genetic relationship while *A. mellifera* is the most distantly related.

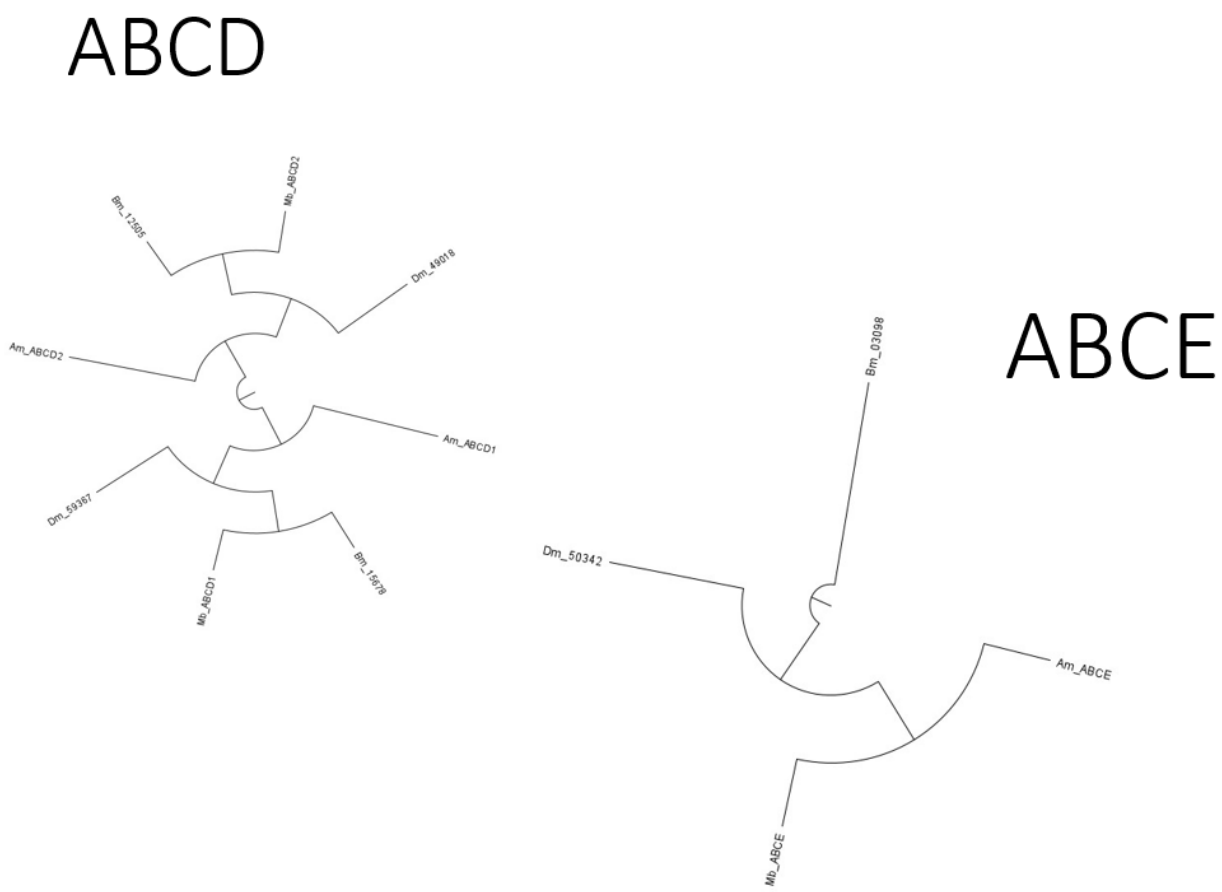


Figure 6.25. Jukes Cantor phylogenetic tree of ABCD and ABCE class transporters expressed in the *M. brassicae* genome. *A. mellifera, D. melanogaste*r and *B. mori* ABCD and ABCE class transporters are also included for comparison.

6.3.7.5 ABCF Class

In the *M. brassicae* genome, three (6% of the total *ABC* transporter complement) ABCF class *ABC* transporters were identified (Figure 6.26). This was also the case for *B. mori* and *A. mellifera.* However, only one gene of this class was found in the *D. melanogaster* genome. This single *D. melanogaster* gene was the most divergent of the ABCF class transporters of the species analysed.

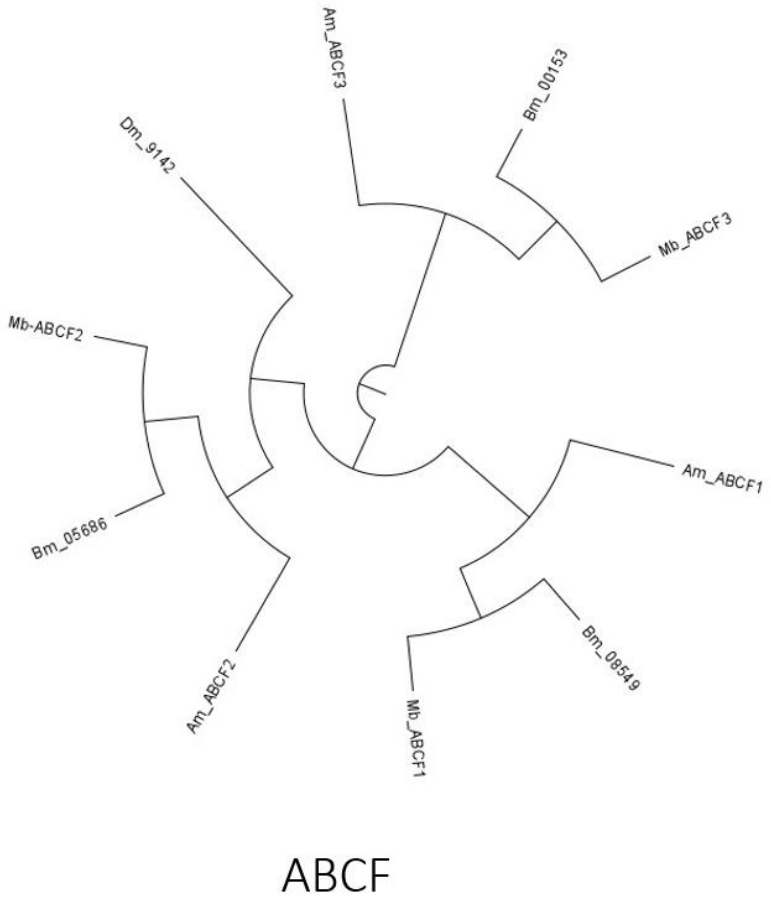


Figure 6.26. Jukes Cantor phylogenetic tree of ABCF class transporters expressed in the *M. brassicae* genome. *A. mellifera, D. melanogaster* and *B. mori* ABCF class transporters are also included for comparison.

6.3.7.6 ABCG Class

The ABCG class was the largest class of *ABC* transporter identified in the *M. brassicae* genome, with 14 (28% of total *ABC* transporter complement) ABCG class transporters identified. The high diversity of this class was also reflected in the genomes of *B. mori, D. melanogaster* and *A. mellifera* in which 13, 15 and 15 ABCG class transporters were present respectively.

The phylogenetic analysis indicated that this class of transporter consists of several clades of four genes. Almost all of these groups contain one gene from each of the four analysed species. However, there are also two clusters of two genes including only *A. mellifera* and *D. melanogaster* genes, suggesting a degree of specialisation of this class in these two species.

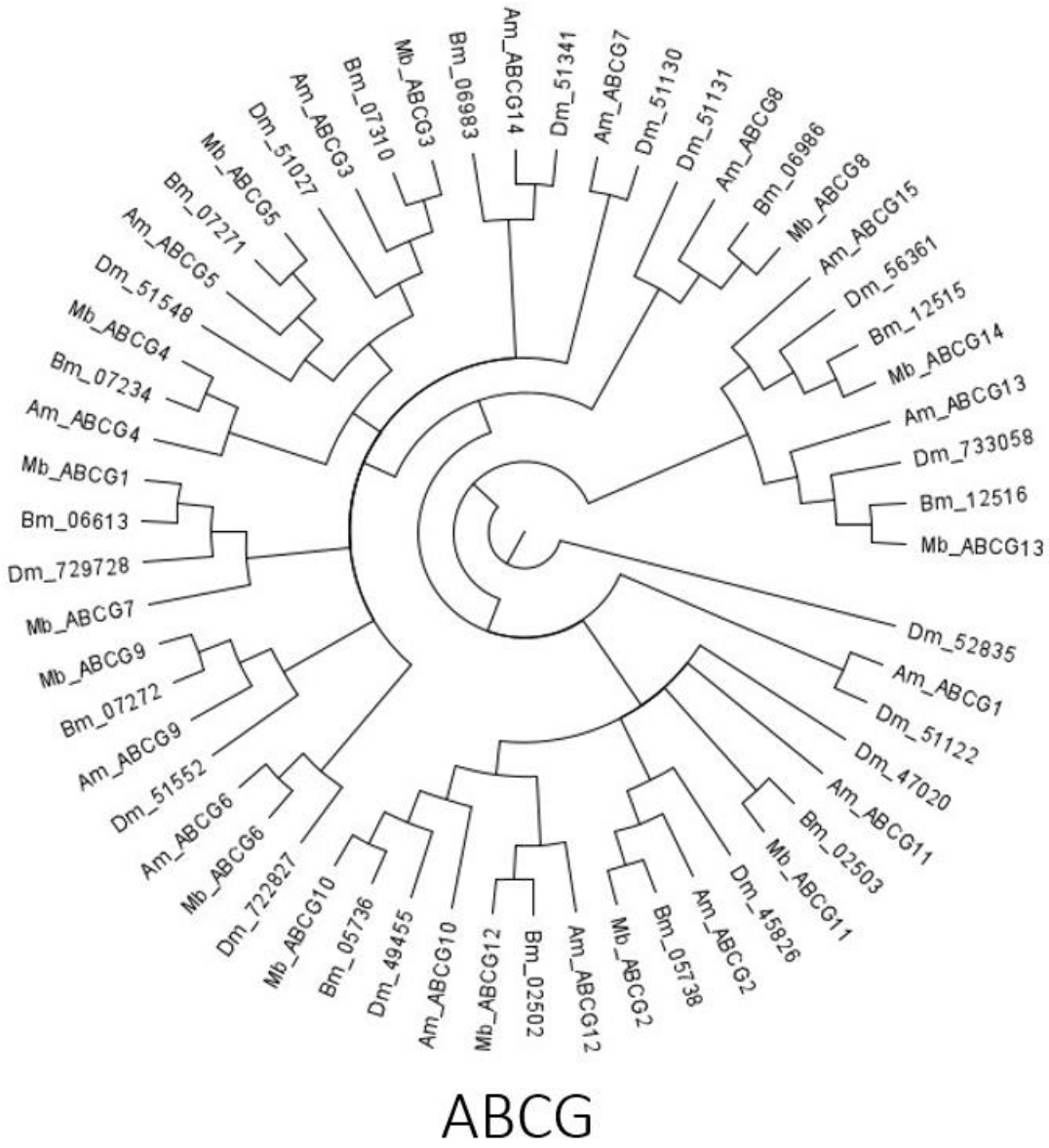


Figure 6.27. Jukes Cantor phylogenetic tree of ABCG class transporters expressed in the *M. brassicae* genome. *A. mellifera, D. melanogaster* and *B. mori* ABCG class transporters are also included for comparison.

6.3.7.7. ABCH Class

Three ABCH class genes (6% of the total *ABC* complement) were identified in the *M. brassicae* genome (Figure 6.28). This number is reflected in the genomes of each of *B. mori*, *D. melanogaster* and *A. mellifera* which contain three, four and three genes respectively. Each of the *M. brassicae* genes

identified here were similar to those of *B. mori*, while those of *A. mellifera* and *D. melanogaster* were more distantly related.

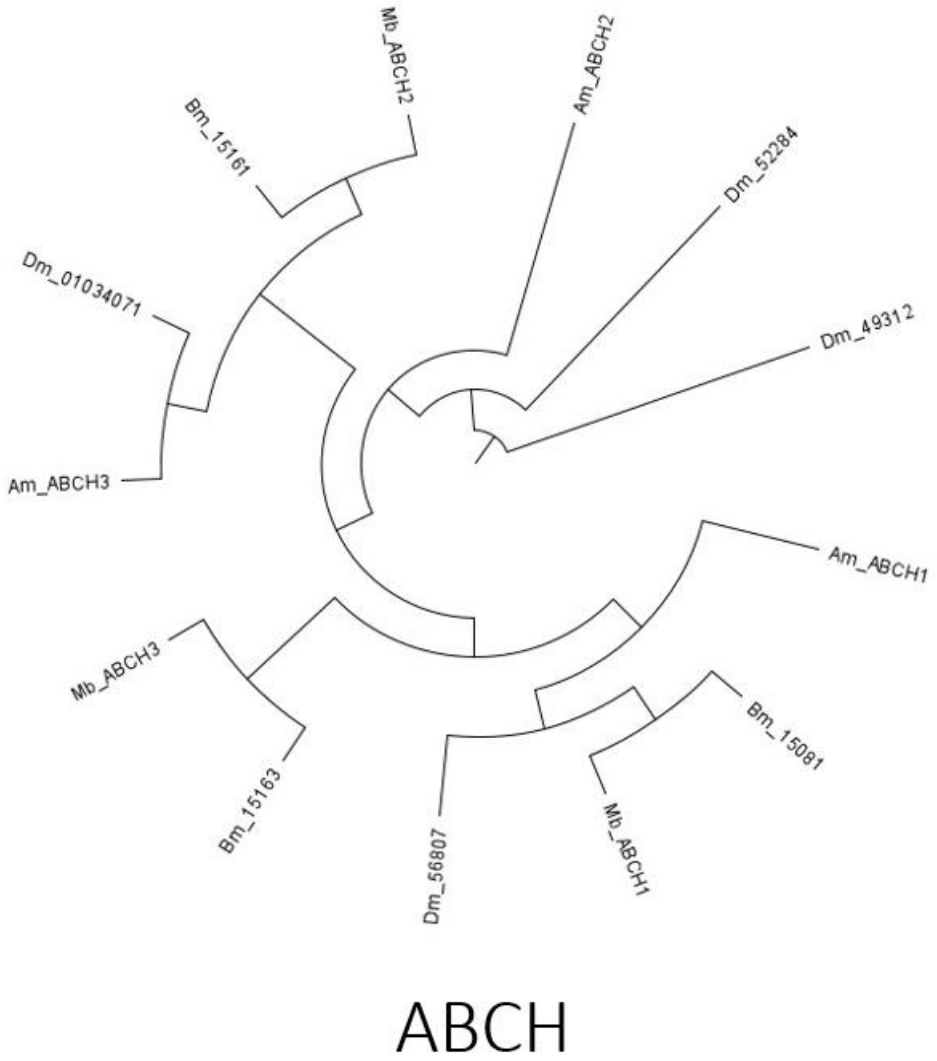


Figure 6.28. Jukes Cantor phylogenetic tree of ABCH class transporters expressed in the *M. brassicae* genome. *A. mellifera, D. melanogaster* and *B. mori* ABCH class transporters are also included for comparison.

6.3.8 *ABC* Transporter Differential Expression

6.3.8.1 ABCA and ABCB Class

Of the six ABCA class genes identified in the *M. brassicae* genome, none showed greater than 10-fold difference with two upregulated presenting greater than 10 upregulation in adults (Figure 6.29). In the ABCB class, two genes were upregulated in adults with greater than 10-fold difference in expression, with notably large difference in expression (>500 fold) for the gene *Mb_ABCB4*.

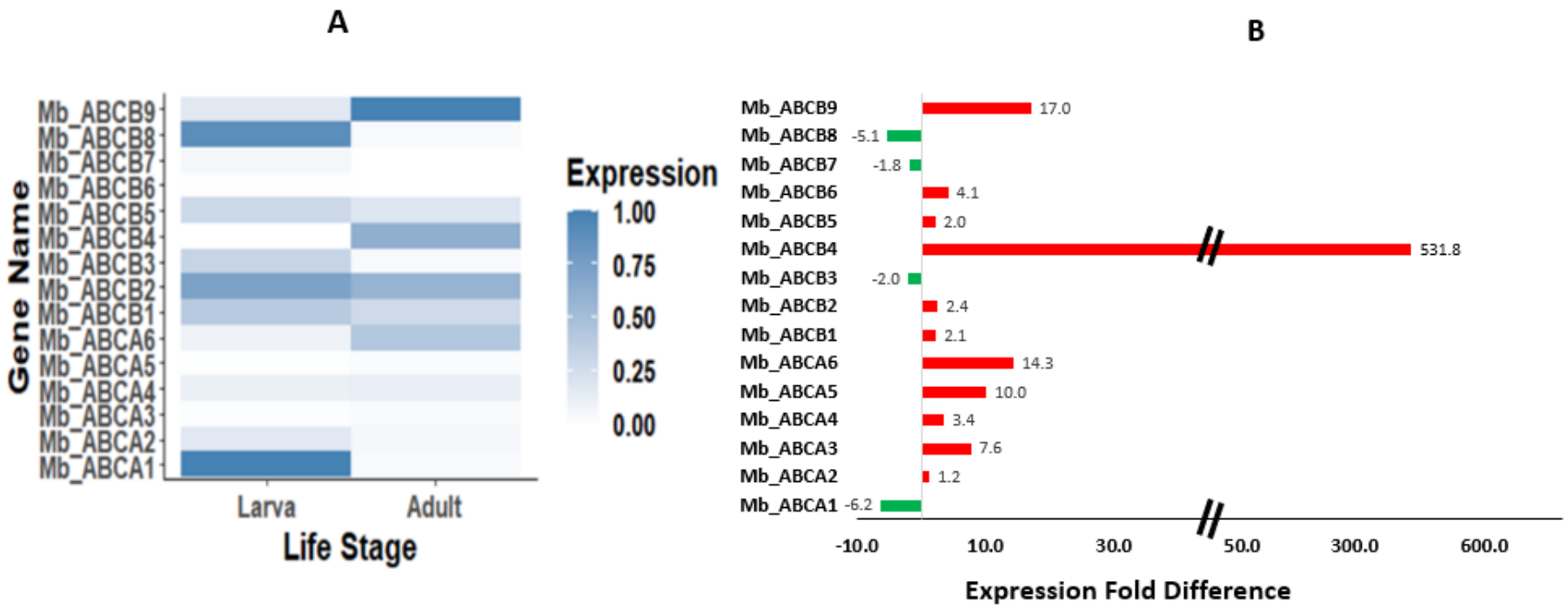


Figure 6.29. Normalised count of expression of ABCA and ABCB class genes of *M. brassicae* adults and larvae (A). Plot B shows fold differences in expression levels of ABCA and ABCB genes. Negative values (shown in green) indicate upregulation in larvae where positive values (red) indicate upregulation in adults.

6.3.8.2 ABCC, ABCD and ABCE Class

A total of 12 ABCC class *ABC* transporters were identified in the *M. brassicae* genome. Six were upregulated in larvae and six in adults (Figure 6.30) but none with a >10-fold difference (Figure 6.20B). With the exception of *Mb_ABCC3* all genes in this class were expressed in moderate or low levels (Figure 6.30 A).

Two ABCD class transporters were identified, one was upregulated in larvae and one in adults. Both differentially expressed genes were expressed moderately highly in both adults and larvae. Only one ABCE class *ABC* transporter was identified; this was expressed highly in larvae and moderately in adults.

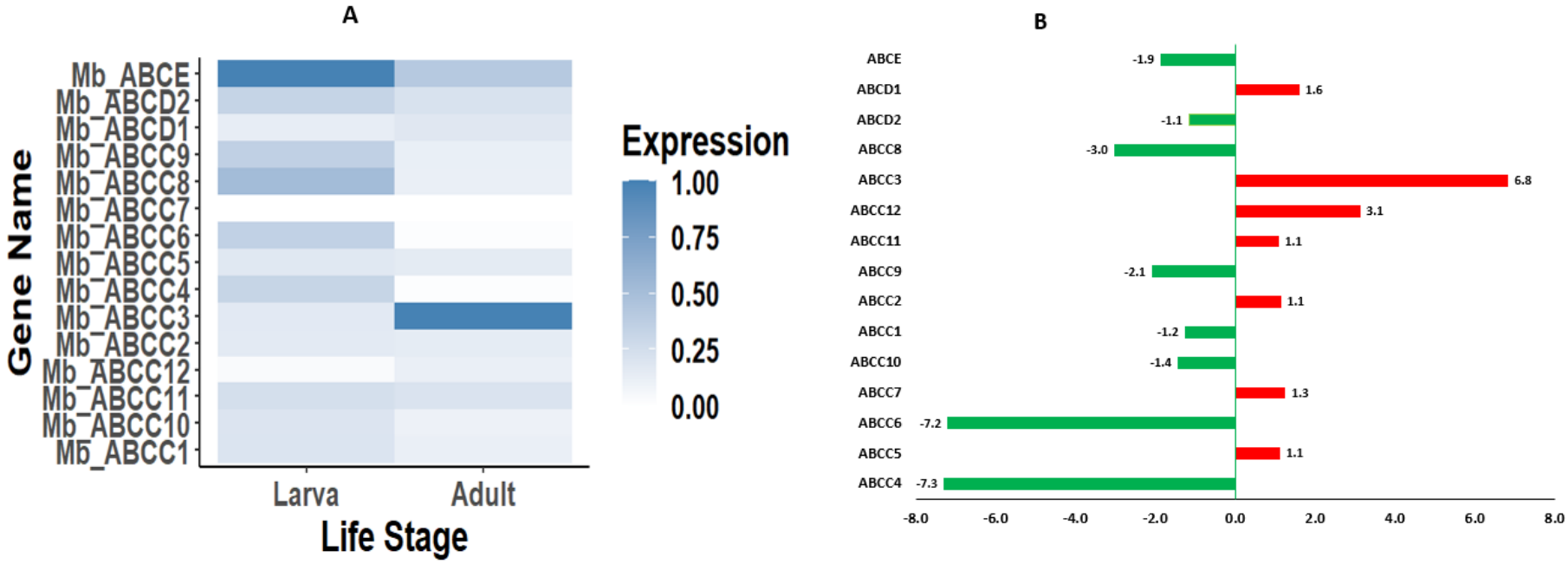


Figure 6.30. Normalised count of expression of ABCC, ABCD and ABCE class genes of *M. brassicae* adults and larvae (left plot). The plot on the right shows fold differences in expression levels of ABCC, ABCD and ABCE genes. Negative values (shown in green) indicate upregulation in larvae where positive values (red) indicate upregulation in adults.

6.3.8.3. ABCF, ABCG and ABCH Class

Three ABCF class *ABC* transporter genes were identified in *M. brassicae*. Of these, *Mb_ABCF3* was expressed highly in both adults and larvae (Figure 6.31). The largest class of *ABC* transporter in *M. brassicae* were the ABCG class. Of these, six were upregulated in larvae, most notably *Mb_ABCG12*, and eight in adults although fold differences were relatively moderate (<20 in all cases). With the exception of *Mb_ABCG5* and *Mb_ABCG4* all genes in this class were

expressed at low levels. Three ABCH genes were identified, each of which were upregulated in larvae but none with >10-fold expression difference. However, none of these genes were expressed highly in adults or larvae except for *Mb_ABCH1* which was expressed highly in the larval stage.

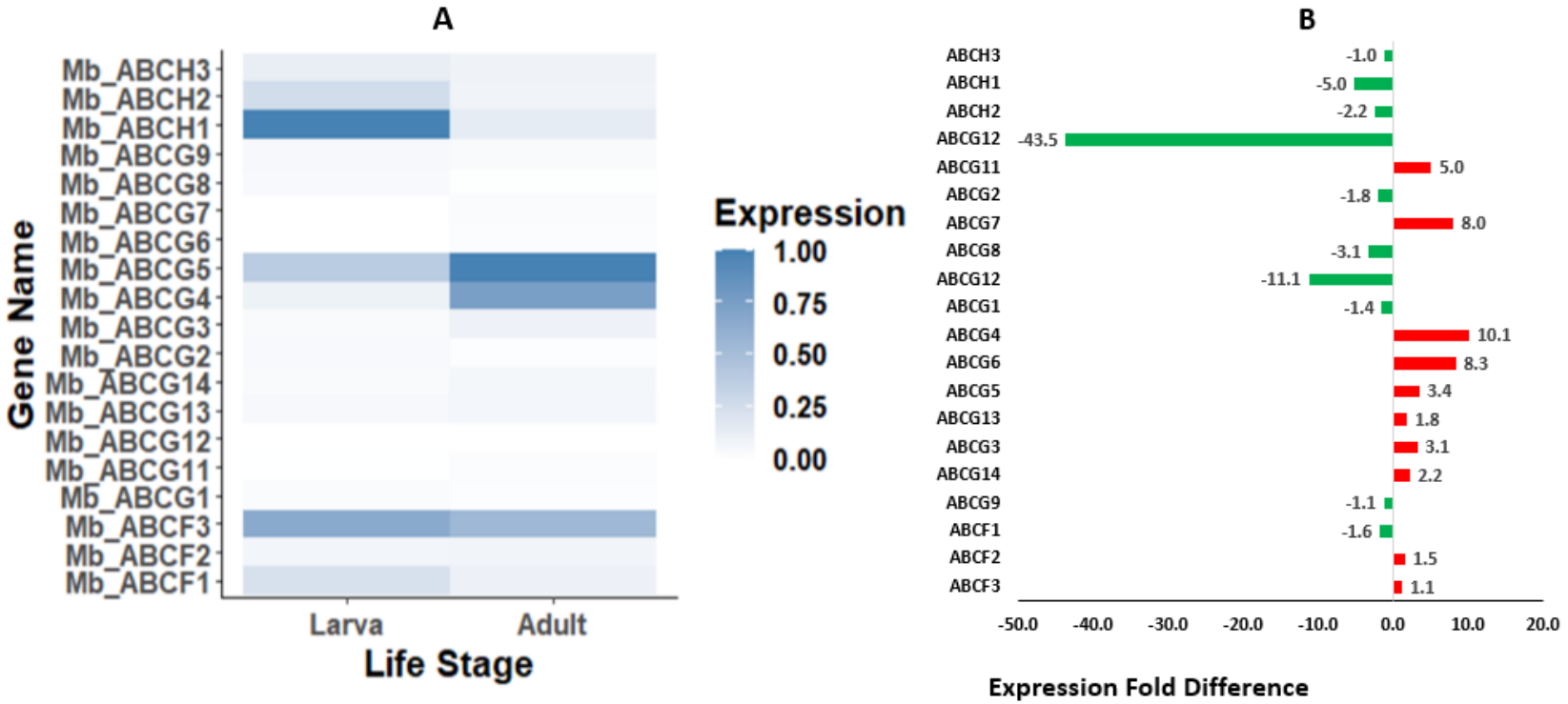


Figure 6.31. Normalised count of expression of ABCC, ABCD and ABCE class genes of *M. brassicae* adults and larvae (left plot). The plot on the right shows fold differences in expression levels of ABCC, ABCD and ABCE genes. Negative values (shown in green) indicate upregulation in larvae where positive values (red) indicate upregulation in adults.

6.4. Discussion

6.4.1 nAChR Sequence and Expression

In the previous chapter, differential sensitivity to the neonicotinoid insecticide imidacloprid was found in larvae and adult *M. brassicae*. The GUTS_RED model explored the TK mechanisms behind this but here, the genome complement of *M. brassicae* is explored to consider potential TD mechanisms of this toxicity. The first, and potentially most obvious genetic cause for differential sensitivity between the adult and larval stages, is differential binding affinity for imidacloprid to the target nicotinic acetylcholine receptor (nAChR). Changes to the sequences of nAChR subunits can influence this binding affinity, enhancing or reducing the impacts of this chemical to organisms (Matsuda, 2005; Ihara *et al*. 2008; Matsuda *et al.* 2020).

Before investigating the influence of xeno-metabolic enzymes on *M. brassicae* adults and larvae, it was decided to analyse the sequences and expression patterns of nAChR α and β subunits in both life stages. Not much expression difference was found in α and effectively none at all in β subunits between adults and larvae. In general, the three α subunits associated with the subtle expression changes (5%-7% difference in expression proportions) did not differ in amino acid identities within the critical interaction loops (Short *et al* 2021), that are predicted to influence imidacloprid binding. The one exception is position 222 (numbering using the *D. melanogaster* alpha 2 gene). Structural evidence (Ihara *et al* 2008) suggests that a tyrosine (Y) at this location rather than a phenylalanine (F) in conjunction with a non-acidic residue at position 242, will enhance imidacloprid binding. The three alpha subunits presenting subtly altered expression possess non-acidic residues at position 242 and fluctuate between an F and Y at position 222. However, the 'higher binding' combination (i.e., a Y at 222 combined with non-acidic at 242), seen in *Mb_nAChR_alpha_2_like*, makes up a lower proportion of nAChR subunit expression in adults relative to larvae. As such, it does not predict a higher imidacloprid-nAChR binding affinity in the more sensitive stage, i.e., adults. Overall, the expression and sequences of larval and adult nAChR subunits appear incapable of explaining the differential sensitivity observed between the larvae and adult *M. brassicae* life stages, shown in Chapter 5.

6.4.2 *CYP450* Complement and Expression in *M. brassicae*

The remaining putative functional *CYP450* genes found in *M. brassicae* were arranged into phylogenetic clans. Each clan was structurally unique and performs a variety of functions within the insect. The smallest clan in *M. brassicae* was clan 2. *CYP450*s in this group are responsible for biosynthesis and metabolism of endogenous compounds (Shi *et al.* 2022). The Halloween CYPs (*Spo, Spok* and *Phm*), for example, are involved in biosynthesis of ecdysteroids *(Ono et al.* 2006; Niwa and Niwa 2014). In addition, *CYP18a1* is responsible for steroid hormone inactivation and plays an essential role in metamorphosis (Guittard *et al*. 2011).

In *M. brassicae*, six *CYP450*s were identified in clan 2, consistent with the genome of *D. melanogaster*, *B. mori* and *P. xylostella*. The *M. brassicae* genes in this group were contained within one clade, indicating a high level of conservation of sequences within this family. In comparison to the *D. melanogaster* genome, no orthologue for the gene Spookier (*Spok*) was found in *M. brassicae*. There were, however, two variants of the gene *Mb_CYP18a1*, both found clustered with *Dm_CYP18a1*. This was also found in the genome of *B. mori*, in which there was an orthologue for the Halloween gene Spook (*Spo*)(*Bm_CYP307a1*), but not *Spok* (Ono et al. 2006). The Halloween genes *Spo* and *Spok* in clan 2 are essential for cuticle deposition and embryonic viability in dipteran species. The function of these genes is still largely unknown, although in *D. melanogaster*, this gene is expressed highly in ovarian tissue and early embryos. However, this clan has not been implicated with insecticide detoxification in previous studies and thus was not analysed further in terms of life stage expression.

The Mito clan plays a similar role to clan 2 *CYP450*s in insects and is also responsible for ecdysteroid metabolism (Calla *et al.* 2017). Studies have hypothesised there are two types of *CYP450* in the Mito clan, those that perform essential physiological functions and those that have rapidly evolved to become taxon specific (Yu *et al.* 2015). In the *M. brassicae* genome, nine genes were identified from the Mito clan. This is compared to 11 in *D. melanogaster*, six in *A. mellifera* (Berenbaum and Johnson 2015), 10 in *B. mori* (Ai et al. 2011) and 13 of *P. xylostella* (Yu *et al*. 2015). Like clan 2, these genes all consisted of a single clade, however, this clan formed clusters which appeared species-specific between *M. brassicae* and *D. melanogaster*; most notably, the CYP12 family.

The CYP12 family has expanded to include seven genes in *D. melanogaster* but has remained conserved in *M. brassicae* consisting of only three genes. This family is completely absent in *A. mellifera* and is likely represented by the closely related CYP333 family in lepidopterans as determined by phylogeny. Both of these two *CYP450* families are implicated in insecticide xeno-metabolism. Specifically, in *B. mori*, a CYP333 gene was implicated in detoxification of imidacloprid and permethrin in the fat body (Yamamoto *et al.* 2010). However, as Mito clan CYP450s are not often implicated in insecticide metabolism, it was decided that expression would not be analysed between larvae and adult *M. brassicae.*

The enzyme *CYP301a1* is involved in ecdysteroid metabolism and cuticle formation (Sztal *et al.* 2012). In the diamond back moth, *CYP301a1* is was found to be upregulated in larvae exposed to pesticides (Gao *et al.* 2018). Leading to the suggestion that this gene is important in regulating water loss that occurs with pesticide exposure (Gao *et al*. 2018), increasing the desiccation tolerance of larvae or reducing insecticide penetration. This example highlights potential toxicodynamic mechanisms of insecticide resistance. In the case of *M. brassicae* larvae, expression of this gene may reduce the capability of pesticides to penetrate the cuticle; decreasing uptake to the larval body and potentially contributing to larval insensitivity to imidacloprid. Given the relatively high water solubility of imidacloprid, this may be a relevant mechanism for the life-stage differences in toxicity seen. Furthermore, this may explain the unexpected sensitivity of second instar larva seen in Chapter 4 in comparison to the lack of mortality with 4th instar larva in Chapter 3. Synergistic potential recorded in Chapter 3 and 4 could also be explained by fungicide exposure inhibiting *CYP301a1,* this could increase absorption rates of an insecticide it is co-exposed with. However, further research on uptake rates would be needed to determine if this is the case.

Clan 3 is the largest and most diverse of the *M. brassicae CYP450* clades. In lepidopterans the size of this clan is correlated to the size of the total CYPome (Calla *et al.* 2017). Members of this clan are involved in insecticide resistance and xenobiotic metabolism (Feyereisen 2006). *M. brassicae* have 43 *CYP450* genes pertaining to this clan split into two families, CYP6 and CYP9. Of these, the CYP9 family consists of one clade, all mapped to the *D. melanogaster* gene CYP9f2. However, when analysed alongside a species that shares a closer evolutionary relationship to *M. brassicae*, *B. mori*, it is seen that two of these genes are possible orthologs of lepidopteran genes; *Mb_CYP9f2_6* is very closely related to *Bm_CYP9a22* and *Mb_CYP9f2_2* is phylogenetically similar to *Bm_CYP9AJ*. In addition, in the *D. melanogaster*

genome, there is a clade of *CYP450*s from the CYP28 family and a small cluster including *CYPS* 309 and 308 that are absent from the *M. brassicae*, *A. mellifera* and *B. mori* genome, suggesting a dipteran origin.

In insects, CYP9a enzymes are known to have a role in detoxification processes of pyrethroid insecticides, such as cypermethrin (Yang *et al.* 2006; Ishak *et al.* 2017; Shi *et al*. 2021). However, as this subfamily is not represented in the *D. melanogaster* genome, it is uncertain how many of these genes are present in *M. brassicae*. In bee species, the subfamily CYP9Q has been identified as detoxification enzymes of neonicotinoid insecticides (Hayward *et al.* 2019). However, this gene is found in a separate cluster to all of the CYP9s of *M. brassicae*. Therefore, it is unclear if any of the CYP9s reported in this genome are detoxicants of neonicotinoids. This is further highlighted by the expression patterns of *CYP450*s in this family as many are upregulated in adults rather than larvae, even though the former have higher sensitivity to imidacloprid (although this aligns with the marginally decreased sensitivity of the adult stage to cypermethrin). In contrast, it is feasible that the gene CYP *Mb_CYP9f2_2* does impact differential life-stage imidacloprid sensitivity as this gene is upregulated almost 3000-fold in larvae consistent with their lower sensitivity. This suggests that this gene should be putatively selected for further study in mechanisms of sensitivity to imidacloprid in *M. brassicae.*

In the putative nomenclature system used, the largest family in the *M. brassicae* clan 3 was the CYP6s. This family was also reported to be the most diverse in other lepidopteran species by Calla *et al* (2017). In *M. brassicae*, this family consisted of 28 members of the CYP6a subfamily and three from the CYP6g subfamily. The CYP6 family is also reported to play a role in the detoxification of pesticides, notably pyrethroids, carbamates (Zhang *et al.* 2019) and neonicotinoid insecticides (Karunker *et al.* 2008), as well as plant toxins (Han *et al.* 2022). One particular example of this gene which in *D. melanogaster* conveys multi-pesticide resistance, including imidacloprid resistance, is CYP6g1 (Daborn *et al.* 2007; Fusetto *et al.* 2017). In *M. brassicae* the CYP6g subfamily is found in a distinct cluster to *D. melanogaster* and lacks the related *CYP450*s, *CYP6w1, CYP6v1, CYP6t1* and *CYP6t3*. In comparison to this, the *M. brassicae* CYP6gs cluster with *Bm_CYP324a1* and *Bm_CYP332a1*, suggesting again a dipteran evolutionary clade with a distinct origin to Lepidoptera. In this cluster, *Mb_CYP6g2_2* is closely related to B*m_CYP332a1*, potentially identifying this *M. brassicae* gene as an orthologue. This *CYP450* plays a role in the detoxification of the pyrethroid

deltamethrin (Brun-Barale *et al.* 2010). However, this gene, and indeed none of the *M. brassicae* CYP6g subfamily are expressed highly in larvae but are all slightly upregulated in the adult stage; which again may explain higher sensitivity to the pyrethroid cypermethrin, but not the sensitivity of adults to imidacloprid.

The subfamily CYP6a is wide and diverse in the *M. brassicae* genome. The genes of the *Mb_CYP6a13* group are all found in a single cluster, alongside the single *CYP6a2*, *Mb_CYP6a2_12*. However, *CYP6a13* was found in a different cluster in *D. melanogaster*, showing a strong degree of speciation. One variant of CYP6a13 (*Mb_CYP6a13_6*) was found to be distantly related to all other clan 3 CYP450s, sharing a common ancestor between clan 3 and clan 4. The role of CYP6a13 is unknown, however it has a putative role in pesticide and plant toxin metabolism (Mamidala *et al.* 2012; Li *et al*. 2019; Sureshan *et al.* 2022). Further, this gene was also found to be highly expressed in ovarian tissues of fruit fly (*Bactrocera dorsalis*) (Wei *et al.* 2019) and differentially expressed in diapause and non-diapause locust eggs (Hao *et al*. 2017). In *M. brassicae* this gene may also be expressed in non-ovarian tissues as test subjects were male and kept in controlled temperature conditions and, therefore, not affected by diapause. Based on expression characteristics it is, however, unlikely that this subfamily is responsible for the differential sensitivity to imidacloprid as it is only expressed in low levels in larvae.

The two CYP6s with the biggest upregulation in larvae were *Mb_CYP6a17_2* and *Mb_CYP6a2_2.* This large difference between larvae and adult expression could play a role in the differential sensitivity seen to imidacloprid. Bahia (2021) found that honeybees surviving exposure to clothianidin expressed high levels of this *CYP450*. Furthermore, this *CYP450* family is implicated in resistance to the pyrethroid, deltamethrin (Battlay *et al.* 2018). Thus, deltamethrin and imidacloprid resistance has also been reported with increased expression of *CYP6a14* (Zhang *et al.* 2021). In honeybees, expression of this *CYP450* also increased with concentrations of the neonicotinoid thiacloprid (Fent *et al*. 2020). This study also found that thiacloprid exposure also impacted Phase II metabolic enzymes. Therefore, it is possible that imidacloprid sensitivity is a product of both *CYP450* and *GST* expression in *M. brassicae*.

The final clan found in *M. brassicae* was the clan 4 *CYP450*s containing 39 genes within three clades. This complement is greatly expanded from the four members of this clan in *A.*

mellifera, and slightly larger than the 32 genes in *D. melanogaster* and 29 of *B. mori* (Nelson 2006). Surprisingly in this clan, a total of 28 variants of *CYP4c3* (26 after removal of pseudogenes) were found. Although these *CYP450*s were named putatively, by mapping to *D. melanogaster*, this evolutionary duplication does suggest this gene performs an important function within *M. brassicae*.

Members of the CYP4 clan are linked to odorant or pheromone and insecticide metabolism (Feyereisen 2006). When phylogenetically organised, *Mb_CYP4c3* variants belong to each of the clades that make up the CYP4 clan. In the cockroach, *CYP4c7* appears to metabolise juvenile hormone (Sutherland *et al*. 1998). However, (Wang *et al.* 2019a) identified a role in cuticle shedding in spiders. Furthermore, *CYP4c3* is also implicated in resistance to pyrethroids and organophosphates (Li *et al*. 2015; Lien *et al.* 2019). This wide array of functionality is perhaps indicated in *M. brassicae* by the distribution of Mb_CYP4cs across all of the different CYP4 clades.

In the *M. brassicae* transcriptome, all *Mb_CYP4cs* are expressed at low to medium levels at both the adult and larval life stage. However, several were noticeably upregulated in adults and three in larvae. This could be potentially indicative of the requirement to suppress juvenile hormone in the adult *M. brassicae*, allowing reproductive systems to activate (Sutherland *et al*. 1998). The highest expression count of *Mb_4c3* was found in *Mb_CYP_4c3_24*. In comparison to the genome of fellow Lepidoptera, *B. mori*, this *CYP450* was identified as sharing a close relationship with *Bm_367B1,* indicating that it may be a putative orthologue. However, the function of the *CYP450* in *B. mori* is unknown.

One further interesting find from the CYP4 clan is in expression patterns between adults and larvae. On the whole, this clan shows low to medium level expression of each gene except for *Mb_CYP4g15_9* which is expressed very highly in both life stages. Most insect orders contain at least two paralogues of *D. melanogaster* CYP4g, although this can be greater in Lepidoptera (Feyereisen 2020). In the lepidopterans, *B. mori*, *M. sexta* and *S. litura*, *CYP4g1s* form a cluster of three genes (Feyereisen 2020). In *M. brassicae* this cluster is made of four individual genes, one of which (*Mb_CYP4g15_9*) is a more distant relative and another is found within a separate clade. In *D. melanogaster*, *CYP4g1* plays a role in hydrocarbon production, thereby preventing desiccation if found within oenocyte cells (Wang et al. 2019b). However, this gene can sometimes be expressed in the brain, where its function is

unknown. This phenomenon has been recorded in *B. mori*, *M. sexta* and *D. melanogaster* (Niwa *et al.* 2011). Therefore, without tissue specific expression data, it is impossible to know if *Mb_CYP4g15_9* is involved in hydrocarbon production but given the high expression in both adults and larvae it is likely that its role is in such a generalised metabolic function.

A final observation for the *CYP450* clan 4, is the presence of a clade containing *CYP311* and *CYP313* genes in *D. melanogas*ter but not *M. brassicae*. This clan is also absent from the *B. mori* genome; suggesting its function is specific to Diptera. This is also found in the *B. mori CYP450*s where there is a cluster containing *Bm_CYP341s*. However, as the *Mb_CYP4d* is part of this cluster, it is likely these genes are paralogues as there is no comparable gene *CYP341* in *D. melanogaster.*

6.4.3 *GST* Complement and Expression in *M. brassicae*

*GST*s are arguably the most important superfamily of xeno-metabolic enzymes involved in Phase II metabolism (Pavlidi *et al* 2018). They are responsible for the conjugation of compounds to glutathione to create a more polar molecule to cross membrane barriers. Approximately 200 *GST*s have been identified in insects divided into the six major classes of cytosolic *GST*; delta, epsilon, omega, sigma, theta and zeta (Enayati *et al.* 2005). In the *M. brassicae* genome, 48 *GST*s were identified. This is comparable to *D. melanogaster* (40 *GST*s) and the red flour beetle (*T. castaneum*) (41 *GST*s) (Shi *et al.* 2012). However, is much higher than the lepidopteran *B. mori*, in which only 23 cytosolic *GST*s have been identified to date (Yu *et al.* 2008), it is possible that with advances in metabolomic technology on re-evaluation this number could increase. The amount of *GST*s identified in *M. brassicae* is also much higher than those seen in the hymenopterans *A. mellifera* (10), *Bombus huntii* (11) and alfalfa leaf cutting bee (*Megachile rotundata*) (9) (Berenbaum and Johnson 2015), suggesting a large degree of expansion in dipteran and lepidopteran insects or loss in the Hymenoptera order.

The most diverse of the six cytosolic *GST*s of *M. brassicae* was the epsilon clan. In all three species, this was the largest clan, containing 26 members, a large expansion to the 8 of *B. mori* and 14 of *D. melanogaster* (Shi *et al.* 2012). The epsilon clan consists of separate *M. brassicae* and *D. melanogaster* clades (Figure 6.17), suggesting a high degree of speciation between the two species. Indeed, the only *D. melanogaster GST* gene that clustered with *M.*

brassicae was *Dm_gfzf* and the *M. brassicae GST* containing zinc. In *D. melanogaster*, this protein is the first to be identified with roles as both a transcriptional coactivator and *GST* (Baumann *et al.* 2018). However, despite its range of functions, this enzyme is not expressed highly in either larvae or adult tissue. In *B. mori,* the gene with the closest relationship to the zinc containing *GST* is an unclassified *GST Bm_GST_U1*, a gene that is absent in *A. mellifera.*

The epsilon *GST* clan has been implicated in DDT and pyrethroid insecticide resistance (Enayati *et al.* 2005). As suggested by the phylogenetic tree (Figure 6.18), this clan is absent in some species. It has been proposed that genes in this family may be important in adapting to environmental pressures (Ayres *et al.* 2011). Therefore, it is possible that this gene family is expanded in *M. brassicae*, as a response to the polyphagous lifestyle of this species, which exposes it to a variety of dietary chemical exposure. This exposure compares to that for a specialist feeder species, such as bees, which lack any members of the epsilon clan within their genome.

Transcriptomic analysis highlighted that many of the epsilon clan *GST*s were moderately highly expressed. *Mb_GST_E3_9* was very highly expressed in both the larval and adult stages. This enzyme is known to play a role in detoxification of pyrethroids in Diptera (Vontas *et al.* 2012; Pavlidi *et al.* 2018; Kouamo *et al.* 2021; Messenger *et al.* 2021) and recently has also been reported in detoxification of neonicotinoid insecticide exposure and the diamide compound chlorantraniliprole (Mao *et al*. 2019; Wang *et al.* 2020) in *B. mori*. The upregulation of *Mb_GST_E3_6* seen in larvae may at least in part explain the differential life stage sensitivity seen to imidacloprid. Further *GST*s implicated with insecticide resistance within this clan are *GSTE2, GSTE4, GSTE5* and *GSTE6* (Kouamo *et al.* 2021). However, in the *M. brassicae* genome, there were no orthologues for *GSTE4, GSTE5* or *GSTE6,* but *GSTE2*s were expressed relatively highly in larvae and adults, suggesting no role in differential sensitivity.

The delta clan is contained within the epsilon clan in all the phylogenetic trees created with the *M. brassicae* data set. This clan is small in Lepidoptera, consisting of only four genes in *M. brassicae* and five in *B. mori* (Shi *et al.* 2012). Comparatively, this family is more diverse in dipterans *A. gambiae* and *D. melanogaster* which contain 17 and 11 genes respectively. Like the epsilon clan, the delta clan is also reportedly involved in resistance development to organophosphate insecticides and DDT (Enayati *et al.* 2005; Hassan *et al.* 2021). Delta class

*GST*s are involved in the degradation of odorants and have been reported to be expressed highly in the antennae of the lepidopteran navel orangeworm (*Amyelois transitella*). In the *M. brassicae* transcriptome, these *GST*s were expressed in very low to moderate levels in both adults and larvae but were largely upregulated in larvae.

The most conserved of the insect *GST* clans are those from the theta and omega *GST*s (Yamamoto *et al.* 2005; Yamamoto *et al.* 2009a). This is confirmed by the results seen in this chapter, all theta and omega genes consist of a single clade across all species. Analysis of the *M. brassicae* genome identified one theta *GST* and two omega clan members. This is comparable to the one theta and four omega *GST*s of *B. mori* (Shi *et al.* 2012). The *D. melanogaster* theta clan is slightly more diverse and contains four members. Theta *GST*s are associated with metabolism and detoxification of lipid peroxidation processes, protecting from oxidative stress (Yamamoto *et al.* 2005). Therefore, perhaps it is unsurprising that as there is only one member of the theta clan found in *M. brassicae*, it is expressed highly in both adult and larval stages.

The omega clan is one of the smaller clans in many insects (Shi *et al.* 2012). Omega *GST*s are upregulated in response to heat-shock, UV and heavy metal exposure (Meng *et al.* 2009; Yamamoto *et al.* 2009a). For example, Kim *et al* (2010) found that omega and zeta class *GST*s were induced in pufferfish on exposure to cadmium. This *GST* class is thought to protect from oxidative stress by scavenging reactive oxygen species (ROS) by peroxidase activity (Gullner *et al.* 2018). In *D. melanogaster* this class has expanded in comparison to *M. brassicae*, suggesting a degree of species specificity. The *M. brassicae* transcriptome indicated that the omega class *GST*s show low level expression in adults. Although, *Mb_GST_O3_1* is expressed in moderate levels in larvae and is not present in adults. *GST*_O3 is implicated in the detoxification of pyrethroids, neonicotinoids and organophosphates (Yamamoto *et al.* 2009a; Balakrishnan *et al.* 2018). Therefore, given this critical function, it is possible that genes from this family are involved in the differential sensitivity between life stages. This suggests that this family should be explored further in response to the differential sensitivity recorded in Chapter 5.

The zeta class were also well conserved among the species analysed in this chapter and consistently contained one clade. The *D. melanogaster* gene *Dm_GST_Z2* appears to be an orthologue of the *M. brassicae* gene *Mb_GST_Z2_1.* However, the other zeta *GST*

Mb_GST_Z2_2 is the most highly divergent of this class, even when compared to the other *M. brassicae* zeta *GST*. When compared to *B. mori* it appears that *Mb_GST_Z2_1* is an orthologue of *Bm_GST_Z1* and *Mb_GST_Z2_2* is an orthologue of *Bm_GST_Z2*. Two zeta class *GST*s are consistent among Lepidoptera (Shi *et al.* 2012; Liu *et al.* 2015). It is suggested that this enzyme in *B. mori* acts as a detoxicant for xeno-metabolics containing chloride, including permethrin (Yamamoto *et al.* 2009b). The transcriptome of *M. brassicae* indicates that one of these *GST*s (*Mb_GST_Z2_1*) is expressed in larvae, but not adults and the other (*Mb_GST_Z2_2*) is moderately expressed in larvae and upregulated in adults. Therefore, it is unlikely that differential sensitivity was solely as a result of expression of zeta *GSTs*.

The second largest and a diverse class of *M. brassicae GST*s belong to the sigma class. This class was perhaps surprisingly diverse, as the *D. melanogaster* genome only contained one member of this class and *B. mori* only two (Shi *et al.* 2012). This suggests a large degree of divergence between lineages within this class. Sigma class *GST*s have several functions, including, detoxification of products of lipid peroxidation, acting as signalling molecules against pathogens (Huang *et al.* 2011) and in insecticide detoxification (Wang *et al.* 2021). In the lepidopteran the eastern spruce budworm (*Choristoneura fumiferana*), a *GST* from this class was reported to show low level expression in the early larval stages, but reach very high levels by sixth instar, suggesting a role in plant allelochemical detoxification (Feng *et al.* 2001). The expansion of this family seen in *M. brassicae* may be reflective of the range of allelochemicals encountered by generalist feeder species such as *M. brassicae* particularly when compared to the specialist feeder *B. mori.*

The *D. melanogaster* genome only contains one member of the sigma *GST* class. Therefore, all *M. brassicae* sigma *GST*s were named *Mb_GST_S1*. When compared with a lepidopteran species *B. mori*, all of these *GST*s were clustered by species. This suggests a strong divergence in this group between the different species. Such differences may again highlight strong differences between generalist and specialist feeding strategies of *M. brassicae* and *B mori*. Many genes within this *GST* class showed high expression in adults or larvae but never both. Enayati *et al.* (2005) suggested that this class of *GST* was highly expressed in indirect flight muscles and, therefore, the function was structural. This potentially explains the differential expression between adult and larvae of *Mb_GST_S1_9*. Given that this gene family is so expanded, it is plausible that it covers the range of functions reported by other studies, including insecticide resistance.

Microsomal *GST*s were identified in *D. melanogaster* and *A. mellifera,* but these are not found in *M. brassicae* or *B. mori*. In *D. melanogaster* this enzyme has been implicated in protecting cells from oxidative stress but also in processes related to aging, although these are still unclear. Shi *et al.* (2012) suggested that microsomal *GST*S are absent in *B. mori*, as a greater amount of gastric acid is needed to digest mulberry leaves, which would damage the structure of microsomal *GST*s. Digestion of plant matter may also explain the absence of this enzyme from *M. brassicae*. Although some microsomal *GST*s have been identified in other lepidopteran species (Shi *et al.* 2012).

6.4.4 *ABC* Transporter Complement and Expression in *M. brassicae*

ABC transporters function as protein carriers and remove products of phase I and II metabolism from the cell. Upregulation of *ABC* transporter genes can convey resistance to insecticides (Dermauw and Van Leeuwen 2014; Pan *et al.* 2020). In the *M. brassicae* genome, 50 putative *ABC* transporters were identified. This is comparable to the 51 identified in *B. mori* and the 41 of *A. mellifera* (Liu *et al.* 2011; Berenbaum and Johnson 2015). For some species, this class is comprised entirely of full transporters. However, in some lepidopterans such as *B. mori* and *M. sexta*, half transporters have also been identified (Liu *et al.* 2011; Xie *et al.* 2012).

ABCA transporters are poorly characterised in insects. Therefore, these genes were difficult to identify. For this reason, it is possible that further analysis could uncover more members of this gene class. In mammals, the ABCA class has a function in the control of cellular lipid transport (Quazi and Molday 2011). As lipid metabolism is required for the flight capacity of insects, it is also possible that the ABCA class of transporter is beneficial to Lepidoptera (Wu *et al.* 2019); potentially explaining the upregulation in adults compared to juveniles of all but one ABCA genes. The class has also been implicated in the detoxification of pyrethroid insecticides (Kalsi and Palli 2017), with the adult upregulation potentially contributing to the differential sensitivity seen to pyrethroids between adults and larvae in Chapter 5.

The ABCB class of *ABC* transporters contained both half and full transporters in *M. brassicae.* This pattern is also seen in many insect species, such as *B. mori* and *A. mellifera* (Wu *et al.*

2019). This transporter class is also relatively unstudied in insects and the functions of these enzymes are largely unknown. In mammals, these genes are often implicated in specific functions to this group, such as bile transport (Annilo *et al.* 2006). This class of transporter are further thought to play a role in detoxification of both plant toxins and synthetic insecticides, including chlorpyrifos, deltamethrin and imidacloprid (Sun *et al*. 2017; Kowalski *et al.* 2020). This class of insecticides saw the largest fold difference of expression between adults and larvae in the *ABC* transporter *Mb_ABCB4*. In this instance, this large upregulation was in the adult life stage and therefore, it is unlikely that this is involved in the enhanced detoxification of imidacloprid seen in larvae in Chapter 5.

The half transporters of class ABCB are said to control homeostasis of iron in mitochondria of humans and prevent against oxidative stress (Zutz *et al.* 2009). This class of transporters are also relatively understudied. However, it is thought that the human genes may be orthologous of the insect sequences (Merzendorfer 2014). For example, in *D. melanogaster*, a role in cellular iron stasis has been previously identified (Merzendorfer 2014). The half transporters of *M. brassicae* and *B. mori* were closely related phylogenetically, suggesting a close relationship of Lepidoptera regarding this gene. However, the function of these genes in lepidopterans is unknown at this time and is cause for further study.

Most insect genomes contain between 9 and 16 ABCC class transporters (Wu *et al.* 2019). This is also the case for *M. brassicae* for which 12 were identified. The ABCC class is the largest class of the *ABC* transporters in the *B. mori* genome. It has been suggested that the wide diversity of genes within this class have contributed to a wide range of function such as xenobiotic detoxification, ion transport and signal transduction (Labbé *et al.* 2011; Dean *et al.* 2022). In the *M. brassicae* analysis, half of this class were upregulated in larvae and half in adults. It is recommended that the function of these *ABC* transporters is explored in more detail to determine if their role in xeno-metabolism is toxicant specific, thereby implicating the expression of these *ABC* transporters in the differential sensitivity between the adult and larval stages.

The three classes of *ABC* transporter, ABCD, ABCE and ABCF are well conserved between insects. This pattern is also reflected in the *M. brassicae* genome. Most insects contain two ABCD genes, one ABCE and three ABCFs (Wu *et al.* 2019). The ABCE gene, also named ‘Pixie’ in *D. melanogaster*, plays a role in ribosome formation and assembly. Deficiency of this

protein can result in malformed wings, specifically the small wing phenotype (Coelho *et al.* 2005; Kashima *et al.* 2014). In *M. brassicae,* this gene was expressed highly in both life stages but was slightly upregulated in larvae. In the Lepidoptera *B. mori* and *P. xylostella* ABCE was the highest expressed of all *ABC* transporters, suggesting involvement in a critical function (Qi *et al.* 2016).

The ABCF class is thought to be structurally similar to ABCE. Neither class act as classic transporters. Instead it is thought that ABCF is also involved in ribosome synthesis, translational control and the export of mRNA from the nucleus (Qi *et al.* 2016). Genes from classes E and F have not been implicated in insecticide resistance and as such it is unlikely that they are responsible for the differential sensitivity seen between the larval and adult stages.

In *M. brassicae* the largest class of *ABC* transporter was the ABCG class. In insects, this class commonly contains between 2 and 24 genes (Wu *et al.* 2019), meaning the number found in *M. brassicae* (15), is unremarkable. In humans, this gene is involved in lipid transport (Merzendorfer 2014). The best studied genes of this class are White (*DmelW*), Scarlet (*St*) and Brown (*Bw*) in *D. melanogaster* where they are involved in eye pigmentation (Borycz *et al.* 2008). In *B. mori*, an orthologue of White has been located and this plays a role in transportation of ommochrome precursors. Further, in lepidopteran and hymenopteran species, this gene may dimerise with brown to become a urate transporter (Kômoto *et al.* 2009; Merzendorfer 2014).

The lepidopteran, *P. xylostella*, has 15 ABCG genes, several of which have been attributed to resistance to the diamide insecticide chlorantraniliprole (Shan *et al.* 2021). In *M. brassicae,* several of these ABCG genes are upregulated in the larval stages, potentially contributing to the differential sensitivity found between adults and larvae to imidacloprid. Additionally, this gene has also been reported to play a role in ecdysone responses and transport of lipids to the epidermis preventing water loss (Hock *et al.* 2000; Broehan *et al*. 2013). In this case, expression patterns would mirror those seen of the Mito Clan *CYP450 Mb_301a1* which also functions in moulting and prevention of desiccation.

The ABCH class of *ABC* transporter is structurally similar to the ABCG class present in mammalian species. Liu *et al.* (2011) and Merzendorfer (2014) studied the structure and

organisation of ABCH transporters in the genome of five insect species. Their analysis found that each species had three ABCH genes: as seen here in *M. brassicae*. The function of this class requires further study. However, Rösner *et al* (2021) suggested a non-specific stress response on exposure to insecticides.

Of the three class of genes investigated here (*CYP450, GST* and *ABC* transporter), the ABC-transporters are the least functionally characterised. While the availability of metabolomic testing techniques and data has opened access to the study of enzyme families such as *ABC* transporters, the function of many of these genes and families is unknown in insects. For this reason, it is recommended that this enzyme family becomes a topic of future research in order to better integrate knowledge of the functions of each class into predictions of species sensitivity.

It is also important to recognise that the genes described in this chapter are not the sole enzymes responsible for insecticide detoxification. This work could also have been expanded to cover UDP-glucuronosyltransferase (UGT), N-acetyltransferase (NAT), and sulfotransferase (SULT). However, this chapter aimed to identify genes from one representative enzyme family from each stage of the metabolic process. From this analysis, it is recommended the genes *Mb_CYP_6a2_2, Mb_CYP_6a17_2, Mb_CYP_9f2_2* and *Mb_GST_O3* are prioritised in future *M. brassicae* studies with the hope that they can explain mechanistically the differential sensitivity of larvae and adults to imidacloprid.

6.5. Conclusion

An understanding of toxicant metabolism is vital for revealing the pathways that led to damage within organisms. Thus, toxicity of a chemical to an organism may be predicted by the xeno-metabolic capabilities of these species and by extension to the xeno-metabolic gene complement. The diversity of genes in the main xeno-metabolic families may underpin the transformation and in doing so reduce the level of toxicity. It is recognised that exposure to plant toxins has aided the development of detoxification processes in phytophagous insect species. However, it is unclear how this has affected the evolutionary divergence of Lepidoptera from other insects given the largely herbivorous nature of the juvenile stages and nectar feeding strategies of some adults.

In this chapter, three phases of metabolism were studied in detail in the *M. brassicae* (cabbage moth) genome by the identification of three enzyme superfamilies representing a specific detoxification pathway; *CYP450*s (Phase I), *GST*s (Phase II) and *ABC* transporters (Phase III). These enzymes were isolated by comparing to the *D. melanogaster* genome and BLAST searching comparable annotated genes. Genes were then named putatively by their associated *D. melanogaster* enzyme name (except for *ABC* transporters which were named by their putative association to *A. mellifera*). The identified enzyme superfamilies were then compared phylogenetically against genes in the *D. melanogaster* genome and also those in the closer related lepidopteran species, *B. mori.* The Western Honeybee (*A. mellifera*) was also used as a basis for comparison between taxa. Finally, a transcriptome generated from a larval and adult stage identified expression patterns of these enzymes at different life stages.

Chapter 5 highlighted differential sensitivity within the life stages to insecticides, most notably imidacloprid which was relatively non-toxic to larvae but caused a high mortality in adults. The gene compliment and expression analysis showed that only one clan CYP4 gene was expressed highly in both adults and larvae – *Mb_CYP4g15_9*. The CYP3 clan showed much greater variance of expression between the adult and larval stages. This data may highlight potential genes of interest that differ in expression between life-stages, perhaps driving the observed difference in sensitivity. As adults were more susceptible to imidacloprid than larvae in Chapter 5, here, genes of interest were selected on the condition

that they were upregulated in larvae and were part of a class of *GST*s, ABCs, or clan of *CYP450*s implicated in neonicotinoid resistance (Table 6.4). This criterion indicates that the *CYP450* genes *Mb_CYP6a17_2*, *Mb_CYP6a14_3, Mb_CYP6a2_2, Mb_CYP9f2_2* may have an impact on the susceptibility of *M. brassicae* to imidacloprid, all being less expressed in adult. Additionally, the *GST*s *Mb_GSTE3_6* and *Mb_GSTE2_3* also show reduced adult compared to larval expression. To validate hypotheses concerning the functional role of these gens in insecticide sensitivity, further research such as CRISPR could be undertaken to knockdown these genes to understand how far they are implicated in neonicotinoid resistance.

Table 6.2. Genes most likely to be identified as influencing differential sensitivity between adult and larval stages. All of these genes have >100-fold upregulation in larvae, those highlighted in green are >1000-fold upregulated. No ABC transporters are represented here as none show a >100-fold upregulation in larvae.

Gene Family	Gene of Interest
CYP450	*Mb_CYP_6a2_2*, *Mb_CYP_6a2_1*, *Mb_CYP_6a17_2*, *Mb_CYP_9f2_2*, *Mb_CYP_4d2_3, Mb_CYP_4c3_2*
GST	Mb_*GST*_Z2_1, Mb_*GST*_O3, Mb_*GST*_D1_2, Mb_*GST*_D1_2, Mb_*GST*_E7_2, Mb_*GST*_E7_5

The function of genes within many of the *ABC* transporter classes was unknown and therefore, were difficult to attribute to the differential sensitivity between the larvae and adults in Chapter 5. The gene *Mb_ABCB4* was upregulated by a ~500 fold-difference in adults and was potentially involved in the decreased adult sensitivity to cypermethrin. Although several genes of the ABCG class were upregulated in larvae, except for *Mb_ABCG12*, this difference was never greater than 20-fold.

This chapter represents the first attempt to identify the super enzyme families *CYP450*s, *GST*s and *ABC* transporters in *M. brassicae* and associate their expression with differential sensitivity to pesticides between life stages. Although more research is needed to understand the effect and function of many of these individual genes, this work has shown that this species' genome is comparable to other lepidopterans. Furthermore, comparative

analysis of the transcriptome between the larvae and adult stage has provided an insight into the potential metabolic causes of differential sensitivity in the two phenotypically distinct life-stages of this species. In highlighting the differential expression of xeno-metabolic enzymes, it may in future be possible to predict the susceptibility of an organism based on the presence and expression of specific genes. This may have an implication for protecting non-target species, by targeting risk assessments to the most susceptible life stages.

CHAPTER 7 - CONCLUSION

7.1. Summary

This thesis, 'Defining species sensitivity and synergism potential for pesticides and pesticide mixtures through physiological traits analysis', aimed to develop an understanding of the sensitivity of insects, specifically Lepidoptera, to a range of different pesticides (insecticides and fungicides) and investigate any potential interactions between binary mixtures of these. Further, this work explored mechanistic causes for such interactions using a multifaceted approach of toxicity testing, TKTD modelling and bioinformatics.

To achieve this, the following objectives were met:

1. Selection of a model species (*M. brassicae*) for sensitivity testing
2. Development of a high-throughput testing method capable of assessing sublethal and mortality effects of pesticides over 96 hours with this species; including TKTD modelling analysis
3. Expansion of this testing method to a range of insecticides and fungicides representing a range of modes of action or target receptors/pathways
4. Adapting of the bioassay method and model to test binary mixtures of pesticides
5. Identify additivity, synergism or antagonism in insecticide/fungicide binary mixtures
6. Expand testing of a mixture identified as synergistic over a full larval growth phase (i.e. from instar 2 to 6)
7. Comparative testing of adult *M. brassicae* to determine cases of differential sensitivity between life stages
8. Generation of genome and transcriptome of *M. brassicae* to i) identify xeno-metabolic enzymes and ii) compare expression between larvae and adult stages

In this thesis, TKTD modelling of survival (GUTS modelling) was demonstrated to be a suitable tool for assessing the sensitivity of lepidopteran (*M. brassicae*) to a range of insecticides and fungicides. Further, this analysis was also extended to simulate the effects of pesticide mixtures, providing a more realistic exposure scenario faced by Lepidopterans in field settings. TKTD models are an alternative to previous regression models (such as probit) and

mixture models such as Concentration Addition and Independent Action as they account for the temporal aspect of toxicity. Using TKTD models, processes such as uptake, distribution, metabolism and elimination are considered within the time aspect which allows for meaningful comparisons between both chemicals and species.

Using the novel bioassay developed in Chapter 2, the sensitivity of *M. brassicae* was analysed on exposure to several insecticides and fungicides, representing a range of target receptors and MoAs. Perhaps unsurprisingly, given their target taxa and receptor, acute toxicity was never recorded in 4th instar larvae exposed to any fungicides. Although, sublethal effects on weight were reported for the strobilurin fungicide azoxystrobin and the azole fungicide propiconazole. However, acute toxicity was found in three of the six insecticides tested. The GUTS model calculated the lowest LC_{50} values for the organophosphate chlorpyrifos and the pyrethroid cypermethrin in both larvae and adult *M. brassicae* bioassay testing.

One surprising result from the toxicity screening of larvae *M. brassicae* was the low sensitivity observed to neonicotinoid insecticides. When tested in concentrations close to maximum acetone solubility, only 16.6% mortality was recorded with exposure to imidacloprid. Further, no mortality or sublethal effects were found with sulfoxaflor exposure, which although not a neonicotinoid, targets nAChRs and therefore, suggests that further research is needed into the binding potential of neonicotinoid insecticides to Lepidopteran nAChRs. In a clear contrast to these results, imidacloprid exposure had an acute effect on adult *M. brassicae* survival. The LC_{50}s between the adult and larval life stage were compared in a ratio which found that survival of adult: larva was 148 : 1782 over 96 hours.

The GUTS model provided robust and reliable analysis for the single chemical survival data but here was also expanded and adapted to simulate the effects of chemical mixture exposure. Although this method was not quantifiable, it allowed the identification of chemical interactions such as synergy and antagonism through an under or over-prediction of observed mortality. Of the 13 mixtures tested to *M. brassicae* larvae, synergistic interactions were found between six chemical pairings. One of these synergistic mixtures was between two insecticides, cypermethrin and cyantraniliprole. Indeed, the diamide insecticide cyantraniliprole interacted synergistically in every pairing tested. However, it was found in the single chemical bioassay tests that this insecticide had a significant effect on weight in all tested concentrations, suggesting that this chemical had such a profound effect

on larval physiology that any further stress on metabolic processes would result in a greater mortality effect. Mixtures involving cyantraniliprole were the only chemical to interact with the fungicide azoxystrobin. All of the remaining synergistic mixtures contained an insecticide and fungicide.

In concordance with previous studies, this thesis reported synergy in pyrethroid and azole fungicide mixture combinations cypermethrin and prochloraz and cypermethrin and propiconazole (Rösch et al. 2017; Kretschmann et al. 2015; Cedergreen 2014). Synergy was also found in the adult bioassay of cypermethrin and prochloraz, suggesting that all life stages could be vulnerable to co-exposure to cypermethrin and prochloraz. This was further confirmed by the study of the effects of cypermethrin exposure on the growth, development and survival of *M. brassicae* throughout their whole larval stage in Chapter 4. Contrary to single chemical testing, here, an effect of prochloraz applied as a single chemical was seen as larvae were exposed at an earlier instar. Further, the time between moults and time to pupation was significantly impacted by these chemicals, applied both singly and as a mixture. These effects have wide implications for lepidopteran populations in terms of broods per year and seasonal occurrence.

The model species (*M. brassicae*) was selected as an indicator of the toxicity of pesticides to Lepidoptera. However, at the onset of bioassay experiments, it was not known how representative the culture of *M. brassicae* used was to other Lepidopteran species. To investigate this, the genome of *M. brassicae* larvae was sequenced and genes mapped to the highly studied and annotated genome of *Drosophila melanogaster*. This genome could be used to investigate many facets of toxicity, including receptor structure and xeno-metabolic capabilities. While it is recognised that there are many gene families involved in the metabolism of toxicants, time constraints meant that only a fraction of these could be studied in detail. To represent the three stages of metabolism, here the Phase I metabolic enzymes CYP450s were studied, along with GSTs of Phase II metabolism and ABC transporters representing Phase III.

Insect CYP450s belong to one of four clans, *CYP2, CYP3, CYP4* and the mito clan. Analysis of the *CYP450* superfamily found that this gene family was largely expanded in *M. brassicae* in comparison to the honeybee *A. mellifera* (122 in *M. brassicae* and 46 in *A. mellifera*). However, was loosely comparable to the 87 *CYP450*s identified in the *D. melanogaster*

genome. The CYPome size of M. brassicae was also closely comparable to that of fellow *Noctuidae* family members *H. armigera* and *S. litura* (in which 112 and 138 *CYP450*s were identified respectively). This highlighted that at least in terms of CYP450 expression, there was a fair amount of genomic conservation in the *Noctuidae* family but are widely diversified from several other insect species.

The large divergence seen in the *M. brassicae* genome compared to insect species such as honeybees has also resulted in the evolution of pseudogenes. In this thesis, pseudogenes were defined as lacking a region seen in all other genes of this type. For example, in *M. brassicae* pseudogenes were identified as those missing any of the following motifs; the WxxxR motif, the GxE/DTT/S motif, the ExLR motif, the PxxFxPE/DRF (PERF) motif, and the PFxxGxRxCxG/A motif. This criterion excluded 24 genes from the *M. brassicae* CYP450 analysis. It is unclear from the analysis undertaken in this thesis if the excluded pseudogenes are functional and this is recommended for further study.

One further rationale behind the decision to investigate the CYP450 complement of *M. brassicae* was also that they have been implicated in many studies of insecticide resistance; often of neonicotinoids. CYP450 clans with implications in insecticide resistance are the CYP3 and CYP4 clans. These two clans were found to be widely expanded in *M. brassicae* and were the two largest of the four CYP450 clans. Potentially, explaining the low sensitivity seen for neonicotinoid exposure and indeed many of the chemicals tested in Chapter 3.

Analysis of GSTs identified 48 GSTs split across six classes, delta, epsilon, omega, sigma, theta and zeta. The sigma class was largely expanded from the *D. melanogaster* genome (13 in *M. brassicae* and 1 in *D. melanogaster*), potentially the evolutionary product in response to the generalist diet of *M. brassicae*. Further, the other class of GST implicated in insecticide resistance, epsilon, was also expanded in *M. brassicae* and in the majority of cases was upregulated in larvae; suggesting a potential contribution to the differential sensitivity reported between the adults and larvae.

The final family of metabolic enzymes analysed in this thesis were the ABC transporters. This superfamily of enzymes is poorly characterised and annotated in other arthropod species, so the function of many of these genes is unknown. However, a total of 50 ABC transporters were identified in *M. brassicae* across eight classes, ABCA to ABCH. This number is

representative of other lepidopteran species. The largest of these families was the class ABCG, which has been linked to pesticide resistance in several studies (Shan et al. 2021; Rösner et al. 2021; He et al. 2018; Liu et al. 2011), this expansion again may be in response to plant toxins ingested by generalist feeder species.

7.2 Further Work

Although the work undertaken in this thesis has provided an insight into the sensitivity of lepidopterans exposed to pesticides and pesticide mixtures, it has also highlighted several areas recommended for further study.

One criticism of the work undertaken in this thesis is the lack of internal chemistry data. This meant that the uptake and distribution of chemicals within test organisms was unknown. Whilst the reduced GUTS model used allows for analysis of chemical data with no internal residue data, this information would conclusively exclude differences in bioavailability as a cause for the differential sensitivity seen between adults and larvae exposed to imidacloprid. Further, this data would confirm the extent of the impact of heat degradation when using the artificial diet bioassay method. The developed bioassay methods for adults and larvae both allowed for the calculation of feeding rates. However, this data was not analysed in this thesis. Coupled with chemical analysis, this information would give a more accurate dose range for pesticide bioassays.

In this thesis, a study was conducted to assess the synergism potential of an azole fungicide and pyrethroid insecticide over the full larval growth cycle of *M. brassicae* larvae (Chapter 4). However, this work began at the second instar larvae and ended at the pupal stage. To study the effects of exposure over the whole life of *M. brassicae* (from egg to adult) an expansion of the DEBkiss study would need to be designed beginning with exposure of eggs. Exposing eggs to pesticides or mixtures would be a relatively simple process, eggs could be added onto spiked filter paper, but considerations must be made for handling deaths more likely to occur with frequent testing of smaller instars of larvae.

The greatest challenge with designing a full lifecycle study of Lepidoptera would be that it would not be easy to design a method suitable for DEB analysis as this species has distinctly different feeding strategies before and after pupation. Further, during the egg stage and pupation, no food is assimilated or excreted and therefore, no energy is gained in this stage. A potential solution to this problem could be topical applications but this method may lack the realistic nature of exposure provided by the experiments undertaken in this thesis.

Using DEB models, or simplified versions can also study the reproductive outputs of organisms. As the experiment conducted in Chapter 4 only considered the larval stages, the impacts of cypermethrin and prochloraz on reproduction were not analysed. This should be a relatively easy process involving the daily counting of eggs. This data could allow a simulation of reproductive rates over the time course of exposure and perhaps highlight sublethal synergistic effects of mixture treatments.

One of the more interesting findings of the work undertaken in this thesis was the differential sensitivity to pesticides and the associated expression of xeno-metabolic enzymes in the adult and larval life stages. This finding has large implications for pest management strategies and could lessen the impacts of pesticide application on non-target species by targeting the most sensitive life stage of Lepidoptera therefore minimising the risk to beneficial species. A recommended advance of the data collected in this thesis would be to prepare a transcriptomic analysis of all life stages, from egg, all larval stages, pupa and adult. Further observations could be a comparison between male and female and tissue-specific expression patterns. For example, Yu et al (2015) found that a family of CYP450s in *P. xylostella* (CYP367s) were found specifically in adult heads, suggesting a specific role, potentially in odorant processing. This data would help identify the roles of each xeno-metabolic enzyme in the metabolism of xenobiotics.

In comparing differential sensitivity between life stages and expression levels, several genes of interest were identified. These were selected due to their >100-fold upregulation in larvae and association with neonicotinoid resistance in previous studies. The genes Mb_CYP6a17_2, Mb_CYP6a14_3, Mb_CYP6a2_2, Mb_CYP9f2_2 and GSTs Mb_GSTE3_6 and Mb_GSTE2_3 and ABC Mb_ABCG12 were identified using this criterion. To determine the impact of these genes in resistance to imidacloprid, an experiment could be designed to knock down genes using CRISPR (clustered regularly interspaced short palindromic repeats)

(Fusetto et al. 2017). This technique would allow a comparison of sensitivity between organisms with and without expression of these specific genes.

Genomic and transcriptomic data provided insight into the metabolic capacity of the model species chosen, *M. brassicae*. This data highlighted that like other lepidopterans, the gene families of CYP450s, GSTs and ABC transporters were largely diverse and had expanded from dipteran and hymenopteran species. However, at present, it is unknown how representative of other Lepidoptera our *M. brassicae* culture, or indeed wild-caught *M. brassicae* are. In this thesis, *D. melanogaster* was used as a comparative species but several clades were identified which appeared either Lepidoptera or Diptera specific. Therefore, it is recommended that as metabolomic techniques and studies become more accessible and molecular profiles of further species are annotated, this data is revisited and mapped to a species which shares a closer evolutionary link to *M. brassicae*. Perhaps this information could then be correlated to the feeding strategies of other lepidopterans i.e. generalist and specialist to uncover a link between xeno-metabolism and host plant range.

It is hoped that in future research, the multi-faceted approach used throughout this thesis of bioassays, TKTD modelling and metabolomic analyses can be used to assess the impacts of pesticide use on both beneficial and pest species. By understanding both the impacts and mechanisms of toxicity may in future allow pest management strategies to limit adverse effects on beneficial species by targeted application to more sensitive life stages. Further, understanding chemical interactions such as synergy allows them to be used either advantageously or avoided in plant protection policies by maximising plant yield while minimising pesticide application.

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