Long-term effects of insecticides on environmental health – how does exposure of a female egg-laying insect to an insecticide affect subsequent generations?

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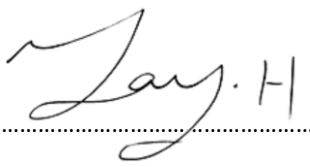
The author confirms that this research project contains no unacknowledged work or ideas from any publication or work by any other author.

Student signature 

CONTENTS

FIGURES

TABLES

Abstract

Since early crop domestication, there has been an arms race by humans vying for optimal crop yields and quality, while insect pests continued to cause damage through feeding. Insecticides are a prominent class of pesticides, developed for the purpose of killing and dissuading insects from feeding on target crops. Despite abundant research on the effects of insecticides on various aspects of the (target) insect life cycle, we know surprisingly little about any possible transgenerational effects, especially in beneficial non-targets. Such effects could be of concern in an environmental and conservation context, whereby non-target insects could pass exposure-effects on to subsequent generations. Therefore, in this study we have investigated the effects of exposure to sub-lethal doses of widely used pyrethroid and neonicotinoid insecticides in an egg-laying butterfly; not only to her directly, but also the offspring's development, survival, and host plant detection ability.

In *Bicyclus anynana* (a popular butterfly model system), we have shown that LC_{50} doses varied greatly depending on the pyrethroid (deltamethrin, cypermethrin) and neonicotinoid (thiacloprid, imidacloprid, thiamethoxam) they were topically exposed to, and that sublethal doses may take time to have an effect. Furthermore, males appeared to be more sensitive, with reduced lifespans, which could affect those species that display protandry.

We found that of all the insecticides tested, only deltamethrin significantly decreased female longevity. Additionally deltamethrin and thiacloprid also limited the number of eggs laid due to a shortened laying-period, however reproductive rate did not differ among all treatment groups. A transgenerational effect was observed, as offspring from thiamethoxam exposed females had a delayed development time, with offspring from deltamethrin and imidacloprid treated females having significantly reduced hatching success. All offspring from insecticide-treated females (except imidacloprid) were not effective at locating a hostplant. These results showed that whilst some effects could be observed after initial direct exposure to females (namely from deltamethrin), the most substantial sublethal effects were actually transgenerational rather than direct.

Overall, sublethal doses of insecticides mostly did not seem to affect the females themselves in terms of longevity and egg-production, yet their offspring did appear to display consequences to their long-term fitness. Given that Lepidoptera inhabit a broad range of habitats, a substantial decline in population from a heterogeneous ecosystem could theoretically result in habitat degradations, due to the reduction of ecosystem services.

1. Introduction

1.1 How does sublethal exposure of widely used insecticides affect insects and their offspring?

1.1.1 The advantage of insecticides

Since the beginning of crop domestication there has been an arms race, of humans vying for optimal crop yields and quality, with insect pests causing damage through feeding (Melo *et al.,* 2022; Parvin *et al.,* 2021). Without proper protection and control of insects, disease, weeds, and other pests, some crops could see up to a 100% loss, and up to a 45% loss of the global food supply (Parvin *et al*., 2021; Sarwar *et al*., 2013). This persistent arms race led man to seek out new practises of deterring insects where not desired, subsequently leading to the first pesticide (flea repellent made of natron) by c.1550 B.C. (Costa, 1987).

Insecticides are a subclass of pesticides, with the objective of controlling pest insect populations and killing insects, to preserve target crops from feeding damage (Botías *et al*., 2015; Long and Krupke., 2016; Olaya-Arenas *et al.,* 2020). Nearly 600,000 tons of insecticides are applied globally per year to meet climbing crop demands, which is expected to escalate with the projected increase in the global population to 9.8 billion, by 2050 (Kumar and Mozumdar., 2014; Müller, 2018; Parvin *et al*., 2021; Sarwar *et al*., 2013).

1.1.2 Widely used “low-risk” insecticides

The chemical dichlorodiphenyltrichloroethane (DDT), was one of the earliest and most widespread commercial insecticides used in agriculture in the 1940s, until it was associated with catastrophic ecological damage and harm to non-target species resulting in its widespread ban (Turusov *et al*., 2002). The public and agricultural consensus began to favour safer alternatives, which consequently led to the widespread implementation of ‘reduced-risk’ insecticides in agriculture (DiBartolomeis *et al*., 2019; Tudi *et al*., 2021; Turusov *et al*., 2002). This is essentially a substance that could deter and control pest insects whilst having a low direct toxicological effect on other animal classes including humans (Chrustek *et al*., 2018), and is therefore considered safe for use in agriculture (Yang *et al*., 2016). The most frequently used insecticide classes include organophosphates, carbamates, spinosyns, with pyrethroids and neonicotinoids being the most popular ‘reduced-risk’ classes, leading to their wide use in agriculture (DiBartolomeis *et al*., 2019; Tudi *et al*., 2021).

Pyrethroids are a class of synthetic insecticides designed and optimised from the structure of the naturally occurring compounds called pyrethrins, which are insecticidal substances produced in some plant species like *Chrysanthemum cinerariaefolium* (Bhardwaj *et al*., 2020; Elliott, 1977; Soderlund, 2010). It was first developed in the United States of America and reintroduced as an insecticide by the early 1970`s (Scott, 2017; Wirtz *et al*., 2009). Although different pyrethroids can vary in their exact mode of action, principally these insecticides interfere with insect nerve impulses, caused by the pyrethroid compounds binding to voltage-gated sodium channels (VGSC) (Table 1), inhibiting transmission of the channel from an activated (ion-conducting) state to an inactive (non-conducting) state, that are fundamentally required for movement and function of the insect (Field *et al*., 2017). Exposure in high enough quantities, results in cell membranes of electrically amenable cells to become constantly polarised, resulting in a lack of movement, paralysis, quickly followed by death (Field *et al*., 2017). Pyrethroids are recorded as being lethal to all insect orders and therefore the label “reduced-risk” is misleading, as beneficial insects may inadvertently be affected as well, resulting in ecosystem-wide effects, thereby indirectly harming other organisms that were considered safe from negative effects (Bhardwaj *et al*., 2020; Braak *et al*., 2018; Gibbons *et al*., 2015) (Table 1). As a contact insecticide, it primarily kills via topical exposure (theoretically in addition to ingestion), and is typically applied to crops in a variety of methods depending on the target crop and insect type (e.g. foliar spray for leaf feeding insects, or seed treatment for protection on fruits and leaves) (Dunbar *et al*., 2019).

Table 5: Mode of actions of some of the most commonly used pyrethroids against insects in agriculture (based on Rehman *et al.,* 2014; further references indicated).

Pyrethroid	Mode of action	Reference
Deltamethrin Fenvalerate	Type II pyrethroid: contains α-cyano phenoxybenzyl group, acting on the insect’s VGSC and possibly γ-amino butyric acid (GABA) mediated chloride channels. This causes irreversible damage to the insect nervous system, disarming areas of high nerve activity like the brain.	Taylor-Wells *et al*., 2015
Cypermethrin Cyhalothrin	Type II pyrethroid: targets the sodium channels of the insect nerve membrane. In doing so causes over-activity of the insect VGSC, resulting in loss of nervous system function and death.	Reegan *et al*., 2021
Permethrin Bifenthrin	Type I pyrethroid: causes hypersensitivity of the nervous system, with exposed nerves initiating over activity of impulses because movement of sodium ions is blocked, stopping the VGSC from becoming inactive. Resulting in paralysis and death.	Gammon *et al*., 2019

Neonicotinoids comprise a systemic class of synthetic insecticides, developed from the natural compound nicotine (Yang *et al*., 2016). Their physiochemical characteristics (octanol water partition coefficient and dissociation constant) allow the compound to be absorbed and translocated to plant tissues, making them toxic to insects regardless of their crop application method (e.g. seed treatment, spraying) (Bass and Field., 2018; Simon-Delso *et al*., 2015; Wu *et al*., 2019; Yang *et al*., 2016). Whilst the exact mode of action differs based on the particular neonicotinoid (Table 2), principally they act by binding to nicotinic acetylcholine receptors (nAChRs) in the insect nervous system, resulting in persistent nerve simulation in low doses but full receptor blockage, paralysis and death at high doses (Kundoo *et al.*, 2018; Taillebois *et al*., 2018). Neonicotinoids are therefore considered lethal to all insect orders. Neonicotinoids are a globally used class of insecticides, which has seen consistent use in agriculture since its development in the 1980`s and introduction in the 1990`s (Krupke *et al*., 2012). Neonicotinoids have a stronger affinity in binding to insect specific nAChRs rather than to comparable vertebrate receptors, consequently are lower in risk to vertebrates (Kundoo *et al*., 2018). Subsequently they have been considered 'reduced-risk insecticides', which, does not take into account indirect and sublethal ecosystem-wide effects (Braak *et al.,* 2018; Gibbons *et al*., 2015; Sánchez-Bayo, 2019; Yang *et al*., 2016).

Table 6: Mode of actions and their effects on insects upon exposure of some of the most commonly used neonicotinoids in agriculture.

Neonicotinoid	Mode of action	Reference
Imidacloprid Thiamethoxam	Postsynaptic antagonism makes the compound a partial agonist of nicotinic acetylcholine receptors (nAChRs), and so interferes with neurotransmission causing paralysis and death.	Taillebois *et al*., 2018; Talcott, 2013.
Clothianidin	Full agonist of insect nAChRs and internal regulation of cyclic adenosine monophosphate (cAMP), causing an irreversible depolarisation of insect interneuron synapses. This causes paralysis and death.	Calas-List *et al*., 2013
Thiacloprid	Antagonist of nAChRs in the post-synaptic membrane, inducing neuronal hyper-excitation resulting in paralysis and death.	Van der Sluijs *et al*., 2013
Nitenpyram	Inhibit postsynaptic nAChRs, which interferes with synaptic transmission in the central nervous system causing paralysis and death.	Mao *et al*., 2015

For nearly a century, these two classes have had mixed legalities (globally) coupled with alternating popularity in agriculture.

After the ban of DDT (organochlorine insecticide), pyrethroids were introduced as an alternative in the 1970`s, partly because of similar insecticidal properties (yet not as ecologically destructive) as sodium channels are the primary target of action of DDT and pyrethroid insecticides (Vijverberg *et al*., 1982). Although the use of DDT is largely banned, it is still one of the recommended insecticides for malaria control in Africa (Rehwagen, 2006). Pyrethroids are used extensively in the control of insect pests and vectors of numerous human diseases. The pyrethroids were then replaced by many neonicotinoids by the 1980-1990`s due to its popular effectiveness and increased perceived safety profile in agriculture (Selvam and Srinivasan, 2019; Turusov *et al*., 2002).

It soon became apparent that the advertised “low-risk” effects ignored indirect effects to the ecosystem, thereby affecting vertebrates, coupled with the direct effects on beneficial insects (Braak *et al*., 2018; Gibbons *et al*., 2015). Indeed, by the 2000’s, frequently used neonicotinoids (Table 2) were linked with a number of deleterious ecological effects to beneficial fauna (e.g. their associated link to colony collapse disorder in Hymenoptera, resistance in populations of insects) (Chensheng *et al*., 2014), resulting in the international ban of their use in agriculture in areas like the European Union (EU) in 2013 (Epstein *et al*., 2022; Jactel *et al*., 2019; Warren., 2021). After this ban, the most immediately applicable alternative was to reinstitute the use of pyrethroids in agriculture, because of its availability and high selectivity to insects over vertebrates like mammals (more so than classes like organophosphates or carbamates) (Jactel *et al*., 2019; Selvam and Srinivasan, 2019). Implementing newer neonicotinoids was not feasible at the time, because they still employed the same modes of action, which could result in the persistence of resistant insect populations, posing similar risks to the environment in quick succession (Jactel *et al*., 2019). However, since leaving the European Union in 2020 (and so had flexibility to the EU agricultural laws), the British government legalised the emergency use of neonicotinoids to protect sugar beet (*Beta vulgaris*) against pests such as aphids that were resistant to pyrethroids (Warren, 2021). Many countries across Africa, Asia, and the Americas still use neonicotinoids in agriculture due to different agricultural regulations and legislations (Bass *et al*., 2015; Nimako *et al,* 2021). To summarise, their use differs depending on variability in legal status around the world.

Both insecticide classes have similar agricultural functions, often being used interchangeably (Douglas and Tooker, 2016; Dunbar *et al*., 2019; Yang *et al*., 2016). Therefore, a

comprehensive comparison of their effects on both target and non-target insects can reveal the extent of their impact and highlight undocumented effects.

1.2 The effects of insecticides on Insects

1.2.1 Routes of insecticide exposure

Over 95% of all pesticides are produced for agriculture (Baghel *et al.,* 2022). Insects within or adjacent to agricultural sites can become exposed to insecticides by numerous vectors, depending on insecticide type, crop application method, and the feeding/pollinating behaviours of the insect (Botías *et al*., 2012; Braak *et al*., 2018; Krishnan *et al*., 2021; Müller, 2018).

Direct exposure

Direct exposure refers to topical, or ingested contact with an insecticide after it has been applied to a specific surface or crop type, and any present residue can become potentially absorbed into insect spiracles, cuticles, or membranes (Botías *et al*., 2015; Long and Krupke, 2016; Olaya-Arenas *et al.,* 2020; Paramasivam and Selvi, 2017). Insecticides are most often applied to crops in the form of crop dusting, spraying, fogging, with smaller areas sprayed with the insecticide via means of aerosol spray, handheld spray, fogging/misting (Cilek *et* al., 2008; Manica *et al*., 2017). Foliar spray applications are the most popular application method in agriculture because the can cover large areas in a short timeframe (Braak *et* al., 2018; Dewer *et al*., 2016; Oberemok *et al*., 2015). Spray treatment of crops can also expose adjacent habitats from the target site (e.g. hedgerows and meadows), by traveling downwind (Braak *et al*., 2018; Botías *et al*., 2015; Krishnan *et al*., 2021a; Krishnan., 2021b; Long and Krupke, 2016; Olaya-Arenas *et al.,* 2020). Systematic insecticides (i.e. translocated through plant tissues) are often applied to crops in the form of root spraying, seed treatment, or spraying, therefore insects feeding on treated plant matter absorb insecticides (e.g. through the digestive system) (Cloyd and Bethke, 2011).

Indirect exposure

Systematic insecticides are absorbed and translocated through plant tissues (Braak *et al*., 2018), and thus have been recorded to be present in pollen via nectar and guttation droplets, therefore pollinators may be exposed to these compounds when feeding on flowers of treated plants (Bass and Field, 2018; Braak *et al*., 2018). This has frequently been observed to be destructive in colony-living insects (i.e. Hymenoptera), like honey bees (*Apis mellifera*), where accumulation of neonicotinoid residues like imidacloprid can build up in

hives (Michlig *et al*., 2023). Braak *et al* (2018) and references therein, found after application to crops, water-soluble insecticides, such as neonicotinoids, can become washed into adjacent surfaces exposing nearby habitats and water systems, and thus exposing aquatic insects (Krishnan *et al*., 2021b). It has been estimated that 40% of all land agricultural sites could be at risk of washing insecticides into nearby water systems (Ippolito *et al*., 2015; Krishnan *et al*., 2021b).

Conditions could arise where insecticides are transovarially transmitted from the mother and passed into her eggs during oogenesis (i.e. maternal transmission of insecticides). Meaning the subsequent generation (F_1) may still become affected even if they have not been directly exposed themselves (Braak *et al.*, 2018; Neuparth *et al*., 2020; Paula *et al*., 2014; Shaw *et al*., 2017). This is because offspring phenotypes can be significantly affected by the mother's external environment (abiotic and biotic), as well as her own phenotype (i.e. maternal effect) (Carter *et al*., 2013; Telfer, 2009; Ziegler and Van Antwerpen, 2006).

During oogenesis in Lepidoptera, proteins and mRNAs (derived from maternal effect genes) are transferred into eggs in order to carry out vital roles in development (Carter *et al*., 2013). Theoretically insecticides present at the site of oogenesis could also become transferred in similar mechanisms. The females may also regulate egg production and early embryogenesis of their offspring differently, as the presence of an insecticide during oocyte patterning could theoretically alter maternal gene expression or incorporation of resources (i.e. yolk deposited in the egg), consequentially affecting the development of the offspring (Lim and Lee, 1982; Perveen and Miyata, 2000; Santorum *et al*., 2021).

1.2.2 Effects of pyrethroids on insects

In total, data for nearly three-quarters of insect species had shown that direct exposure to pyrethroids can affect fitness-related traits and survival (Table 3; see appendix table A1). Of the studies that recorded direct effects of pyrethroid exposure, 11 were conducted on adults, 2 on pupae, 21 on larvae, and 3 on eggs, suggesting that larvae were the primary focus of many lethal and sublethal pyrethroid investigations (Table 3).

Lepidoptera have been widely studied (comprising of 13 species examined), with a majority of the selected studies being moths (Table 3). Only one study was conducted on pupae and eggs (Krishnan *et al*., 2021a), two were on adults (Hahn *et al*., 2015; Krishnan *et al*., 2021a), with the remaining seventeen studies being on larvae (Table 3; see appendix table 1). Approximately 70% of all agricultural pests are from the order Lepidoptera (Braak *et al*., 2018), and although some butterfly species are considered pests (e.g. *Pieris spp*), the

majority are moths. Among these pest species, larval stages are considered the most destructive to crops, which therefore is the focus of many studies (Braak *et al*., 2018; Guan *et al*., 2018; Ryan *et al*., 2019).

The Coleoptera order has been well-studied for the effects of pyrethroids. Not many studies have examined the effects of pyrethroids on the pupal stage (Fogel *et al*., 2016), with most focusing on the larval (five) and adult (six) stage (Table 3; see appendix table 1). Numerous species of Coleoptera have the potential to damage crops, at both the larval stage mainly causing root damage, while adults harm mainly to stems, flowers, and foliar surfaces (Moorhouse *et al*., 1992). Subsequently this is why these two development stages were the focus of agricultural research.

Direct effects of exposure to deltamethrin and permethrin have been observed in four Dipteran species, with most species being examined during the adult stage, and one species examined during the larval stage (Table 3). The species were used as a model systems for studying resistance following prolonged use (Martins *et al*., 2012).

The orders Hymenoptera and Neuroptera (Table 3) were not typically associated as agricultural pests and therefore are not usually targets of insecticide use (Havard *et al*., 2019; Mulligan *et al*., 2010). Rather, the context for many studies of these orders was on how non-target species are affected after exposure (Dai *et al*., 2010; Siegfried, 1993; Mulligan *et al*., 2010). As for Neuroptera, they are a beneficial natural enemy of many agricultural pest insects (such as aphids and Lpidopteran larvae), and so are at the focus of conservation efforts, as they can become exposed in agricultural sites (Mulligan *et al*., 2010; Su *et al*., 2022).

Table 7: A summary of insect species (not all pest), development stages and effects observed after exposure to pyrethroids. Age of exposure can be categorized as: A = adult, P = pupa, L = larva, E = egg. This table is a summary of main findings based on observations and evidence combined in appendix table A1.

Order	Species	Age of exposure	Pyrethroid	Exposure	Effect
Coleoptera	*Sitophilus zeamais, Eriopis connexa, Coccinella septempunctata, Leptinotarsa decemlineata, Phaedon cochleariae*	A, P, L	Cyhalothrin Deltamethrin Cypermethrin	Direct: (topical, chronic, ingested) Indirect: (inherited, transovarial)	Direct: Reduced longevity, Impaired movement behaviour, impaired sexual behaviour, improved survival, increased mortality, increased development time Indirect: reduced offspring survival, impaired development
Diptera	*Aedes aegypti, Culex quinquefasciatus, Anopheles albimanus, Diamesa zernyi*	A, L	Deltamethrin, Permethrin	Direct: (topical, chronic, ingested)	Direct: Reduced longevity, reduced reproductive output, insecticide resistance, impaired movement behaviour
Hymenoptera	*Apis mellifera ligustica, Telenomus busseolae*	A	Bifenthrin, Cyfluthrin, Deltamethrin	Direct: (topical, chronic, ingested)	Direct: Impaired movement behaviour, reduced longevity, reduced reproductive output
Lepidoptera	*Spodoptera littoralis, Spodoptera frugiperda, Spodoptera eridania, Spodoptera exigua, Danaus plexippus, Helicoverpa zea, Helicoverpa armigera, Agrotis ipsilon, Hadena bicruris, Chloridea virescens, Cydia pomonella, Plutella*	E, l, P, A	Deltamethrin, Cyfluthrin, Cypermethrin, Permethrin, Etofenprox, Bifenthrin	Direct: (topical, ingested) Indirect: (inherited, transovarial)	Direct: Increased mortality, delayed development time, no pupal eclosion, reduced growth, reduced reproductive output, increased resistance, impaired movement behaviour Indirect: Reduced offspring survival, reduced offspring fertility

	xylostella, *Bicyclus anynana*				
Neuroptera	*Chrysoperla carnea*	A	Cypermethrin	Direct: (topical)	Direct: Reduced reproductive output
Ixodida	*Dermacentor reticulatus*	E, L	Deltamethrin, Cypermethrin	Direct: (topical)	Direct: Reduced survival, Increased development time

1.2.2.1 Direct effect: Reproductive output

Representative studies from our literature survey found that reproductive output effects can be seen as a consequence of pyrethroid exposure (Table 3). Pyrethroids are known to have ovicidal activities against insects, consequently reducing numbers of (viable) eggs laid, because changes in the insect nervous system can affect normal function of the endocrine system, interfering with production of ecdysone and juvenile hormone (Dai *et al*., 2010; Friesen and Kaufman, 2003; Hahn *et al*., 2015; Maund *et al*., 2012).

Reproductive output was seldom the only effect observed after exposure, often being accompanied by reduced longevity and hatching success of eggs laid (D'Ávila *et al*., 2018; Martins *et al*., 2012) (See appendix table 1). Interestingly, *Cimex lectularius* (Hemiptera) had a reduction of 34%-73% in egg hatching success and some clutch sizes after Tempid exposure (pyrethroid-neonicotinoid combination insecticide), although had no impact to later development (Crawley *et al*., 2017).

1.2.2.2 Direct effect: Survival and longevity

The mode of actions pyrethroids have on insect nervous systems (Table 1; Table 3) could result in disruptions to normal physiological processes (e.g. feeding, mating, and predator avoidance), and consequently the prolonged physiological stress caused by these chemicals could reduce long-term fitness and survival (Dai et al., 2021; D'Ávila *et al*., 2018; Martins *et al*., 2012). However, in some instances it was found that effects to longevity were sometimes negligible in comparisons to controls, such as in the ladybird beetle *Eriopis connexa* and actually in one species, the Colorado beetle *L. decemlineata*, exposure to pyrethroids improved survival (Fogel *et al*, 2016; Margus *et al*., 2019).

1.2.2.3 Direct effect: Development time

Development time has frequently been recorded to be affected after pyrethroid exposure (Table 3; also see appendix table 1). This was often a consequence of pyrethroids affecting hormonal regulation within insects, so processes like ecdysis, egg, larval, and pupal development can become delayed (Kruger *et al*., 2021; Massot *et al*., 2021; Orchard, 1980). Skouras *et al* (2017), suggested that these effects on development time may also be due to resources being directed away from development and towards costly detoxifying processes and immune system responses. If disruption to development is too severe it can cause greater mortality in insects or the halting of development (shortly followed by death), as was seen in the ladybird beetle, *C. septempunctata* (Afza *et al*., 2023; Buczek *et al*., 2013; Skouras *et al*., 2017).

1.2.2.4 Direct effect: Behaviour

Various effects behaviour have been documented for many insect orders. For example, in the Diptera order, the mosquitoes *A. albimanus*, and *A. aegypti,* had reduced flight speed, spent more time in flight, and turned less frequently when exposed to deltamethrin, and permethrin (Table 3; see appendix table 1) (Cohnstaedt and Allan, 2011). These effects were observed to be a consequence of interference with sodium-ion channels, resulting in altered movement (Cohnstaedt and Allan, 2011).

The Hymenoptera bee species, *A. mellifera ligustica,* had impairment to overall navigation, learning, fecundity, and basic feeding behaviour, following exposure to bifenthrin and deltamethrin (Table 3; see appendix table A1) (Dai *et al*., 2010). It was inferred to be because pyrethroids affected early life history traits (as eggs- pupa), and so changed their physiology as adults (Dai *et al*., 2010).

The Lepidopteran species *H. armigera* (cotton bollworm)*,* had altered reproductive behaviour, in that females avoided or would not oviposit on surfaces exposed to cypermethrin (Achaleke and Brévault, 2010). Insecticide repellence such as this, is more related to sensorial perception rather than neurotoxic responses, which is what resulted in this behavioural modification (Cordeiro *et al*., 2010).

Studies examining the effects of pyrethroids on behavioural traits in Coleoptera documented changes to behaviour, where species like the ladybird beetle *C. septempunctata* had additional and prolonged grooming, indicating irritation following contact to deltamethrin exposed sites (Table 3; see appendix table 1) (Wiles and Jepson, 1994). This hyperactive grooming behaviour was likely a result of reflex actions to chemoreceptors detecting

deltamethrin (Wiles and Jepson, 1994). Whereas the ladybird beetle *E. connexa* had impaired mating behaviour after cyhalothrin exposure and longer oviposition periods following exposure to cypermethrin (D'Ávila *et al*., 2018; Fogel *et al*., 2016). This was inferred to be an effect of a pyrethroids acting on the insect's nervous system, impairing coordination and mating ability (D'Ávila *et al*., 2018).

Overall insect behaviour was clearly affected after pyrethroid exposure, but could greatly vary depending on species and the degree of the behaviour altered (Table 3; see appendix table A1).

1.2.2.5 Direct effect: Resistance

Resistance to insecticides was widely found among insect orders, of those who are frequently exposed to sublethal doses of pyrethroids (Liu, 2012) (Table 3; see appendix table 1). Martins *et al* (2012), suggested this was attributed to modifications and over expression of detoxifying enzymes and pyrethroid receptors in the insect nervous system, reducing sensitivity.

1.2.2.6 Indirect effects: Hatching success

Egg hatching success in Coleoptera and Lepidoptera was reduced when parents were exposed to pyrethroids like lambda-cyhalothrin, cypermethrin, and permethrin (Afza *et al*., 2023; Jallow and Hoy, 2005; Müller *et al*., 2019). Indirect effects to Lepidoptera hatching success were suggested to be from maternal exposure (i.e. maternal effect) to insecticides, and *P. xylostella* exposed to permethrin produced offspring that were even more susceptible (Jallow and Hoy, 2005). Interestingly, the Coleopteran species *L. decemlineata* and *P. cochleariae* had increased hatching success compared to controls, whilst a full mechanisms was not described, it was hypothesised that higher larval body mass may increase long-term survival (Margus *et al*., 2019; Wolz *et al*., 2022).

1.2.2.7 Indirect effects: Offspring development

The only observable indirect effects to offspring development were seen in *P. cochleariae* (Coleoptera), where adults produced offspring with fluctuating levels of antennae asymmetry when exposed to sublethal doses of a pyrethroid. It was speculated this was likely due to a possible reduced maternal transfer of resources into the offspring, induced by lambda-cyhalothrin (Müller *et al*., 2017).

1.2.3 Effects of neonicotinoids on insects

Table 4 contains summarised examples from studies that examined the direct and indirect effects of neonicotinoids on insect fitness-related traits and survival, which showcases the diverse effects commonly used neonicotinoids had on sensitivity and response to insecticides depending on the insect age of exposure and species.

Studies on Lepidoptera were most often conducted on larvae, usually because of their strong ecological presence as agricultural pests, or as non-target species inhabiting agricultural landscapes (Braak *et al*., 2018). Of the representative number of species, a majority were moth (Table 4), with only two studies conducted on eggs, one on pupae (Krishnan *et al*., 2021a; Saour, 2008), seven were on adults, with the remaining fifteen being on larvae (Table 4; also see appendix Table 2).

Of all of our representative Coleoptera species investigated (four species), they focussed on the adult life-stage (see appendix table 2). Many beetle species are highly destructive to crops as adults, and so are the primary target stage of some neonicotinoids, making them of interest for research (Moorhouse *et al*., 1992).

The only Dipteran species observed in this literature survey were *Drosophila spp*, used as a model system for behavioural effects incurred by neonicotinoid exposure, rather than an agricultural pest (Tasman *et al.,* 2021). Alteration of behaviour were found to be a result of modifications to neuropeptides and nAChRs subunits (Tables 2; Table 4) (Tasman *et al.,* 2021).

The ecological significance of adult *Osmia bicornis* and *A. mellifera* in Hymenoptera studies led researchers to focus exclusively on this developmental stage, as it was the only one that demonstrated complex behavioural or fertility effects (Sandrock et al., 2014; Wood et al., 2019; Wu-Smart and Spivak, 2016).

Three Hemiptera species were studied; two non-target shield-bug species *Podisus maculiventris* and *Euschistus heros*, selected for their potential unintentional exposure in agriculture (Santos et al., 2016; Rix and Cutler, 2020; Wang *et al*, 2017). Whereas the polyphagous worldwide pest aphid *Aphis gossypii* was examined for any transgenerational effects and long-term risks to crops (Wang *et al*, 2017).

Table 8: A summary of insect species studied (not all pest), developmental stages and effects observed after exposure to neonicotinoids. Age of exposure can be categorised as: A = adult, P = pupa, L = larva, E = egg. This table is a summary of main findings based on observations and evidence combined in Appendix Table A2.

Order	Species	Age of exposure	Neonicotinoid	Exposure	Effect
Coleoptera	*Coleomegilla maculate, Harmonia axyridis, Hippodamia convergens, Coccinella septempunctata*	A	Imidacloprid, Thiamethoxam	Direct: (Topical, Chronic, Ingested) Indirect: (inherited, transovarial)	Direct: Reduced survival, reduced longevity, reduced fecundity Indirect: Reduced hatching success, increased development time, reduced fecundity
Diptera	*Drosophila spp.*	A	Imidacloprid, Clothianidin, Thiamethoxam, Thiacloprid	Direct: (Topical)	Direct: altered behaviour
Hymenoptera	*Osmia bicornis, Apis mellifera, Scaptotrigona aff Depilis*	A	Clothianidin, Thiamethoxam, Imidacloprid	Direct: (Chronic, ingested) Indirect: (inherited, transovarial)	Direct: Reduced reproductive output, decreases overwinter survival, reduced survival, delayed pupa Indirect: altered sex ratio
Hemiptera	*Podisus maculiventris, Euschistus heros, Aphis gossypii*	L, A	Imidacloprid, Nitenpyram	Direct: (Topical, Ingestion) Indirect: (inherited, transovarial)	Direct: Increased fecundity, reduced fecundity, reduced longevity, impaired foraging, impaired behaviour Indirect: Reduced fertility, reduced offspring survival
Lepidoptera	*Vanessa cardui, Vanessa atalanta, Danaus plexippus, Phthorimaea operculella, Helicoverpa*	E, L, P, A	Imidacloprid, Clothianidin, Thiamethoxam, Thiacloprid	Direct: (Ingested, topical) Indirect	Direct: Reduced survival, arrested ecdysis, reduced reproductive output, increased development time, decreased development time, resistance, reduced growth, additional appendages, reduced

	armigera, Helicoverpa zea, Polyommatus Icarus, Galleria mellonella, Grapholita molesta, Pieris rapae, Agrotis ipsilon, Cydia pomonella, Lobesia botrana, Chrysodeixis includes, Bicyclus anynana				sizes, impaired navigation, altered behaviour, altered pheromone production Indirect: reduced fecundity, reduced size
Neuroptera	*Chrysoperla externa*	A	Thiamethoxam	Direct: (Ingested) Indirect: (inherited)	Direct: Reduced fertility Indirect: Reduced fertility, reduced offspring survival

1.2.3.1 Direct effect: Reproductive output

Reproductive output has been observed to be affected after direct exposure, for instance, from our literature survey we found negative effects seen in Coleoptera (from imidacloprid), Hymenoptera (from clothianidin, thiamethoxam, and imidacloprid), Lepidoptera (from imidacloprid), and Neuroptera (from thiamethoxam) (Table 4). Insects exposed as adults were seen to most frequently elicit effects to reproductive output (Table 4), likely due to the ovicidal activity many neonicotinoids are recorded to have on insects (Hoffmann *et al*., 2008). Though no mechanism was given, it could be speculated to be from the neonicotinoids becoming manifested into the egg from mother via transovarial transmission (Braak *et al*., 2018; Ahn *et al*., 2012).

Different neonicotinoids had varied effects on Hemiptera, as shown by the reduced fecundity and longevity of *A. gossypii* following exposure to sublethal doses of nitenpyram, and the increased fecundity of *P. maculiventris* after exposure to sublethal doses of imidacloprid (Rix and Cutler, 2020; Wang *et al*., 2017). These differences in response are likely due to the varying modes of action of the neonicotinoids, and differences in species resistance to the insecticides (Brevik *et al*., 2018; Krishnan *et al*., 2021b) (Table 2).

1.2.3.2 Direct effect: Survival and longevity

Development time could frequently be seen to affect insects, of this Lepidoptera and Hymenoptera were some of the best represented (Table 4 and references therein; also see appendix table 2). Commonly used neonicotinoids have been associated with harmful effects on insect movement and development due to their modes of action (Table 2), which have been shown to disrupt normal physiological processes, causing long-term fitness and survival consequences in exposed insects (Braak *et al*., 2018; Rix and Cutler, 2020; Krishnan *et al*.,2020a; Main *et al*., 2018).

1.2.3.3 Direct effect: Development time

Development time could frequently be seen to affect insects in our literature survey, of this Lepidoptera and Hymenoptera were some of the best represented (Table 4; also see appendix table 2). Lepidoptera had delays to overall ecdysis, affecting larval and pupal development times after exposure to imidacloprid, clothianidin, thiamethoxam. For Hymenoptera, clothianidin increased development time in the egg and larval stages, while thiamethoxam and thiacloprid decreased development time (Grünewald and Siefert, 2019; Rosa *et al*., 2016; Siefert *et al*., 2020). It was speculated that the insecticide presence during development led to the increased time (Grünewald and Siefert, 2019). Ahmad *et al* (2013), suggested that neonicotinoids such as imidacloprid had been linked to reduced larval

and pupal weight, which in turn delays development through physiological stress due to reduced resources being available for development.

1.2.3.4 Direct effect: Behaviour

Of the cited literature in Table 4, none included behaviour for Coleoptera, Hemiptera, or Neuroptera, but such studies do exists that have investigated cognitive and behavioural effects in these orders (Miao *et al*., 2014; Desneux *et al*., 2007). Diptera displayed several alterations to behaviour after exposure to imidacloprid, clothianidin, thiamethoxam, thiacloprid, where disruptions could be observed to sleeping behaviour, circadian rhythms, and impairment to their memory (Tasman *et al.,* 2021; see appendix table A2). This was caused by neonicotinoids disrupting neuron function and knockdown of nAChRs (Dα1 / Dβ2 subunits), resulting in poor memory.

Movement and egg laying ability was observed to be impaired in some Hymenoptera when exposed to imidacloprid (see appendix table 2). This was suggested to be a direct effect of the insecticide acting on crucial sensory and motor functions of the central nervous system, impairing movement ability and incurring physiological stress (Wu-Smart and Spivak, 2016). Neonicotinoids have been shown to have short term effects on colony function in some Hymenoptera, where the queen had reduced movement ability, and workers born to an exposed queen had reduced egg laying ability, impaired foraging and hygienic activities (Sandrock *et al*., 2014; Wu-Smart and Spivak, 2016). This resulted in adverse colony development effects, such as lower brood production and pollen stores needed for food (Wu-Smart and Spivak, 2016).

Research on Lepidoptera showed indirect behavioural effects, where exposure to thiacloprid and clothianidin was associated with disrupted navigation, pheromone-guided behaviour, circadian rhythm, chemical communication, calling and mating behaviour, across multiple moth and butterfly species (see appendix table 2). This was caused by production of compounds like codlemone (vital to pheromone production) being impaired by neonicotinoids, and thus interfering with forms of intra-species communication (Navarro-Roldán and Gemeno, 2017; Rabhi *et al*., 2014). These studies provide evidence that neonicotinoids can cause electrophysiological antennal responses, thus interfering with navigation because chemical communication is fundamental to odour source location (Navarro-Roldán *et al*., 2019). Thiamethoxam has also been observed to have a deterrent effect to *C. includes* larvae after exposure, showing that larvae preferred consuming untreated matter (Lee and Davis, 2023).

1.2.3.5 Direct effect: Resistance

It was found that direct exposure of Lepidoptera to low doses of imidacloprid resulted in decreased sensitivity after initial exposure (Table 4). It has been hypothesised that nAChR variability and/or mutations can result in decreased sensitivity after low direct exposure to insects (Achaleke and Brévault, 2010; Clements *et al*., 2016; Rabelo *et al*., 2020). Variation of sensitivity between species was a result of possible toxicokinetic or toxicodynamic factors, however these exact factors were not investigated (Krishnan *et al*., 2021b).

1.2.3.6 Indirect effect: Hatching success

Reduced hatching success can occur when insects are exposed to neonicotinoids (Dai *et al*., 2021; Rix and Cutlet, 2020), such as in Coleoptera (imidacloprid and thiamethoxam-induced), Hemiptera (imidacloprid), and Neuroptera (thiamethoxam) (Table 4). This was likely because insecticide toxicity impaired embryonic development resulting in poor hatching success, for example as transovarial transmission of the insecticide may have affected development or yolk deposition in eggs, and possibly altering maternal gene expression too (Ahn *et al*., 2012; Braak *et al*., 2018; Ziegler and Van Antwerpen, 2006).

1.2.3.7 Indirect effect: Offspring development

After direct parental exposure, increased offspring development time was observed in Coleoptera (from imidacloprid, thiamethoxam), even though the offspring themselves had not been exposed to these neonicotinoids (Table 4). The exact mechanisms for this transgenerational effect have not always been fully explained in the literature (Dai *et al*., 2021; Deans and Hutching, 2022). Yet it was inferred that reduced feeding activity of parents (induced by physiological stress of imidacloprid) reduced the parental resources available to use for their offspring (Sâmia *et al*., 2019; Xiao *et al*., 2016). Reduced feeding efficiency was in all likelihood caused by low doses of neonicotinoids reducing nutrient absorption and hormonal imbalance, resulting in knock-on effects on offspring development (Xiao *et al*., 2016).

1.2.3.8 Indirect effect: Offspring fecundity

The reduced parental feeding efficiency argument has also been made in those instances where offspring displayed reduced fertility. For example, in the beetle, *C. septempunctata,* parents exposed to imidacloprid had reduced fertility as well as for offspring, potentially arising from reduced allocation of nutrients to offspring (Xiao *et al*., 2016). Hymenoptera like *A. gossypii*, had reduced fecundity and longevity after nitenpyram exposure, but their offspring's fecundity was speculated to increase compared to control groups (Wang *et al*., 2017) – but the authors stressed that further work would be required to confirm this result.

1.2.4 Indirect effects of pyrethroids and neonicotinoids on insect behaviour

A considerable body of work has examined how parental exposure (particularly maternal exposure) to 'stressful' environmental conditions during reproduction can generate transgenerational effects, that affect offspring development, growth, and behaviour (Deans and Hutching, 2022; Gibbs *et al*., 2005; Jallow and Hoy, 2005; Kumar *et al*., 2022; Bacca *et al*., 2021; Bhardwaj *et al*., 2020). Interestingly, lambda-cyhalothrin (pyrethroid) exposure in leaf beetles (*P. cochleariae*) resulted in offspring with antennae asymmetry, potentially affecting chemical communication ability in these offspring (Müller *et al*., 2017; Müller, 2018). These effects were observed at doses nearly 60 times lower than field-realistic doses, and therefore these effects have a strong potential to be seen *in-situ* (Müller *et al*., 2017).

It has been hypothesised that pyrethroids and neonicotinoids have the potential to generate transgenerational effects on offspring behaviour, even though the offspring have not been directly exposed themselves. These transgenerational effects may result from indirect effects on the offspring's nervous system (consequently affecting offspring movement), or through changes in maternal gene expression that results in physiological changes during offspring development (Lim and Lee, 1982; Perveen and Miyata, 2000; Santorum *et al*., 2021).

However transgenerational behavioural effects are not frequently tested for in experiments or when assessing ecological impacts of insecticides (specifically pyrethroids and neonicotinoids) (Brevik *et al*., 2018; Hashimoto *et al*., 2020; Shaw *et al*., 2017), and so the extent of effects to offspring behaviour are still unknown in many insect orders like Lepidoptera (Braak *et al*., 2018).

1.3 Summary on sublethal exposure of widely used insecticides affect insects and their offspring?

The majority of research investigating transgenerational effects has been biased towards agriculturally important pest species (e.g. from the orders Lepidoptera, Coleoptera, Hemiptera), beneficial species crucial for pollination services (e.g. Hymenoptera). Dipteran species were mainly studied due to their association with disease transmission vectors, and species from the *Drosphila* genus were used as an *ex-situ* model, to highlight possible modes of action and effects insecticides could have on other insect species (Cohnstaedt and Allan, 2011; Martins *et al*., 2012; Tasman *et al*., 2021). Some species orders were more typically studied because of their beneficial ecological impacts, whether it be from pollinating services frequently seen in Hymenoptera and some Lepidoptera, or how they predate on agricultural pest species, such as in Neuroptera (Duso *et al*., 2008; Mulligan *et al*., 2010; Sâmia *et al*., 2019). A review by Braak *et al* (2018) has stated that there was still a deficit of information surrounding the effects non-target butterflies may receive after sublethal and indirect exposure to widely used insecticides like neonicotinoids.

Examination of direct and indirect effects of commonly used pyrethroids and neonicotinoids were found to be mostly deleterious to the insect's physiology, life history traits and behaviour (see sections 1.2). Sublethal doses of these classes of insecticides also require further attention and investigation, as collated literature has suggested that their non-lethal exposure affects not just the initial generation, but that the overview presented thus far has made clear that such sublethal effects manifest themselves on the developmental level (see sections 1.2). Based on frequently observed modes of actions of our selected insecticides and surrounding research on transgenerational (and maternal) effects, it could be inferred that insecticides may have become incorporated into the eggs during fertilisation and thus manifesting its presence to the next generation (Braak *et al.*, 2018; Neuparth *et al*., 2020; Paula *et al*., 2014; Shaw *et al*., 2017). Alternatively, maternal gene expression may have differentiated in the presence of the insecticide and thus patterns during oogenesis, and thus offspring development (Lim and Lee, 1982; Perveen and Miyata, 2000; Santorum *et al*., 2021).

In some instances, not all incurred direct effects were negative, as sublethal and repeated exposure lessened the susceptibility insects had to many pyrethroids and neonicotinoids, resulting in resistance in a population, which therefore be beneficial to the insect long-term survival and fitness (Achaleke and Brévault, 2010; Braak *et al*., 2018; Krishnan *et al*., 2021b; Rabelo *et al*., 2020; Wang *et al*., 2017).

1.4 An LC_{50} assay on a selection of neonicotinoids and pyrethroids using the model butterfly, *Bicyclus anynana*

Insecticide exposure to insects can often be sublethal, and thus not have the desired effect (Braak *et al*., 2018). This has been well documented in the field of environmental toxicology, as due to a low exposure dose, insecticide resistance, or low susceptibility to an insecticide, the target insect is not always effectively controlled (Braak *et al*., 2018; Krishnan *et al*., 2021a; Krishnan *et al*., 2021b). When measuring the toxicity of a given insecticide (e.g. pyrethroid or neonicotinoid), it is useful to determine a bassline of lethality a substance has to a model system, this is most often represented by determining the level of dose concentration needed to kill 50% of a population (LC_{50}) over a predetermined measurement of time (Krishnan *et al*., 2021a). The use of an LC_{50} helps to determine levels of lethality, in where anything higher than this value would likely have a strong lethality to the population, than any values below the threshold. This is especially useful when evaluating the toxicity of insecticides, in that it helps to standardise what insecticide concentrations should be used when targeting a specific insect species, as well as comparing relative toxicities of insecticides to each other, species and their development stages, allowing for a more thorough understanding of the insecticide's effectiveness (Braak *et al*., 2018; Krishnan *et al*., 2021a; Krishnan *et al*., 2021b).

Due to the prevalence of Lepidoptera among agricultural sites, indiscriminate insecticide use can result in the exposure of both targeted and non-targeted species (Braak *et al*., 2018; Guan *et al*., 2018; Ryan *et al*., 2019; Warren *et al*., 2001). Likewise, many Lepidoptera species adjacent to agricultural sites can also become exposed to sublethal doses of pyrethroids and neonicotinoids, with various dose concentrations depending on proximity (Krishnan *et al*., 2021a). Krishnan *et al* (2021a) found that species such as monarch butterflies (*D. plexippus*) could still theoretically endure an LC_{50} dose (1.3×10^{-3} - 19.9 ng/µl insect) of pyrethroids and neonicotinoids at up to 60 m downwind from the crop application site.

There are multiple ways in which an LC_{50} can be calculated. The most frequently being via exposure (topical, chronic, or ingested) to a range of insecticide concentrations and recording the mortality over multiple time points (e.g. 24- 96hrs). If mortality is high enough then the 50% lethal concentration can be calculated (Krishnan *et al*., 2021a). Alternatively, an LC_{50} can be measured using a survival curve of the total population, rather than measuring mortality at specific times. By basing the calculation taken to kill 50% of the population on a survival curve, it allows for the addition of a more comprehensive

relationship between the substance and organism (Van Herk *et al*., 2008). By analysing the shape of the curves, it allows for insight into the overall trends in survival a population has, rather than just *snapshots* of the mortality, particularly when observing low dose concentrations.

When measuring LC_{50}, various factors are to be considered in order to maintain reliability of its calculation (Amweg *et al*., 2005; Krishnan *et al*., 2021a). This is because age and stage of development of the insect being exposed is crucial to the insecticide's effectiveness, as insects mature, so can sensitivity to many insecticides change (Castro-Janer *et al.,* 2009; Krischik *et al*., 2015; Krishnan *et al*., 2021a; Krishnan *et al*., 2021b). Evidence has suggested that neonicotinoid sensitivity to insects can often decrease with age because as they develop so do the various receptors in the insect nervous system as well as increased expression of detoxifying enzymes, which may reduce sensitivity to these chemicals (Braak *et al*., 2018; Martins *et al*., 2012). Some effects of insecticides can affect sexes differently. This is mostly because mass can be sexually dimorphic in many insect species, where smaller individuals (usually the males in Lepidoptera) will endure a higher dosage of insecticide per gram of bodyweight than the larger individuals exposed to the same concentration (Singer, 1982). The type of application may affect the susceptibility an insect has, because some insecticides are effective when ingested by the insect (e.g. systemic insecticides like neonicotinoids) and are often used to kill feeding larvae and phloem-feeding insects (e.g. aphids) (Bass and Field, 2018; Simon-Delso *et al*., 2015). Whereas other insecticides are contact insecticides like pyrethroids, and thus are designed to be topically absorbed from treated surfaces or crops, often exposing insects within a sprayed/misted area (Bhardwaj *et al*., 2020; Dunbar et al., 2019; also see chapter 1.1).

Topical application of insecticides diluted using a readily adhering chemical (i.e. acetone) to insects offers the benefit of precise measurement of dosage of insecticide given to the insect. By also knowing a standardised weight of male and female insects of a treatment population, it allows for the dose concentration (ng/g male or female) to be known. This is particularly important when measuring dosage per sex, as many Lepidoptera species exhibit sexual dimorphism with males sometimes being smaller (Singer, 1982). Therefore, despite being exposed to the same application dose, males and females may effectively suffer different concentrations due to differences in body mass.

1.5 Transgenerational experiment: How does exposure to commonly used insecticides to *Bicyclus anynana* affect the subsequent generation

It has been found that that the exposure to widely used pyrethroids and neonicotinoids had a direct impact on insect physiology, reproduction, life history traits, and behaviour in the majority of insects studied (reviewed in Chapter 1.1 – 1.3). The complete scope of observable transgenerational effects and their underlying mechanisms in pyrethroids and neonicotinoids is not yet fully understood due to limited investigation in this area (Braak *et al*., 2018; Brevik *et al*., 2018; Hashimoto *et al*., 2020; Shaw *et al*., 2017). Whilst effects to behaviour were inferred to possibly be affected in offspring of Lepidoptera exposed to insecticides, no formal study had taken place.

Theoretically, if a female butterfly was exposed to sublethal doses of a widely used pyrethroid or neonicotinoid, it may elicit effects to not just herself but her offspring's survival development, fitness, and behaviour too. In order to test the degree in which these insecticides affected butterflies, we would need to observe the direct effects that were often already observed in literature such as changes in survival, reproductive output, and development time, followed by hatching success, development time, and movement behaviour of their offspring.

1.6 Aims and objectives

The examination of the direct and indirect effects commonly used pyrethroids and neonicotinoids had on insects, suggested that they had mostly harmful effects on insects and their offspring, with the exception of short-term benefits like resistance and cases of improved fecundity. Conversely the full extent of the transgenerational effects and the mechanisms causing inherited effects are not yet fully understood, yet it is likely that they originate from the mother (i.e. maternal effect). Most notably there is also a clear absence of recorded transgenerational behaviour effects in non-target butterflies, because of the lack of investigation. The majority of the chosen orders of insects were known agricultural pests, yet there was a notable dichotomy in the Lepidoptera order, as of their distinct difference between species that acted as pests (Moths) and those that acted as pollinators (primarily butterflies). With the pollinators being unintentionally exposed because they inhabit within or adjacent habitat site.

To have the most relevance to what could occur *in situ* and corroborate if effects are visible to what was found in literature, we used the frequently used pyrethroids (deltamethrin and cypermethrin) and neonicotinoids (thiacloprid, imidacloprid, and thiamethoxam) as they were observed to elicit prominent effects to Lepidoptera (Chapter 1.1 – 1.3). Imidacloprid and thiamethoxam (neonicotinoids) were permitted once again for controlled agricultural purposes in the United Kingdom (UK) starting from 2020, after their leaving from the European Union (and thus their agricultural legislations) (Warren, 2021). It would therefore be interesting to observe the effects of these recently reintroduced insecticides too.

Based on these findings, we aimed to;

(1) Identify the topical toxicities of a selection pyrethroids and neonicotinoids on adult *B. anynana* and establish the 50% median lethal dose (LD_{50}) of each insecticide for females (Sections; 1.4, 2.2, 3.1, and 4.1).

(2) Identify direct effects of sub-lethal concentrations of insecticides on female reproductive output in the butterfly *B. anynana* (Sections; 1.5, 2.3, 3.2, 4.2).

(3) Identify indirect effects of maternal exposure of sub-lethal concentrations of insecticides on offspring development, movement behaviour, and their ability to successfully find host plants (Sections; 1.5, 2.3, 3.2, 4.2).

2. Methods

2.1 Animals and experimental design

The Squinting Bush-Brown *B. anynana* (Butler, 1879) (Lepidoptera: Nymphalidae) is an East-African species of butterfly not identified as an agricultural pest, yet could hypothetically come into contact with pyrethroids and neonicotinoids, like other butterflies do (Bauerfeind and Fischer, 2005; Brakefield *et al*., 2009; Mulé *et al*., 2017). The key strength of this species for the present study, is the fact that it is a major Lepidopteran model system for ecological, developmental, and evolutionary (genetic) research (Aduse-Poku *et al*., 2022; Bauerfeind and Fischer, 2005; Brakefield *et al*., 2009; Brakefield *et al*., 1998; Brakefield *et al*., 1991; Connahs *et al*., 2022; De Jong *et al*., 2010; Nowell *et al*., 2017; Tian and Monteiro, 2022), including how environmental stressors like air pollution can affect non-target Lepidoptera (Tan *et al*., 2018). Given the extensive knowledge we have on this species and relative ease of rearing consecutive generations all-year round (*ex situ* in lab conditions) it made *B. anynana* an ideal butterfly model species to investigate life history traits after exposure to insecticides (see section 1.4 for aims).

All *B. anynana* butterflies sourced for the present study (i.e. both LC_{50} and transgenerational experiments) were from a continuous outbred stock population at Oxford Brookes University, established in 2017 from a large, outbred stock kept in Cambridge (founders supplied by O. Brattstrom). Experimental animals were reared in netted cages in incubators (Sanyo (now called PHC) Versatile Environmental Test Chamber MLR-352H). Although we did not check the genetic diversity as such for the laboratory stock population, we are confident this was maintained at outbred levels across generations. Therefore, the animals picked at random in each generation to be tested upon, can be considered genetically diverse.

Incubator conditions were set at a constant 70(±5)% relative humidity (RH), with conditions simulating sun-rise (1/4 light; temp 21.5^{o}C (±1), 0530 - 0600), morning (3/4 light; temp 22.5^{-} ^{o}C (±1), 0600 - 0900), mid-day (4/4 light; temp 24.0^{o}C (±1), 0900 - 1700), evening (2/4 light; temp 22^{o}C (±1), 1700 - 2130), and night (0/4 light; temp 22.1^{o}C (±1), 2200 - 0530). Development time from egg to adult took approximately five weeks under these rearing conditions. Maize plants grown from non-pesticide treated seeds were used as egg-laying plants, as well as the plants on which the caterpillars fed (*Zea mays,* Swift F1; supplied by Kings Seeds, plant grown in Levington Advance Seed & Modular F2S Compost with Sand, LEVF2S75). Adults were fed *ad libitum* on mashed, decomposing, bananas kept moist.

In order to keep track of individual butterflies in larger butterfly cages in the LC_{50} experiments, each butterfly was assigned a unique code upon eclosion, which was written on the hindwing using a fine-point Staedtler permanent lumocolor pen (black). These codes were 2-3 digit labels indicating the day (by letter) and the individual (by number) (example in Figure 1; also see Table 5). Within 1 day of mating (mating was observed by researcher), females and males were randomly distributed over single-sex treatment cages (15 of each sex per dose treatment group, with 5 adults per small cylindrical cage (31x26x26cm). Male and females in the transgenerational experiment did not receive any labelling.

The LC_{50} experiments were done on both males and females, but the transgenerational experiments focused on females and how they and their offspring are affected by sublethal insecticide doses. Although incubator conditions were the same as in the LC_{50} experiments, these females were separated from males within 1 day of mating and kept separate.

In order to verify that experimental LC_{50} animals were truly randomly distributed over treatment groups, and thus that no size bias exists, an average weight for each sex was calculated from weighting 15 adults from each sex upon death (collected < 24hr after death) on an ADAM Equipment PW214 Analytical Balance. Adult (dry) weights were then standardised to 0.0182g ($\pm 7.3\times10^{-3}$) for males and 0.0573g ($\pm 1.47\times10^{-2}$) for females (see appendix table A3).

Insecticides were obtained from Sigma-Aldrich International GmbH from the PESTANAL® product line of deltamethrin (45423-250MG, 98.0% purity), cypermethrin (36128-100MG, 90.0% purity), thiacloprid (37905-100MG-R, 98.0% purity), imidacloprid (37894-100MG, 98.0% purity), and thiamethoxam (37924-100MG-R, 98.0% purity). Initial stock concentrations of the insecticides were 5000 ng/µl (3000 ng/µl for deltamethrin) and diluted down with acetone to desired concentrations to ensure that the insecticides fully dissolved, and because many insecticides readily dissolve into the solvent (Krishnan *et al*., 2021a), which evaporates quickly upon contact (Olaya-Arenas *et al*., 2020). Therefore, butterflies were not left with any residue that could be transferred to others.

For all insecticide LC_{50} and transgenerational experiments, immediately after mating, treatment solutions were topically applied (volume of 3 µl) to the ventral side of the abdomen by means of pipetting. The solution was readily taken up by the skin at this part of the body, which has the ovaries and fat body surrounded by haemolymph. This is a likely place of the body for contact with spray residues on leaves, because their abdomens may come into contact with the leaf-surface during egg laying (Bear *et al*., 2010). A young maize plant was placed in each mated-female cage, for egg laying, along with banana slices embedded in a moistened paper towel for food and fluid uptake. Adults were monitored every 24 hours, with

any deaths recorded in the corresponding dose groups to create mortality curves, which were then used to establish the LC_{50} values for each insecticide dose group (Table 5).

Whilst the insecticide treatment groups and acetone controls were handled for treatment, the no-treatment controls were also treated in the same manner to ensure that all adults endured the same amount of handling (and thus stress) so as to not affect transgenerational variables like offspring performance (Gibbs *et al.*, 2018).

For each insecticide, the LC_{50} curve was determined in a single experiment. Meaning, the LC_{50} determination was not repeated in a subsequent experiment, and therefore not replicated at that level. Likewise, testing whether a transgenerational effect occurs for a particular insecticide was conducted once for each insecticide. Therefore LC_{50} and transgenerational experiments were always conducted within a single generation, not across subsequent generations and therefore that level of replication is not present here. Please note, that no LC_{50} curve for acetone was conducted.

2.2 Analysis of mortality data to construct a mortality curve

Published LC_{50} values for other insects displayed variability in doses, depending on species, development age, and application methods (see appendix table A3). LC_{50} values of a selection of pyrethroids (deltamethrin and cypermethrin) and neonicotinoids (thiacloprid, imidacloprid, and thiamethoxam) were surveyed from literature (appendix table A3) to determine a broad spectrum of insecticide doses to use on *B. anynana* (Table 6). Doses started with negligible concentrations, and then increased (by log_{10}) to concentrations that cause complete mortality ($LC_{95\text{-}100}$) (Table 6).

For each of the selected pyrethroids (deltamethrin, cypermethrin) and neonicotinoids (thiacloprid, imidacloprid, thiamethoxam), survival upon topical application was recorded in 24-hour intervals (i.e. daily) until all adults were deceased. From this data, sex-specific mortality curves were constructed for each insecticide using the Prism – GraphPad (version 5.01) software (Motulsky and Christopoulos, 2004). As it proved not possible to calculate an LC_{50} over a predetermined time interval (e.g. cypermethrin treated *H. armigera* larvae were only inspected at 48hr, by Shinde and Kamtikar, 2011), because no LC_{50} had been conducted on an adult *B. anynana* before, which meant it was not known what were the appropritate time intercepts to calculate the LC_{50}. Therefore we constructed mortality curves for each sex and insecticide, in which we could then select an LC_{50} from an appropriate day a reliable dose could be calculated (also conducted on Prism – GraphPad) (as seen in Bacci *et al.*, 2009).

Table 5: Example data format of information registered to each adult ID.

Adult ID	Adult number	Sex	Day eclosed	Treatment group (ng/µl)	Day treated	Day died	Longevity (Days) after Exposure	Total longevity (Days)
A1	1	F	02/05/22	Deltamethrin 0	03/05/22	22/05/22	19	20
A2	2	M	02/05/22	Deltamethrin 300	03/05/22	14/05/22	11	12
A3	3	F	02/05/22	Deltamethrin 0.03	03/05/22	22/05/22	19	20

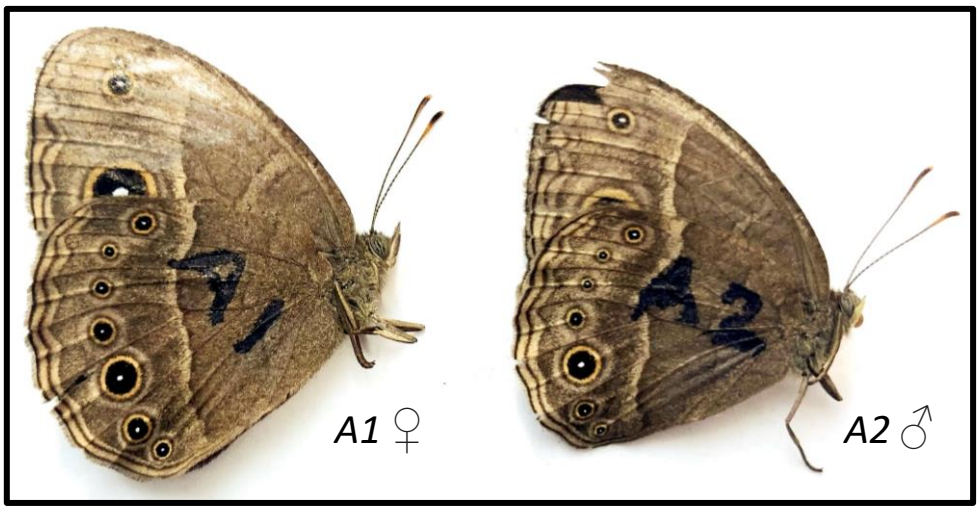


Figure 1: Example *Bicyclus anynana* butterflies. Left = Female labelled 'A1', Right = Male labelled 'A2'.

Table 6: Range of concentrations of insecticides applied to determine a suitable LC_{50} in *B. anynana*. Concentrations per male and females were calculated using an average weight of 0.0182 g for males and 0.0573 g for females, with 3 µl application volumes. Dose ranges were determined from published studies shown in appendix table A3.

Class	Insecticide	Application dose (ng/µl)	Concentration (ng/g males)	Concentration (ng/g females)
Pyrethroid	Deltamethrin	0	0	0
		0.03	4.9	1.6
		0.3	49.5	15.7
		3	494.5	157.1
		30	4945.1	1570.7
		300	49450.5	15706.8
	Cypermethrin	0	0	0
		0.5	82.4	26.2
		5	824.2	261.8
		50	8241.8	2617.8
		500	82417.6	26178.0
		5000	824175.8	261780.1
Neonicotinoid	Thiacloprid	0	0	0
		0.5	82.4	26.2
		5	824.2	261.8
		50	8241.8	2617.8
		500	82417.6	26178.0
	Imidacloprid	0	0	0
		5	824.2	261.8
		50	8241.8	2617.8
		500	82417.6	26178.0
		5000	824175.8	261780.1
	Thiamethoxam	0	0	0
		5	824.2	261.8
		50	8241.8	2617.8
		500	82417.6	26178.0
		5000	824175.8	261780.1

2.3 Transgenerational experiment: Measuring direct and indirect effects of sublethal exposure of widely used pyrethroids and neonicotinoids on female *Bicyclus anynana* and their offspring

2.3.1 Insecticide application

In the transgenerational effect experiments, a virgin female was allowed to mate 24 hrs after eclosion. Mated females were randomly assigned to one of three treatment groups; (1) Insecticide (dissolved in acetone), (2) carrier control (acetone only), (3) no-treatment control (no chemical added). Each treatment group consisted of 15 females (3 cages with 5 per treatment cage, 45 females in total for experiment). The insecticides used were pyrethroids (deltamethrin and cypermethrin) and neonicotinoids (thiacloprid, imidacloprid, and thiamethoxam) (Table 7).

Table 7: Applied sublethal doses of selected pyrethroids and neonicotinoids. Dose concentrations were based upon literature and confirmed by our own LC_{50} experiments (see appendix tables A3 – A8).

	Insecticide	Applied dose (ng/µl)	Concentration (ng/g female)
Pyrethroids	Deltamethrin	3	157.1
	Cypermethrin	5	261.8
Neonicotinoids	Thiacloprid	5	261.8
	Imidacloprid	50	2617.8
	Thiamethoxam	400	20942.4

Every 24 hours, the survival of individual females and quantity of eggs laid per cage was monitored. Eggs were collected for the first 5 days after initial laying to record peak fecundity, after which egg numbers decline as oviposition progresses (Gibbs and Van Dyck, 2010). In order to gauge (daily) egg size variation, a random subset (max of 30 eggs) per cage was imaged with a light dissection-microscope using the software LAS EZ version 3.4.0 (Leica MZ6 with Leica IC80 HD fitted camera). Egg size was determined using Image-J version 1.53k (Gibbs and Van Dyck, 2010), using a custom macro written by Breuker *et al* (2018). These egg counts provided a measure, together with egg size, of daily fecundity per cage. The total number of eggs laid over 5 days was used to calculate the average number of eggs laid per female, per day, per cage, giving a detailed record of egg laying patterns of females per treatment group. These eggs were then placed into Eppendorf tubes (max of 10 eggs per tube to avoid oxygen depletion and eggs being crushed by other eggs on top) covered with muslin wrap at opening, to allow for oxygen circulation whilst impeding escape once larvae hatched.

2.3.2 Movement behaviour assay

Pyrethroids and neonicotinoids have been shown to affect the insect nervous system (see section 1.1.2). This experiment was designed to test the hypothesis that female exposure to insecticides can generate transgenerational effects, impacting the nervous system in their offspring with effects on larval movement behaviour. We used movement behaviour as an indicator trait for an insecticide effect in offspring, using an experimental design modified from Cain *et al* (1985) and Gibbs *et al* (2004) (Figure 2). Sixteen offspring from females of each treatment group were randomly selected and reared to second instar (13 days after hatching: larval stage). Upon reaching the desired instar, these larvae were then placed into individual containers with moistened paper towel (to avoid dehydration), and placed back into incubators, where they were starved for 18 hr to make them more willing to forage and thus move to find host plants to eat (see section 2.1 for incubator conditions). Each larva was placed in the centre of each arena (Figure 2; arenas were made of 1 mm^2 graph paper), equidistant from each plant (i.e. food source). Distance walked and time taken to reach the base of the plant was measured in 5 min increments, and time was capped at 60 min. By recording both distance and time, we could determine how efficient the movement behaviour was for each larva.

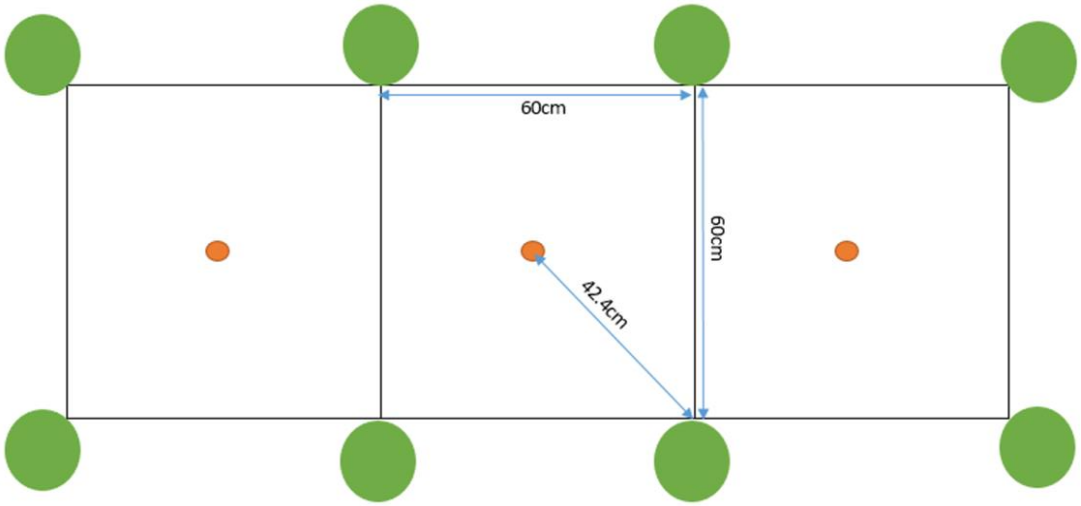


Figure 2: Movement behaviour arena layout used in insecticide experiments. Green dots represent the position of *Z. mays* food plants, whilst orange marks represent larval starting points. Three larvae were tested per 60-minute behavioural assay. Inspiration for the behavioural arena came from Cain *et al* (1985) and Gibbs *et al* (2004).

2.3.4 Female measurement

Butterfly forewing size was used as a proxy for overall body size of the experimental females (Figure 3), as used on butterfly species for Whitehorn *et al* (2018), and in Gage (1994). This was important to quantify as a covariate for measurements of reproductive output, as larger

females are known to lay larger numbers of eggs, and could theoretically respond differently to the insecticide dosage, live longer, and/or have a higher daily reproductive output (Gibbs *et al*., 2005).

The size of the left and right forewings were measured per female (Figure 3). The measuring of the distal side of the female's forewings can be liable to measurement errors, especially in non-pristine wings (Perini *et al*., 2005; Ulijaszek and Kerr, 1999; Van Hook *et al*., 2012). This was then repeated, and averages of the two wing size measurements were made, discarding any unreliable measurements. By repeating the measurements, variability between measurements 1 and 2 could be made, for within an individual (i.e. intra-variation) (standard error of mean), compared to the variability between individuals (inter-variation) (coefficient of determination) (see appendix table A10). If measurements were of the same order, the measurement error was too high, and re-measured.

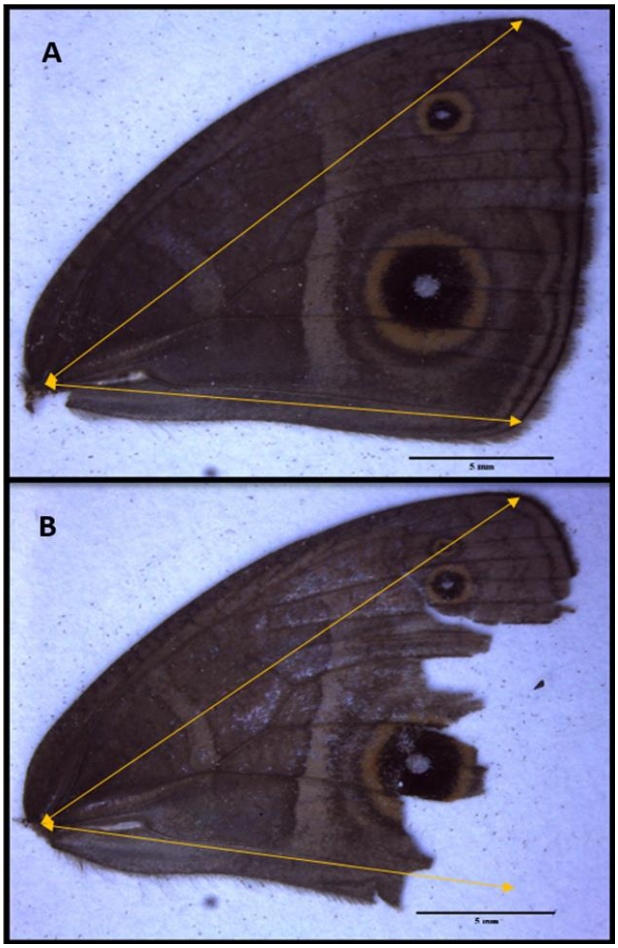


Figure 3: *Bicyclus anynana* right forewing comparison. (A) Wing measurements taken from the base of the humeral vein to the posterior wing-apex (not including wing-fringe), and humeral vein to the posterior wing-apex. (B) In cases where wings have sustained damage, measurements were improvised to achieve an approximate measurement.

2.3.4 Data analysis

Reproductive output and hatching success

Each of the insecticides were tested separately in consecutive generations. All statistical analyses were conducted in R, version 2021.09.0 (R Core Team, 2020). The effects of insecticide treatment on number eggs laid per cage (N = 3), and then repeated with egg size, using female size as a covariate, we analysed by means of a Multivariate Analysis of Covariance (MANCOVA). Such an analysis takes into account that reproductive output on consecutive days is dependent on each other (i.e. resources spent on day 1 cannot be spent on day 2 etc), and that any variability in this multivariate trait can be explained by the treatment as well as female size. Models with an insignificant female size effect were re-run without the covariate (i.e. Multivariate Analysis of variance) (Gibbs *et al.,* 2010; *Van* Dyck *et al*., 1997; Whitehorn *et al*., 2018).

Analysis of Variance (ANOVA) was used to determine variance across means per cage (N = 5 females per cage), among the three treatment groups for; female wing size (mm), female longevity (days), total egg count, egg size (mm^2), development time of eggs (days), as well as hatching success of eggs collected per cage. In cases where variation of cages per treatment groups significantly differed, a Post-Hoc Tukey test was conducted to determine the severity of variation between groups.

Behavioural assay

ANCOVAs were constructed to investigate how each of the two larval movement traits, distance travelled and time taken to reach a host plant, differed across the 3 maternal pesticide treatment groups; (1) Insecticide (dissolved in acetone), (2) carrier control (acetone only), (3) no-treatment control (no chemical added) (i.e. maternal pesticide treatment was a fixed effect). Larval size is known to affect larval movement (Gibbs *et al.,* 2004), therefore in these models larval size was used as a covariate. In cases where variation across maternal pesticide treatment groups significantly differed, a Post-Hoc Tukey test was conducted to determine the severity of variation between groups.

In order to determine the ability for larvae from each treatment group to reach the base of a host plant within the allotted time (60 min), a chi-squared test was used to determine if there was a significant relationship between treatment groups, and the likelihood of their success. If the larvae did not move, did not reach the host plant in the allotted time, or left the arena, they were recorded as being unsuccessful at finding a host plant.

3 Results

3.1 Determining LC_{50} values of neonicotinoids and pyrethroids on the model butterfly, *Bicyclus anynana*

In order to determine what the 50% mean lethal concentration (LC_{50}) of each of our selected insecticides were (aim 1 in section 1.6), mortality curves were constructed (see methodology section 2.2). From each of these mortality curves (Bacci *et al*., 2009), an LC_{50} was calculated from the first day from when a reliable value could be determined.

3.1.1 Pyrethroid: Deltamethrin

Survival curves of both male and females suggested that doses of 30 ng/µl and above, had a greater rate of death among individuals than those applied with doses of 0.03 ng/µl and below (Figure 4). Based on the survival of adults (Figure 4), the LC_{50} values for females was 3.20 ng/µl (50.90 ng/g female) at 4 days, and an LC_{50} of 2.68 ng/µl (441.59 ng/g male) could be observed at 1 day for males after exposure (Table 8; Figure 5). Values for males could be seen to range at 1 – 4 days after exposure (Table 8; Figure 5).

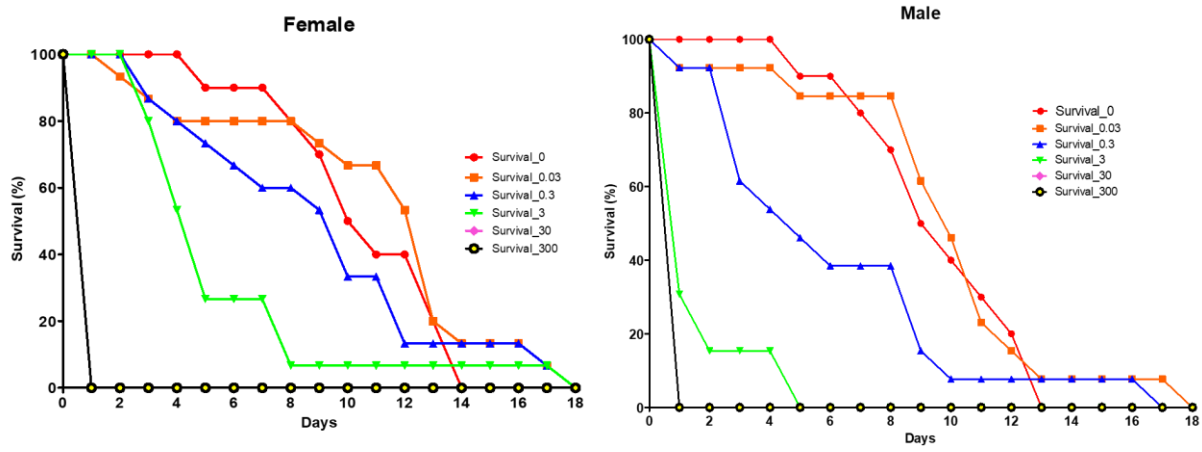


Figure 4: Survival curves for male and female *B. anynana* exposed to varying doses of deltamethrin. All doses are measured in ng/µl. Mortality data points for adults within the 30 ng/µl group overlapped completely with the 300 ng/µl group, as results were identical.

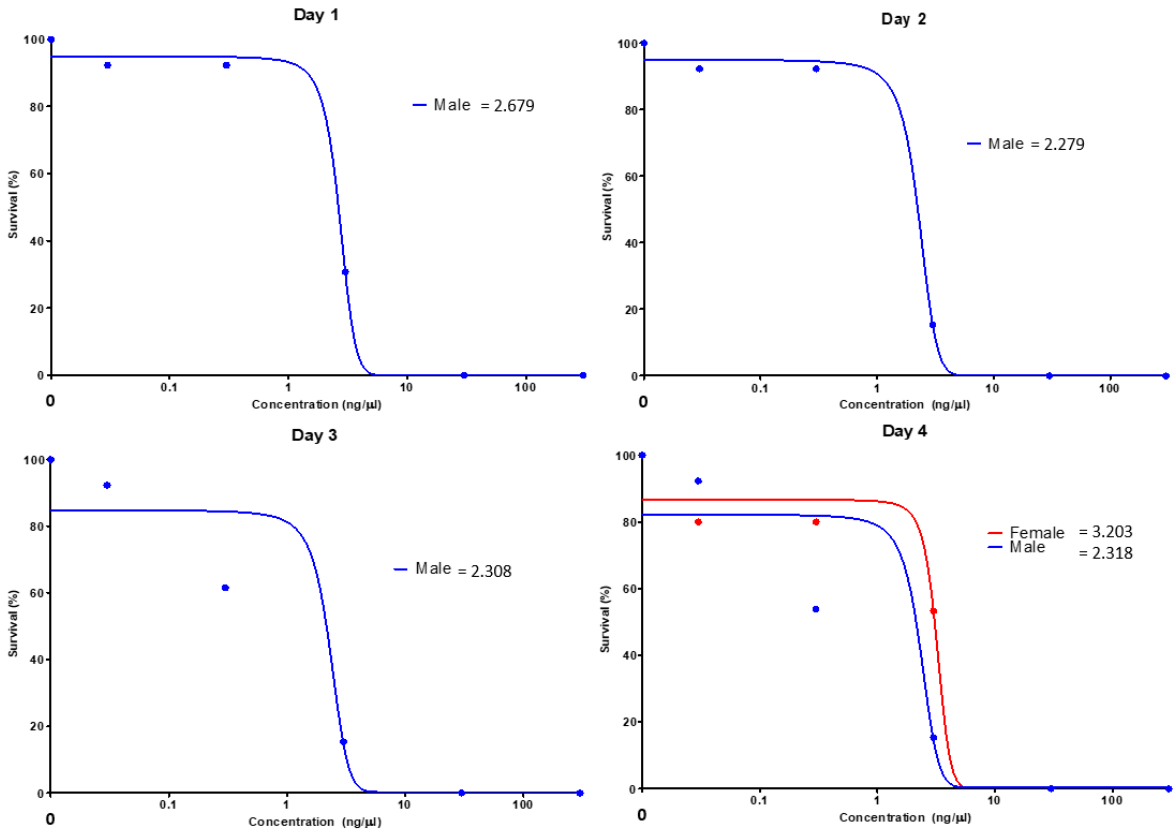


Figure 5: Deltamethrin LC_{50} curves for male *B. anynana* at 1, 2, 3 and 4 days and female *B. anynana* at 4 days. LC_{50} values are shown in ng/µl. Full results can be found on appendix table A4.

3.1.2 Pyrethroid: Cypermethrin

A response curve for both sexes indiciated that doses that were higher than 500 ng/µl had severe lethality in both sexes with all adults dying within 24 hours, whereas doses that were below 0.5 ng/µl had very low lethality relative to the rest of the dosage groups (Figure 6). Based on the survival of adults (Figure 6), reliable LC_{50} values were selected at 8 days after exposure for males, at 4.02 ng/µl (663.13 ng/g male) and females at 4.35 ng/µl (227.59 ng/g female) (Figure 7; Table 8). LC_{50} ranges for both males and females could be observed at 8 – 10 days after exposure (Figure 7).

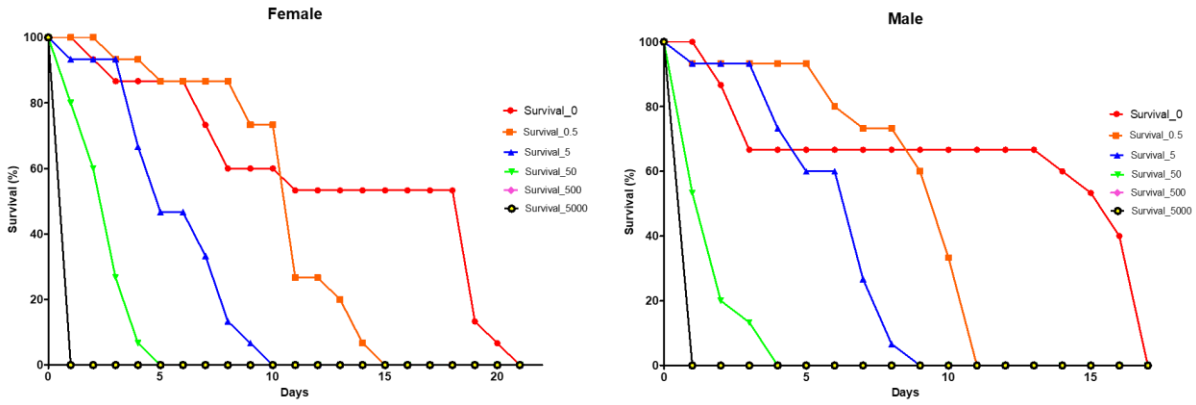


Figure 6**:** Survival curves for male and female *B. anynana* exposed to varying doses of cypermethrin. All doses are measured in ng/µl. Mortality data points for adults within the 500 ng/µl group were overlapped completely by the 5000 ng/µl group, as results were identically plotted.

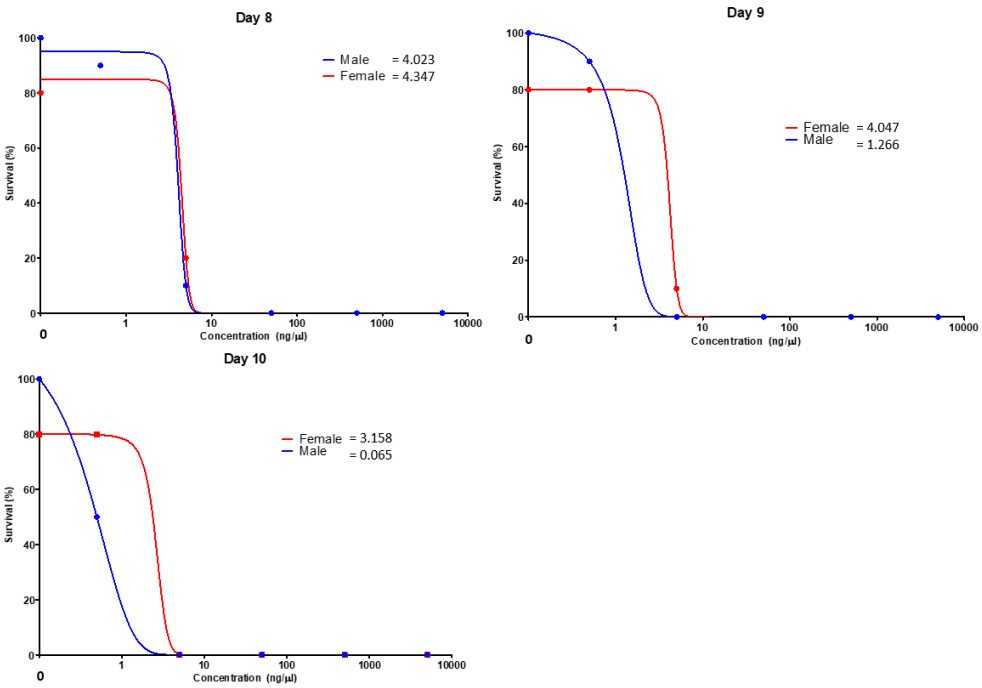


Figure 7: LC_{50} curves for male and female *B. anynana* 8, 9, and 10 days after exposure to cypermethrin. LC_{50} values are shown are shown in ng/µl. Full results can be found on appendix table A5.

3.1.3 Neonicotinoid: Thiacloprid

Doses that were higher than 50 ng/µl had severe lethality in both sexes, with all adults dying from that treatment group within 1 day after exposure, whereas doses that were below 0.5 ng/µl had very low lethality relative to the rest of the dosage groups (Figure 8). The control males and females (0 ng/µl) between 1 – 4 days have a faster mortality rate than some low doses of thiacloprid (i.e. 0.5 – 5 ng/µl) as a result of some individuals dying early in the test period, with the lines coming together around 20 days (Figure 8). Based on the survival of adults (Figure 8), LC_{50} values for males were selected at 8 days after exposure at 4.90 ng/µl (808.19 ng/g male) and at 9 days for females at 5.13 ng/µl (268.32 ng/g female) (Table 8; Figure 9). LC_{50} ranges for males and females could be observed at 8 – 10 days, and females 9 – 10 days after exposure (Figure 7).

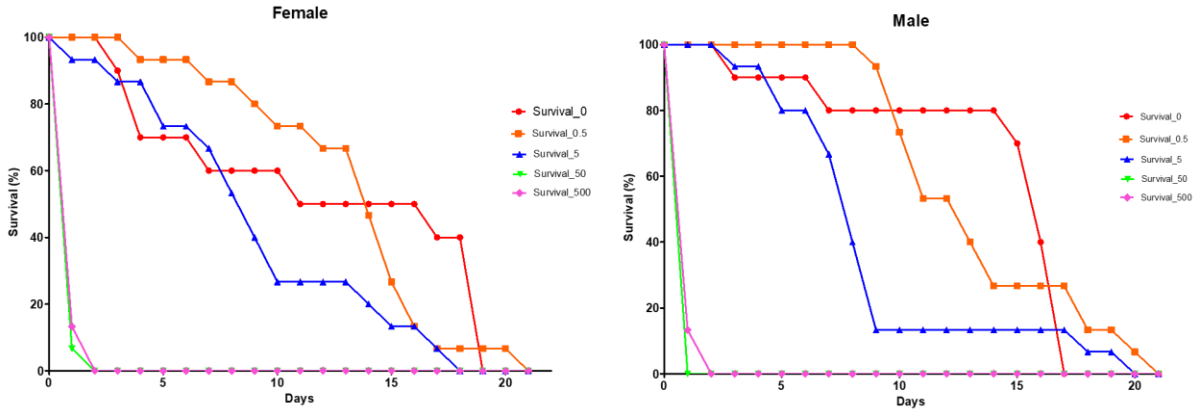


Figure 8: Survival curves for male and female *B. anynana* exposed to varying doses of thiacloprid.

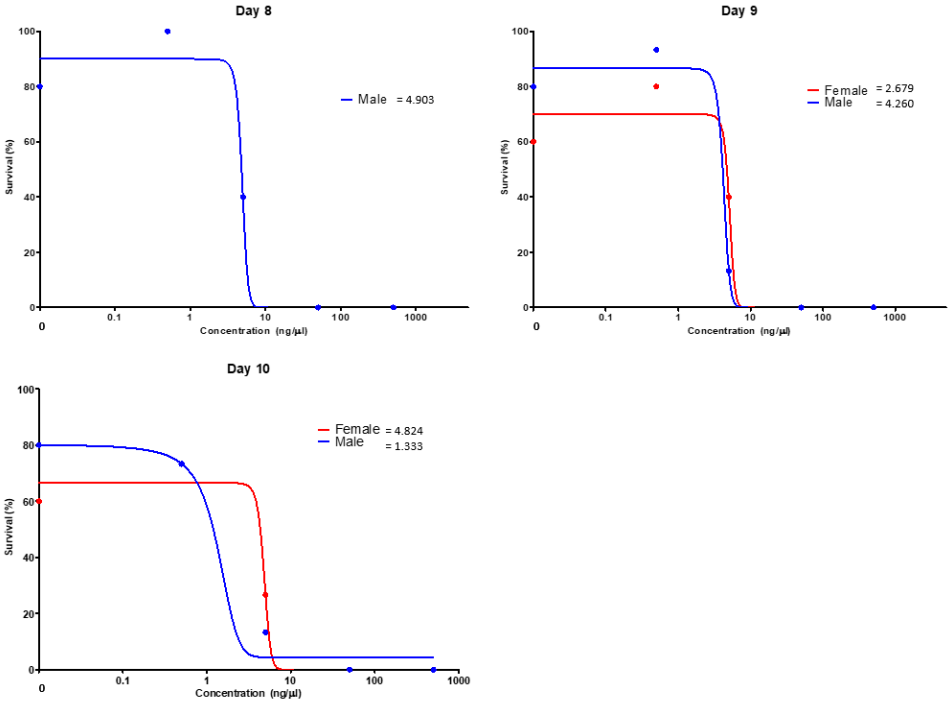


Figure 9: Thiacloprid LC_{50} curves for male *B. anynana* at 8, 9, and 10 days after exposure, and females at 9 and 10 days. LC_{50} values are shown in ng/µl. Full results can be found on appendix table A6.

3.1.4 Neonicotinoid: Imidacloprid

Doses that were higher than 5000 ng/µl had severe lethality, with all adults dying within the first 24 hours after exposure, whereas doses that were below 5 ng/µl had low lethality relative to the rest of the dosage groups (Figure 10). Based on adult survival (Figure 10), a reliable male LC_{50} value was determined 2 days after exposure at 339.30 ng/µl (55928.57 ng/g male) and females were first reliable at 4 days after exposure at 49.97 ng/µl (2616.23 ng/g in females (Table 8; Figure 11).

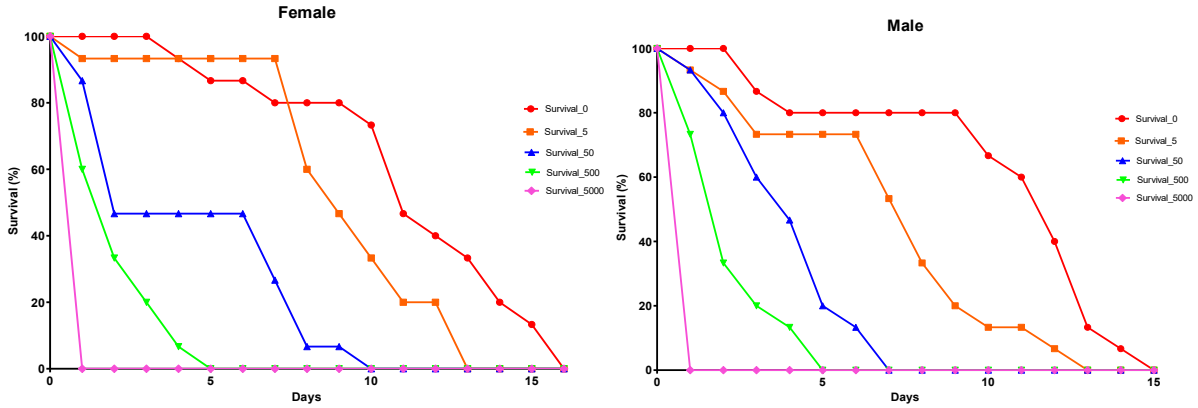


Figure 10: Survival curves for male and female *B. anynana* exposed to varying doses of imidacloprid.

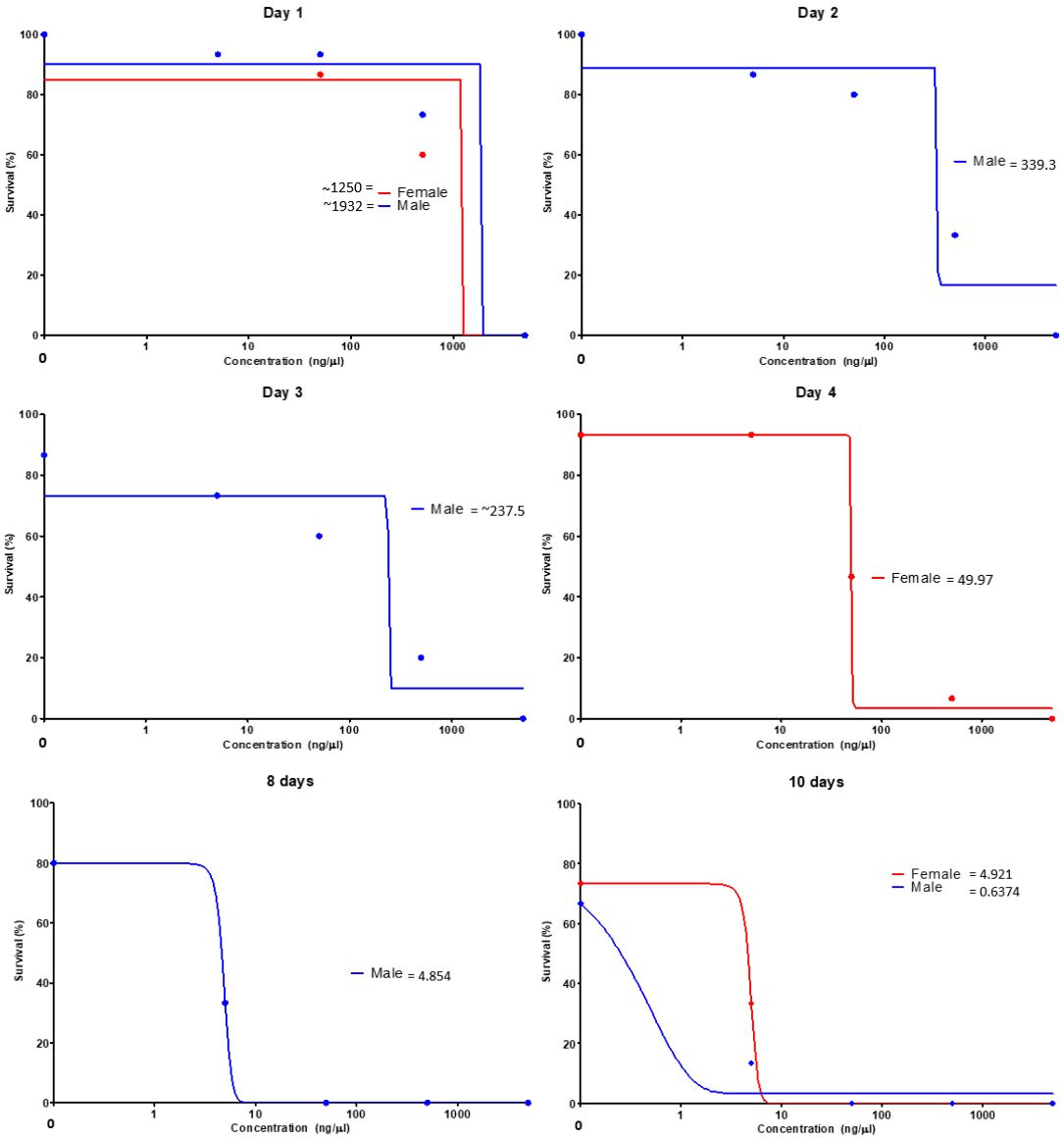


Figure 11: Imidacloprid LC_{50} curves for male *B. anynana* at 1, 2, 3, 8, 10 days after exposure, and females on days 1, 4, and 10 days. LC_{50} values are shown in ng/µl. Full results can be found on appendix table A7.

3.1.5 Neonicotinoid: Thiamethoxam

Doses that were higher than 5000 ng/µl had fast lethality, with all adults dying within the first 24 hours after exposure, whereas doses that were below 5 ng/µl had low lethality relative to the rest of the dosage groups (Figure 12). Based on adult survival (Figure 12), the first reliable LC_{50} for males was at 9 days after exposure at 4.65 ng/µl (766.15 ng/g male), whereas femles were earlier at 4 days after exposure 387.50 ng/µl (20287.96 ng/g female) (Table 8: Figure 13). LC_{50} ranges could be observed broadly between 1 – 10 days in males and females (Figure 13).

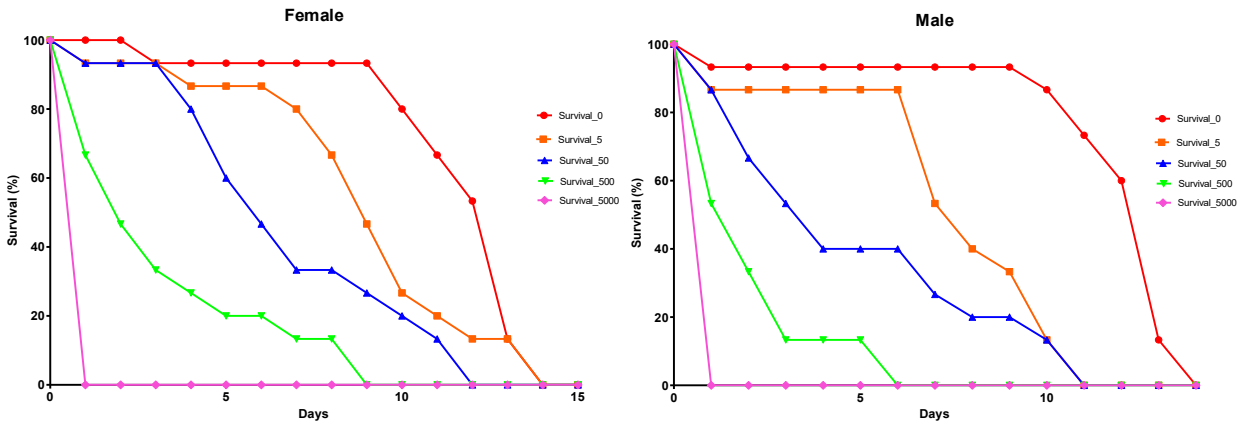


Figure 12: Survival curves for male and female *B. anynana* exposed to varying doses of thiamethoxam. All doses are measured in ng/µl.

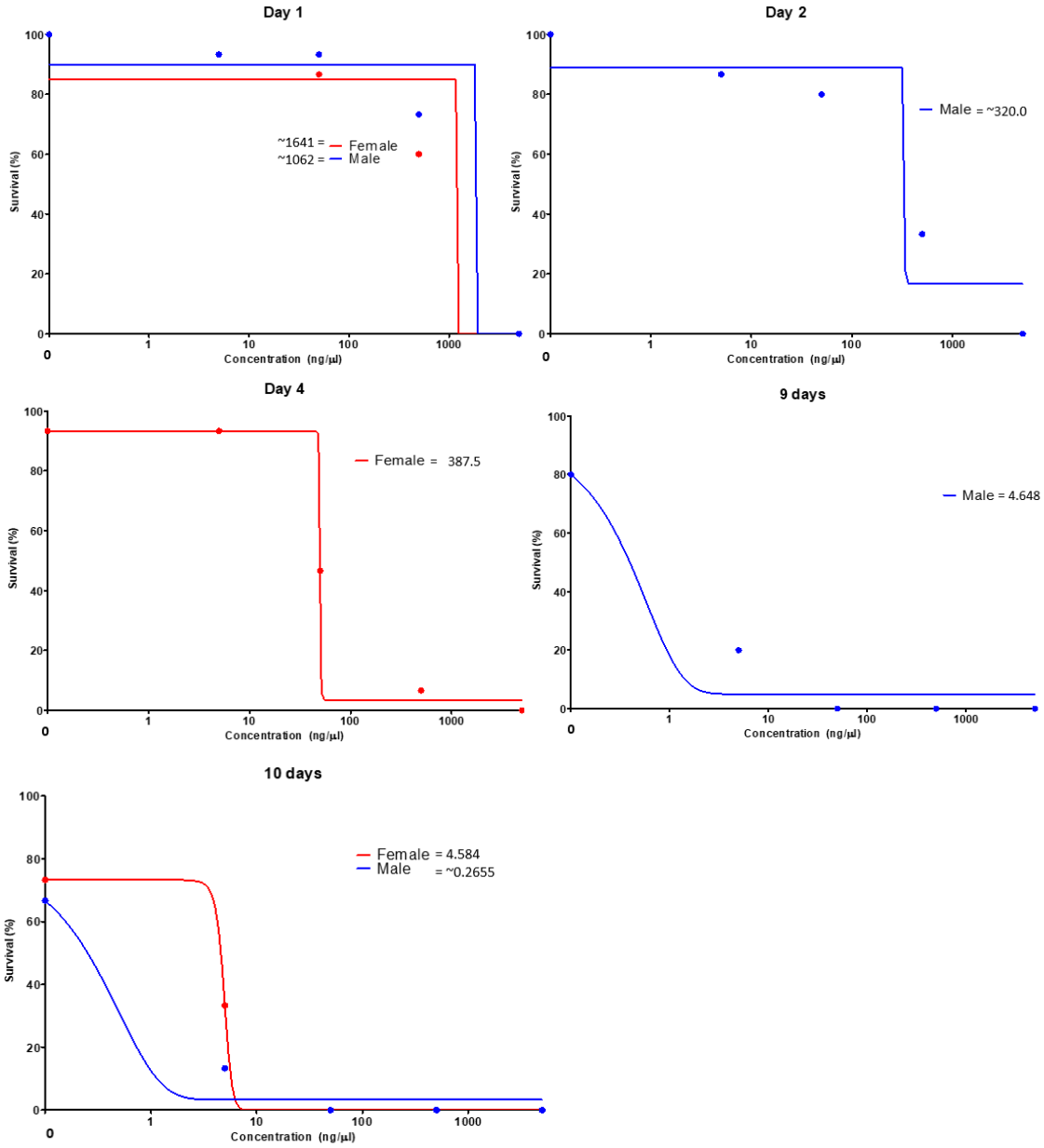


Figure 13: Thiamethoxam LC_{50} curves for male *B. anynana* at 1, 2, 4, 9, 10 days after exposure, and females on days 1, 4, and 10 days. LC_{50} values are shown in ng/µl. Full results can be found on appendix table A8.

Table 8: Compilation of LC_{50} values from all insecticide treatment groups of *B .anynana.* Data is summarised from tables A4 – A8 from appendix.

Insecticide	Male LC_{50} (ng/µl)	Dose concentration ng/g male	Time intervals (days)	Female LC_{50} (ng/µl)	Dose Concentration ng/g female	Time intervals (days)
Deltamethrin	2.68	441.59	1	3.20	50.86	4
Cypermethrin	4.02	663.13	8	4.35	227.59	8
Thiacloprid	4.90	808.19	8	5.13	268.32	9
Imidacloprid	339.39	55928.57	2	49.97	2616.23	4
Thiamethoxam	4.65	766.15	9	387.50	20287.96	4

3.2 Transgenerational experiment: measuring the direct and indirect effects of sublethal insecticide exposure

In order to identify direct effects of sub-lethal concentrations of insecticides on female reproductive output in the butterfly *B. anynana* (aim 2 in section 1.6), various life history traits of the females were examined. This was achieved by conducting statistical tests of variance, covariance, and multiple comparison Post-Hoc Tukey tests, on longevity and reproductive output of females exposed to an insecticide compared to their controls, to determine if there was an effect (see methodology section 2.3). This was then followed by analysis of behavioural traits of offspring of the treated mothers (see methodology 2.3) (aim 3 in section 1.6).

3.2.1 Mother wing size

Wing size was measured of all females used in the transgenerational experiment, to identify if they were of similar size, and thus making sure it was not a factor that could account for variability in the reproductive output. Wing size of all female *B. anynana* did not significantly differ when tested using an analysis of variance (ANOVA: $F_{14,\ 198} = 0.97$, $p > 0.05$) (see appendix tables A9). From this it could be deduced that wing size of all females used within the transgenerational experiments had relatively comparable lengths across treatment groups, and so would not act as a covariate.

3.2.2 Effect on mother longevity after exposure

Female longevity

As larger females may live for longer because more resources are at their disposal (Gibbs *et al*., 2005), female size was taken into account when assessing how an insecticide treatment affected longevity. When tested, we found that wing size was not a factor that could account for variability (ANCOVA, $F_{4, 45} = 3.56$, $p > 0.05$), and so was dropped from the analyses. Of the five insecticide assays, female longevity was significantly reduced as a result of topical deltamethrin exposure (no-treatment (± SE) = 23.33 days ±1.19, acetone (± SE) = 20.80 days ±1.60, deltamethrin (± SE) = 10.53 days ±1.94; ANOVA $F_{2, 9} = 17.81$, $p << 0.001$) (Figure 14). A multi-comparison Post-Hoc Tukey test suggested the two controls did not differ from each other (Tukey $p > 0.05$) but the deltamethrin-treated females significantly differed from both the acetone (Tukey $p < 0.001$) and no-treatment group (Tukey $p << 0.001$) (Figure 14; also see appendix table A11). Thiacloprid also significantly affected longevity (no-treatment (± SE) = 12.10 ±1.29, acetone (± SE) = 9.20 ±1.18, thiacloprid (± SE) = 7.5 ± 1.24; ANOVA: $F_{2,9} = 3.43$, $p < 0.05$), and a multi-comparison Post-Hoc Tukey test identified that the longevity of those females treated with thiacloprid significantly differed from the no treatment control (Tukey $p < 0.05$) but not the acetone control (Tukey $p > 0.05$). There was also no difference between either of the control groups (Tukey $p > 0.05$) (Figure 14). This suggested that the effects of thiacloprid may reduce longevity (Figure 14).

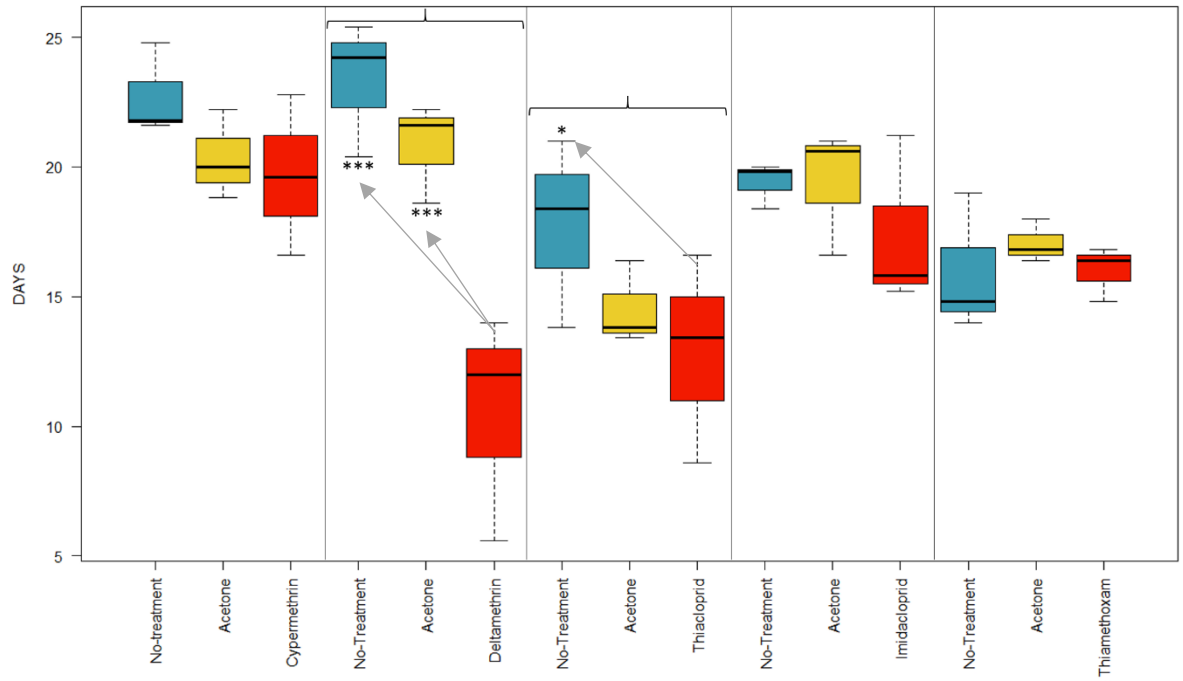


Figure 14: Mother longevity of a selection of pyrethroids and neonicotinoids (N = 3). Brackets indicate an ANOVA with a p<0.05. Post-Hoc Tukey results in relation to insecticide group are; * <0.05, ** <0.01, *** < <0.001. Further details of these results can be found in the appendix tables A11, A14, A17, A20, and A23. Cypermethrin ANOVA, $F_{2, 9} = 1.19$, p>0.05; Deltamethrin ANOVA, $F_{2, 9} = 17.81$, p<<0.001, Thiacloprid ANOVA, $F_{2, 9} = 3.43$, p>0.05; Imidacloprid ANOVA, $F_{2, 9} = 0.77$, p>0.05; Thiamethoxam ANOVA, $F_{2, 9} = 0.20$, p>0.05.

3.2.3 Reproductive output

Egg-laying pattern – egg number

The egg-laying pattern for treated, and non-treated, females was determined by recording the number of eggs laid in each of the cages, per day, over a 5 day period (see section 2.3.1). Patterns of egg-laying indicated that the numbers of eggs laid increased with time before levelling off (Figure 15). Analysing each of the 5 insecticide experiments separately, using a MANCOVA on egg laying patterns (with female wing size as a covariate), showed that there was no difference between treatment groups, indicating that the five insecticides tested had no significant effect on egg output, nor did female size *per se* explain differences in egg production (Cypermethrin MANCOVA: $F_{2, 5} = 5.52$, p >0.05. Deltamethrin MANCOVA: $F_{2, 5} = 1.63$, p >0.05. Thiacloprid MANCOVA: $F_{2, 5} = 0.60$, p >0.05. Imidacloprid MANCOVA: $F_{2, 5} = 0.72$, p >0.05. Thiamethoxam MANCOVA: $F_{2, 5} = 2.23$, p >0.05). Removing female size from the analyses did not alter the fact that the sublethal doses of insecticides appeared not to affect reproductive output (Figure 15).

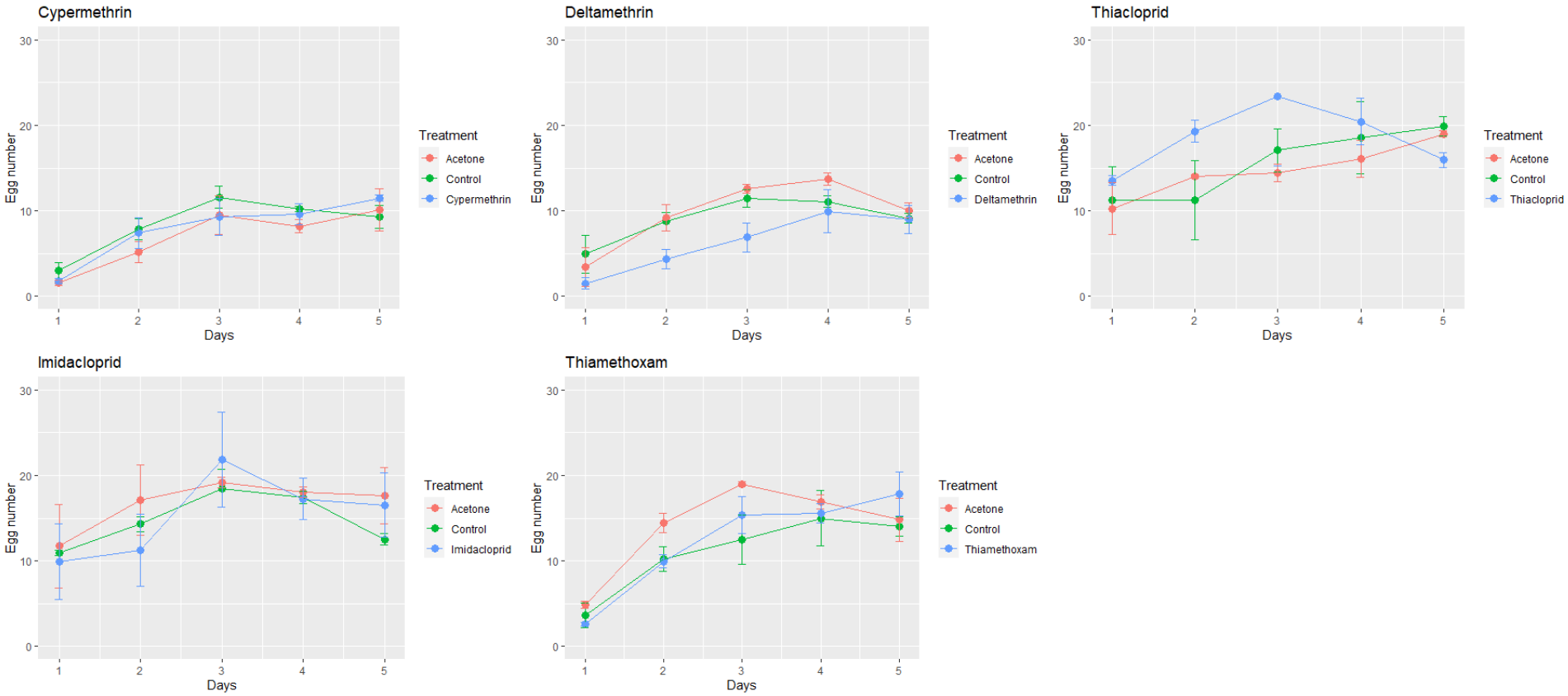


Figure 15: The average number of eggs laid per female between 1-5 days, among a selection of insecticide treatment groups and their controls (N 3). Treatment groups consists of 3 cages of 5 females (∑ 15). Cypermethrin MANOVA: $F_{2,5} = 1.26$, $p > 0.05$. Deltamethrin MANOVA: $F_{2,5} = 1.13$, $p > 0.05$. Thiacloprid MANOVA: $F_{2,5} = 0.85$, $p > 0.05$. Imidacloprid MANOVA: $F_{2,5} = 0.98$, $p > 0.05$. Thiamethoxam MANOVA: $F_{2,5} = 1.35$, $p > 0.05$. Number of females that died before full 5 days of egg collection: Cypermethrin experiment; acetone 2/15. Deltamethrin experiment; acetone 1/15, deltamethrin 7/15. Thiacloprid experiment; control 2/15, acetone 3/15, thiacloprid 4/15. Imidacloprid experiment; acetone 1/15, imidacloprid 2/15. Thiamethoxam experiment; control 3/15, acetone 1/15, thiamethoxam 2/15.

Total egg count

Of the 5 insecticides tested, only deltamethrin significantly reduced the total number of eggs laid (no-treatment (± SE) = 680 ±3.92, acetone (± SE) = 712 ±5.22, deltamethrin (± SE) = 201 ±1.52; ANOVA $F_{2,\,9} = 24.25$, p<<0.001) (Figure 16; also see appendix table A11). Tukey tests showed that deltamethrin was significantly different from both the no-treatment (Tukey p <<0.001) as well as acetone controls (Tukey p <<0.001), whilst the controls did not differ from each other (Tukey p >0.05). This would suggest that whilst the number of eggs laid per female per cage each day did not differ between treatment groups (Figure 15), but the overall quantities of eggs differed for deltamethrin, because female mortality of that treatment group was significantly higher than its controls (Figure 14), and thus less eggs could be laid due to lower female abundance.

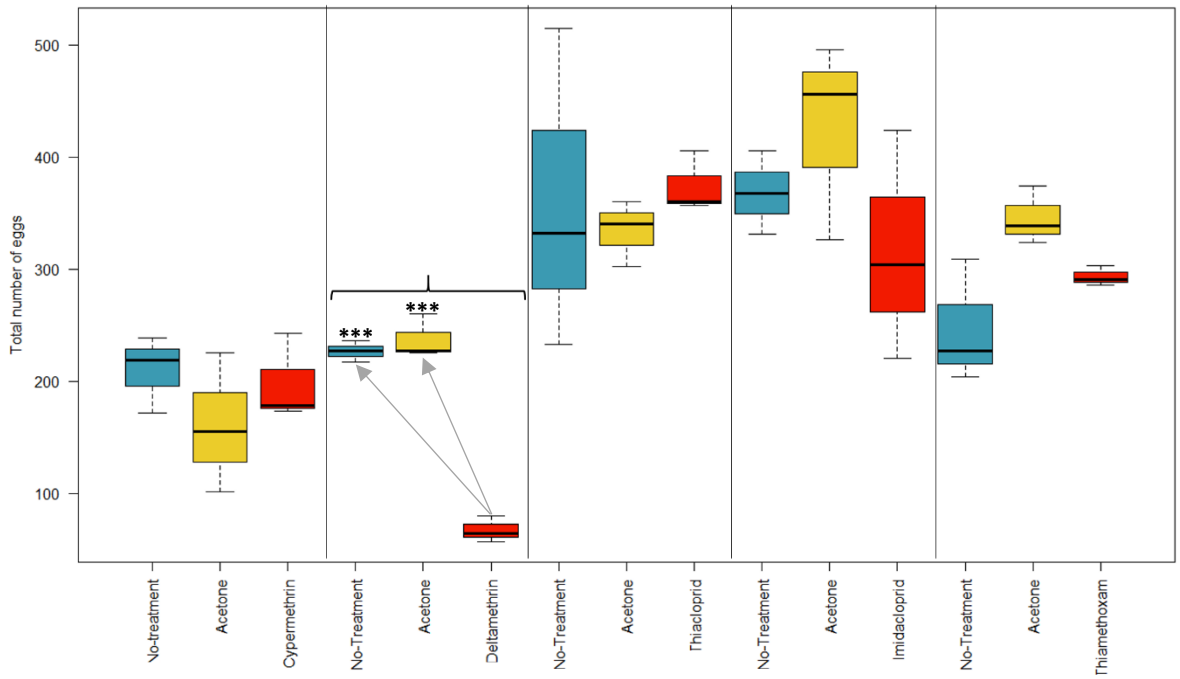


Figure 16: The total number of eggs laid over 5 days for each treatment group (N = 3). Brackets indicate the treatment groups were significantly different (P <0.05) in an ANOVA, bars proceeded with '***' indicate that a Post-Hoc Tukey test was p <0.001. Treatment groups consists of 3 cages of 5 females (∑ 15). Full averages of results can be found in the appendix tables A11, A14, A17, A20, and A23. Cypermethrin ANOVA, $F_{2,\,9} = 1.10$, p>0.05; Deltamethrin ANOVA, $F_{2,\,9} = 24.25$, p<<0.001; Thiacloprid ANOVA, $F_{2,\,9} = 0.45$, p>0.05, Imidacloprid ANOVA, $F_{2,\,9} = 2.87$, p>0.05, Thiamethoxam ANOVA, $F_{2,\,9} = 2.19$, p>0.05.

Egg-laying pattern – egg size

It was found that wing size (as a proxy for female size), was not a factor that could account for variability in egg size (MANCOVA p>0.05). Though egg sizes were diverse between treatment groups and their respective control groups, their variance did not significantly differ (MANOVA p >0.05) (Figure 17). This would suggest that the insecticide exposure to female *B. anynana* did not influence egg size.

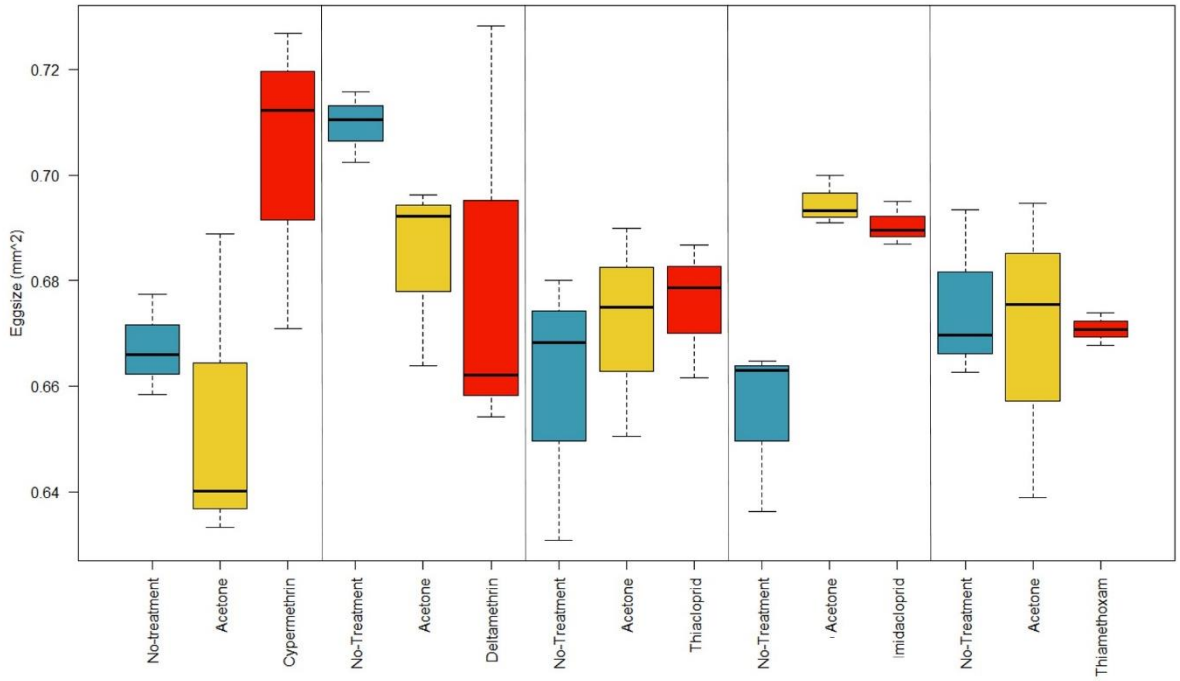


Figure 17: Average egg size of a selection of insecticides and their controls (N = 3). Treatment groups consists of 3 cages of 5 females laying (N total of 9 cages). Full averages of results can be found in the appendix tables A11, A14, A17, A20, and A23. Cypermethrin MANOVA $F_{2,5} = 0.47$, p >0.05; Deltamethrin MANOVA $F_{2,5} = 0.96$, p >0.05; Thiacloprid MANOVA $F_{2,5} = 2.06$, p >0.05; Imidacloprid MANOVA $F_{2,5} = 1.77$, p >0.05; Thiamethoxam MANOVA $F_{2,5} = 0.10$, p >0.05.

3.2.4 Offspring development and hatching success

Development time of eggs

Egg development time was calculated from the time taken at the point they were laid, to the time taken to hatch. Acetone displayed a longer development time than cypermethrin and the no-treatment control (no-treatment (± SE) = 4.65 days ±0.08, acetone (± SE) = 5.23 days ±0.11, cypermethrin (± SE) = 4.83 days ±0.06; ANOVA $F_{2,9} = 6.63$, p<0.01) (Figure 18). A multi comparison Post-Hoc Tukey test showed that the acetone control group differed to the no- treatment control (Tukey p< 0.05), and the cypermethrin group (Tukey p <0.01), whilst cypermethrin and the no-treatment groups did not differ (Tukey p >0.05) (Figure 18).

Acetone also displayed a longer development time than deltamethrin and the no-treatment control (no-treatment (± SE) = 5.08 ±0.08, acetone (± SE) = 5.67 ±0.13, deltamethrin (± SE) = 5.26 ±0.16; ANOVA $F_{2, 9} = 4.41$, $p<0.05$). A multi comparison Post-Hoc Tukey test showed that the no-treatment and acetone controls significantly differed to each other (Tukey $p<0.01$), whilst deltamethrin was significantly different from acetone (Tukey $p <0.05$) and not significant against the no-treatment group (Tukey $p>0.05$) (Figure 18). Inferring that eggs whose mothers were treated with acetone incurred a minor delay to embryonic development, apart from in the thiacloprid experiments (Figure 18).

The average embryonic development time of the no- treatment control was significantly longer than the acetone control and thiacloprid (no-treatment (± SE) = 5.79 ±0.46, acetone (± SE) = 5.50 ±0.14, thiacloprid (± SE) = 5.64 ±0.18; ANOVA $F_{2, 9} = 4.42$, $p<0.05$). A multi comparison Post-Hoc Tukey test identified that Tukey test identified that eggs laid by the no-treatment control took significantly longer to develop than eggs from the acetone control (Tukey $p <0.05$) and the thiacloprid treatment group (Tukey <0.05), yet thiacloprid and acetone did not differ (Tukey $p >0.05$) (Figure 18).

The development time of the thiamethoxam groups was significantly longer than its controls (no-treatment (± SE) = 5.71 ±0.13, acetone (± SE) = 5.65 ±0.11, thiamethoxam (± SE) = 5.92 ±0.08; ANOVA $F_{2, 9} = 3.98$, $p<0.05$). A multi comparison Post-Hoc Tukey identified that thiamethoxam group took significantly longer than the acetone control (Tukey $p<0.05$), but not significantly longer than the no- treatment control (Tukey $p>0.05$) (Figure 18). The no-treatment and acetone controls did not significantly differ in development time ($p >0.05$) (Figure 18). Indicating that of the neonicotinoids, thiamethoxam was the only insecticide to possibly induce a development delay.

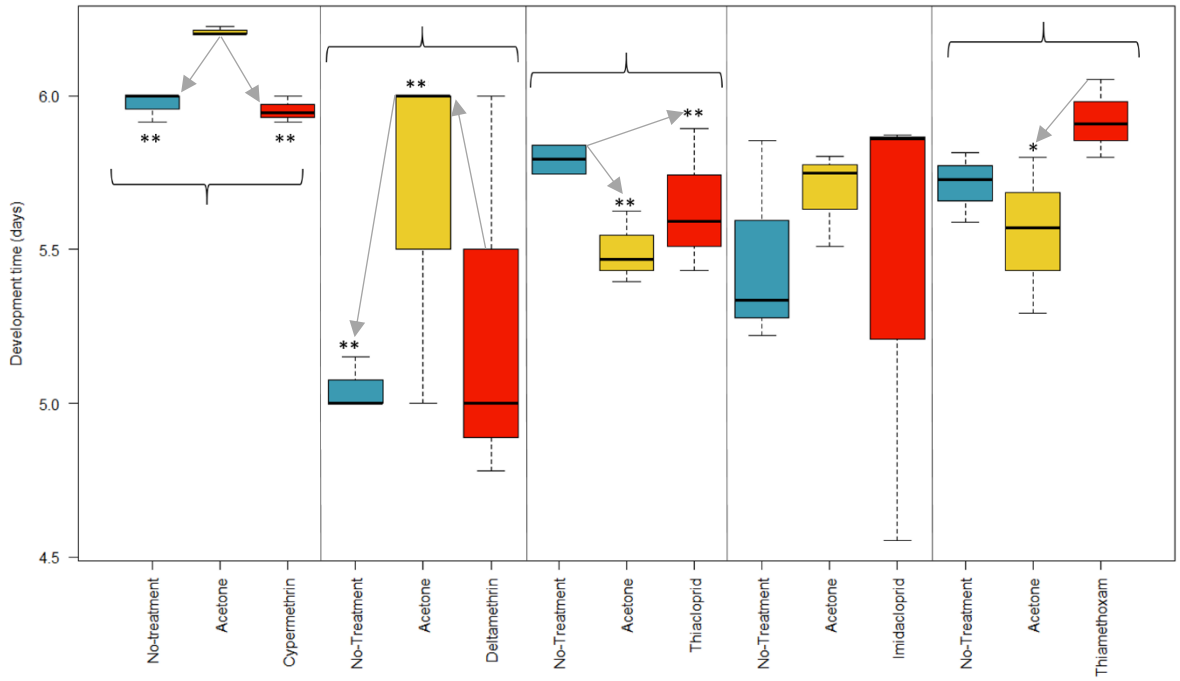


Figure 18: Average egg development time take for eggs whose mothers were exposed to a selection of insecticides, and their controls. Brackets indicate an ANOVA with a p <0.05. Post-Hoc Tukey results in relation to insecticide group are; * <0.05, ** <0.01, *** <0.001. Full averages of results can be found in the appendix tables A11, A14, A17, A20, and A23. Cypermethrin ANOVA, $F_{2, 9} = 6.63$, p <0.01; Deltamethrin ANOVA, $F_{2, 9} = 7.18$, p<0.01; Thiacloprid ANOVA, $F_{2, 9} = 4.42$, p<0.05; Imidacloprid ANOVA, $F_{2, 9} = 1.10$, p>0.05; Thiamethoxam ANOVA, $F_{2, 9} = 3.98$, p<0.05.

Hatching success

The number of offspring hatched per treatment group was calculated, to determine if offspring from the insecticide treated group differed to controls. Of the pyrethroid experimental groups, the average hatching success of deltamethrin was significantly lower than its controls (no-treatment (± SE) = 180.67 hatched ±3.867, acetone (± SE) = 189.00 hatched ±4.14, deltamethrin (± SE) = 33.67 hatched ±0.92; ANOVA $F_{2, 9} = 27.37$, p<<0.001) (Figure 19). A multi comparison Post-Hoc Tukey test showed that deltamethrin was very significantly lower than the no-treatment (Tukey p <<0.001) and acetone (Tukey p <<0.001) controls, whilst the controls did not differ from each other (Tukey p>0.05) (Figure 19). Of the neonicotinoid experimental groups, the average hatching success of offspring from imidacloprid exposed mothers was significantly lower than the control groups (no-treatment (± SE) = 212.67 ±36.77, acetone (± SE) = 209.67 ±16.50, imidacloprid (± SE) = 84.00 ±19.08; ANOVA, $F_{2, 9} = 14.95$, p<<0.001) (Figure 19). A multi comparison Post-Hoc Tukey test showed that the imidacloprid was significantly lower than the acetone (Tukey p<<0.001) and no-treatment controls (Tukey p<<0.001), whilst the controls did not significantly differ

(Tukey p>0.05). It could be concluded offspring of deltamethrin and imidacloprid-exposed females had significantly reduced hatching success.

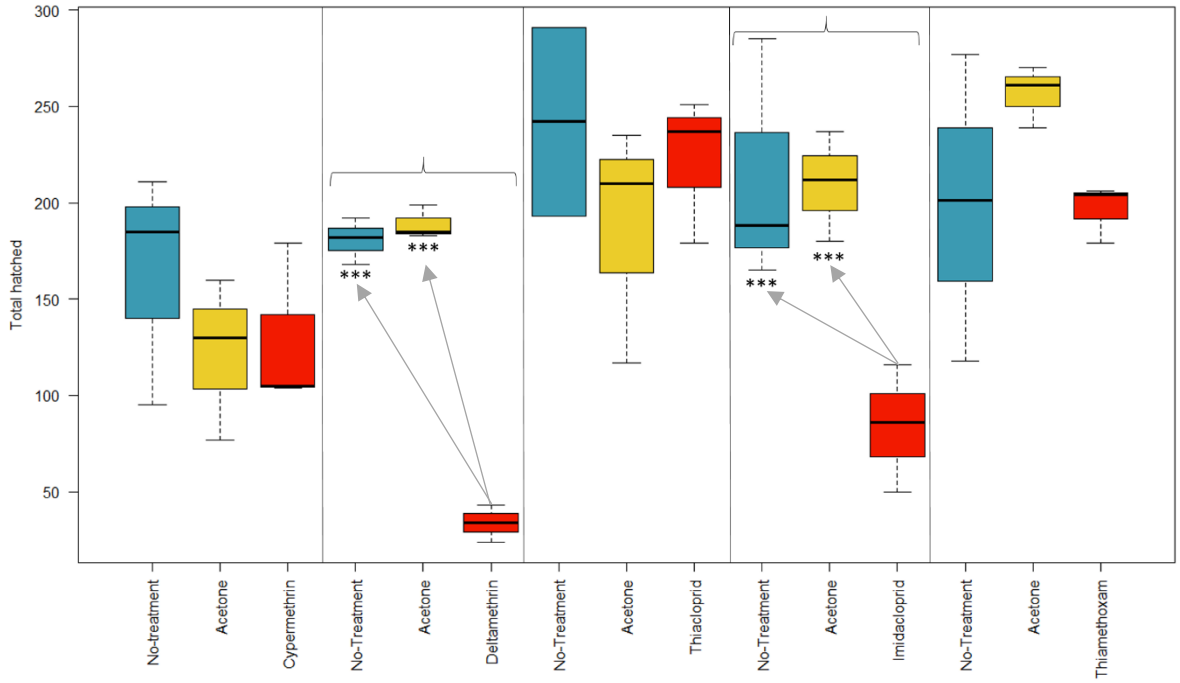


Figure 19: Hatching success per cage, for offspring (pooled across 5 days), whose mothers were exposed to a selection of insecticides, and their controls (N = 3). Brackets indicate an ANOVA with a p <0.05. Post-Hoc Tukey results in relation to insecticide group are; * <0.05, ** <0.01, *** <0.001. Full averages of results can be found in the appendix tables A11, A14, A17, A20, and A23. Cypermethrin ANOVA, $F_{2, 9} = 2.28$, p >0.05; Deltamethrin ANOVA, $F_{2, 9} = 27.37$, p<<0.001; Thiacloprid ANOVA, $F_{2, 9} = 1.35$, p<0.05; Imidacloprid ANOVA, $F_{2, 9} = 14.95$, p<<0.001; Thiamethoxam ANOVA, $F_{2, 9} = 1.23$, p<0.05.

3.2.5 Offspring behaviour

Success to find a host plant

All behavioural assays were shown to be significantly different when tested by means of a chi-square analysis, showing that most insecticide treatment groups had a significantly lower success of reaching the host plant in the allotted time (Table 9; see appendix tables A4, A7, A10, A13, and A16). The imidacloprid behavioural assay was only shown to be significant because the no-treatment larvae were significantly more successful than the acetone and insecticide treatment groups (Table 9). Of the two insecticide classes, pyrethroids had the lowest overall success in larvae (Pyrethroids: deltamethrin and cypermethrin 12.5% successful) whose mothers had been exposed to insecticides (Table 9).

Success time

An analysis of covariance (ANCOVA) (using larval weight as a covariate), showed that each of the insecticide treatment groups and their controls had evenly distributed weights, and so did not influence the success time of any individuals tested (ANCOVA p >0.05) (Figure 20). The time taken of individuals that were able to find a host plant were re-analysed as an ANOVA, which also did not differ between treatment groups (cypermethrin ANOVA $F_{2, 36}$ = 0.97, p>0.05; deltamethrin ANOVA $F_{2, 36}$ = 3.22, p>0.05; thiacloprid ANOVA $F_{2, 36}$ = 0.02, p>0.05; imidacloprid ANOVA $F_{2, 36}$ = 1.76, p>0.05; thiamethoxam ANOVA $F_{2, 36}$ = 2.48, p>0.05) (Figure 20).

Distance covered

An ANCOVA showed that larval weight did not affect distance covered by individuals in the arena for most insecticide experiments (ANCOVA p>0.05), except for thiamethoxam (Figure 21). Therefore the covariate was dropped in those experiments testing as insignificant, and an ANOVA was conducted. Of the pyrethroids, cypermethrin took a significantly longer route than the controls (no-treatment (± SE) = 38.43 cm ±4.57, acetone (± SE) = 44.72 cm ±0.71, cypermethrin (± SE) = 51.80 cm ±0.80; ANOVA $F_{2, 36}$ = 4.46, p<0.05). A multi comparison Post-Hoc Tukey test showed that cypermethrin travelled significantly longer than the acetone control (Tukey p<0.05), but not from the no-treatment control group (Tukey p >0.05), both controls did not differ from each other (Tukey p>0.05) (Figure 21).

Acetone control individuals covered greater distances than the imidacloprid and no-treatment control (no-treatment (± SE) = 48.40 cm ±1.18, acetone (± SE) =57.10 cm ±5.03, imidacloprid (± SE) = 46.80 cm ±0.97; ANOVA $F_{2, 36}$ = 3.57, p<0.05). A multi comparison Post-Hoc Tukey test showed that neither the imidacloprid nor acetone differed (Tukey p>0.05) and did not significantly differ from the no treatment group (Tukey p>0.05), whilst both controls also did not differ from each other (Tukey p>0.05) (Figure 21). The ANOVA indicated a significant difference in means, but the Post-Hoc Tukey test revealed that the individual pairwise comparisons were not different enough to be statistically significant.

An ANCOVA highlighted that weights and distance travelled was larger in individuals whose mothers were exposed to thiamethoxam (Weights for no-treatment (± SE) = 0.0559 g ±0.008, acetone (± SE) = 0.0605 g ±0.005, thiamethoxam (± SE) = 0.0670 g ±0.012; Distance for no-treatment (± SE) = 46.43 cm ±1.00, acetone (± SE) = 46.19 cm ±0.74, thiamethoxam (± SE) = 50.68 cm ±2.73; ANCOVA $F_{2, 36}$ = 3.76, p<0.05) (Figure 21; also see appendix tables A12, A15, A18, A21, A24). A multi comparison Post-Hoc Tukey test showed that thiamethoxam travelled significantly longer than the acetone group (Tukey p<0.05) and the no-treatment group (Tukey p<0.05), whilst both controls did not differ (Tukey p>0.05).

Indicating that weights may have theoretically influenced the success of the acetone and thiamethoxam groups.

Although a substantial proportion of larvae in the insecticide treatment groups were not successful in the behavioural assay, only a small percentage showed no movement at all (Table 9). This suggests that while many larvae were able to move, insecticides impaired movement abilities (Figure 21; see appendix tables A5, A8, A11, A14, and A17).

Table 9: The success of larvae (%) able to reach a host plant within the given time (N = 16). The larvae were categorized on their outcomes and are shown as percentage values and corresponding blue circles: successful arrival at the host plant (Success), larvae that moved but failed to locate the plant within 60 minutes (Failed), larvae that did not move (D_N_M), and those that left the arena (Left). Full averages of results can be found in appendix tables; A12-A13, A15-A16, A18-A19, A21-A22, and A24-A25.

Percentage of successful larvae

	Success	Fail	D_N_M	Left
No-treatment	81.3	18.8		
Acetone	62.5	37.5		
Deltamethrin $X^2_{2,36} = 40.10$, p = <<0.001	12.5	62.5	6.3	19.0
No-treatment	75.0	25.0		
Acetone	68.8	25.0		6.3
Cypermethrin $X^2_{2,36} = 50.45$, p = <<0.001	12.5	75.0	6.3	6.3
No-treatment	68.8	31.3		
Acetone	81.3	6.3	12.5	
Thiacloprid $X^2_{2,36} = 45.85$, p = <<0.001	37.5	62.5		
No-treatment $X^2_{2,36} = 43.12$, p = <<0.001	75.0	25.0		
Acetone	56.3	37.5	6.3	
Imidacloprid	62.5	31.3	6.3	
No-treatment	81.3	18.8		
Acetone	62.5	37.5		
Thiamethoxam $X^2_{2,36} = 51.74$, p = <<0.001	31.3	68.8		

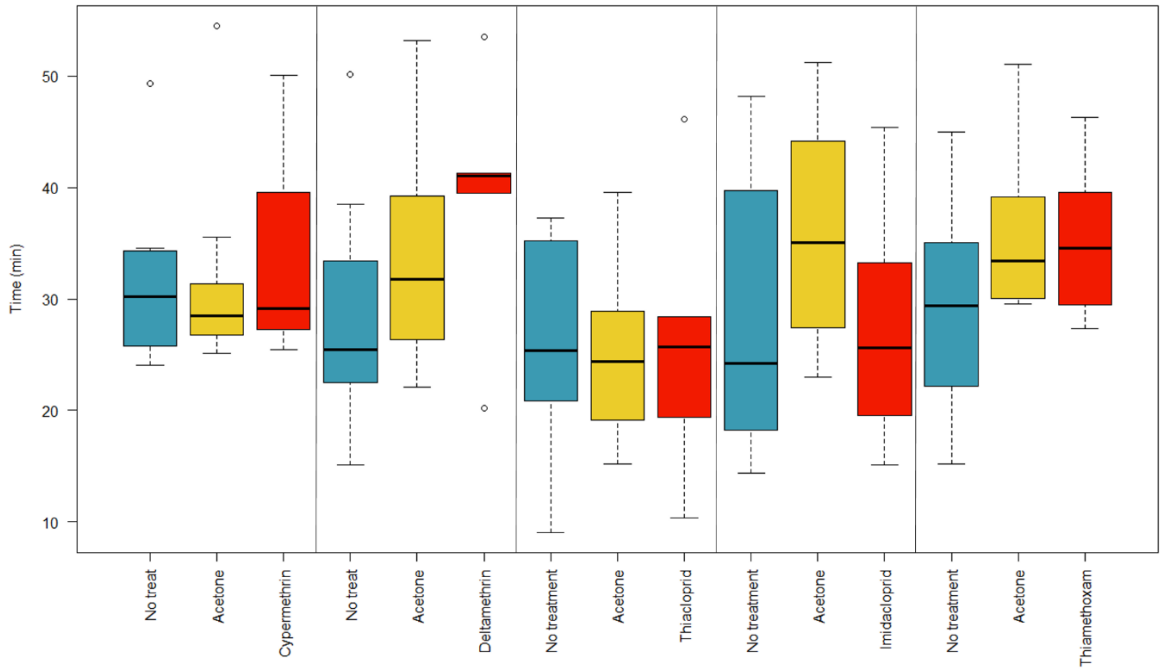


Figure 20: Time at which all successful larvae were able to reach a host plant in the behavioural assay. Summary statistics of these results can be found in appendix tables; A12-A13, A15-A16, A18-A19, A21-A22, and A24-A25. All ANCOVA results were not significant, therefore no additional symbols were added; Cypermethrin ANCOVA, $F_{2,\ 9} = 0.93$, $p > 0.05$; Deltamethrin ANCOVA, $F_{2,\ 9} = 4.14$, $p > 0.05$; Thiacloprid ANCOVA, $F_{2,\ 9} = 0.02$, $p > 0.05$; Imidacloprid ANCOVA, $F_{2,\ 9} = 1.90$, $p > 0.05$; Thiamethoxam ANCOVA, $F_{2,\ 9} = 2.38$, $p > 0.05$.

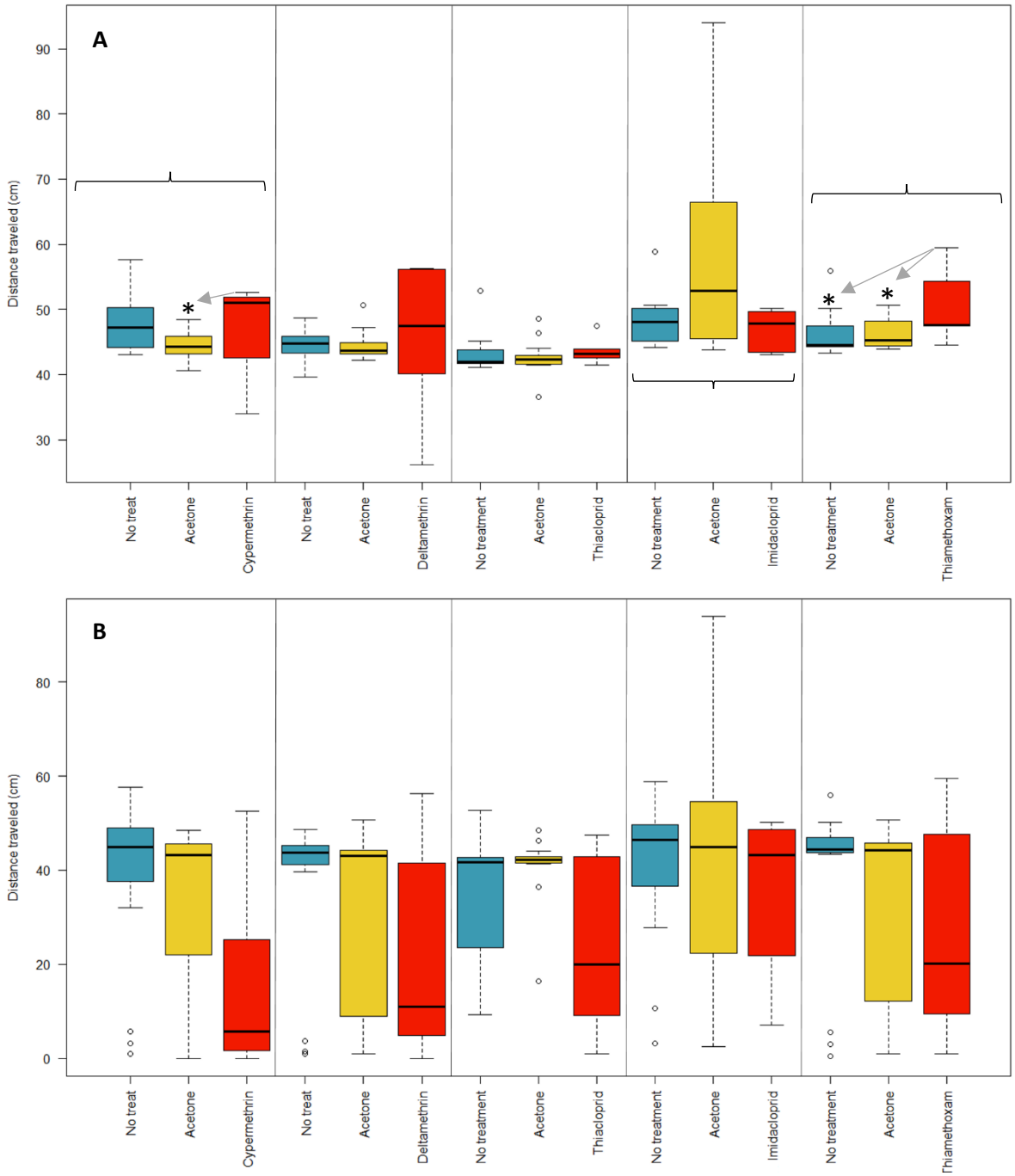


Figure 21: (A) Distance (cm) covered by those larvae that reached a host plant within 60 min. Brackets indicate an AN(C)OVA with a p <0.05. Post-Hoc Tukey results in relation to insecticide group are * <0.05. Summary results can be found in appendix tables; A12-A13, A15-A16, A18-A19, A21-A22, and A24-A25. Cypermethrin ANOVA, $F_{2,\ 36} = 4.46$, p <0.05; Deltamethrin ANOVA, $F_{2,\ 36} = 1.31$, p>0.05; Thiacloprid ANOVA, $F_{2,\ 36} = 0.09$, p>0.05; Imidacloprid ANOVA, $F_{2,\ 36} = 3.57$, p<0.05; Thiamethoxam ANCOVA, $F_{2,\ 36} = 3.76$, p<0.05. (B) Distance (cm) covered by all larvae during the behavioural assay. An ANCOVA was not conducted for all larvae, as the figure served as a point of reference, highlighting that even many unsuccessful larvae still moved. Additionally, measurements of individuals were stopped upon leaving the arena, therefore to include their records in an ANCOVA would generate a false statistic.

4 Discussion

4.1 *Bicyclus anynana* LC_{50}

Overall, males were observed to have lower LC_{50} values compared to females, however it is possible that this was not because males were simply smaller, thus enduring a relatively higher dose concentration (Table 8), but may have been because of age or sex-specific differences in sensitivity. Whilst such differences have been observed in other insects (e.g. bumble bee, *Bombus impatiens* (Mobley and Gegear, 2018), and the moth *P. xylostella* (Banazeer *et al*., 2021; Sayyed *et al*., 2008), the mechanisms underlying such differences were suggested to be species dependant (Banazeer *et al*., 2021), but that they could be mostly attributed to increased expression of genes related to major biological functions (like immunity) (Mobley and Gegear, 2018), and target-site mutations altering insecticide binding opportunities for the insecticides (Banazeer *et al*., 2021). In our investigation, we found males in general had a shorter lifespan (section 3.1; Figures 4, 6, 8, 10, 12), yet it is important to consider that male *B. anynana* are recorded as living shorter than females in other studies, because of the transfer of nuptial gifts and reproductive behaviours were seen to shorten lifespans after mating (i.e. males that invest more in reproduction, have less resources to allocate to their own survival) (Huq *et al*., 2019; Ng *et al*., 2017). Suggesting a shorter lifespan in males could be influenced by reproductive investment, and not just insecticide exposure. Females also have a higher body mass (and thus body fat), which in insects allows for increased ability for chemical detoxification and metabolic functions (Zhao *et al*., 2020), because fat acts as an intermediate metabolism-organ to detoxify harmful chemicals (e.g. insecticides) through enzymatic activity (Jiang *et al*., 2005; Zhao *et al*., 2020). Consequently, due to the aforementioned variation in resource allocation and life history strategies between males and females, they may exhibit different susceptibilities to insecticides, independent of size differences *per se*.

Like our model system *B. anynana*, the females of many UK butterfly species mate shortly after eclosion (Ng *et al*., 2017), and males therefore tend to emerge before females to be best-placed to secure matings (i.e. protandry) (Wiklund and Fagerström, 1977). However, if male lifespan is shortened and fertility affected as a result of persistent (sublethal) insecticide use, protandry may become risky, in particular under certain adverse environmental conditions. Not only may individual fitness be affected, ultimately this may have negative impacts upon the health to populations. As Lepidoptera is known for inhabiting a broad spectrum of habitats and niches (Fox, 2013), any form of genetic

degradation to a population could have greater consequences to the beneficial services these butterflies have, thus causing habitat degradation.

Published studies, such as those reviewed earlier in the thesis (Section 2.2; also see appendix table A3) did not always state the weight (even approximately) of the insecticide-treated individuals, only the dose applied *per se*. Furthermore, often life-stages and application methods differ between studies. This can make conclusions from insecticide sensitivity study comparisons, both intra- and interspecifically, difficult. We would therefore advise to include weight measurements as well, which would facilitate further studies of effects of (sublethal) doses in a variety of contexts. Moreover, such data would prove beneficial for both insecticide application strategies as well as conservation efforts. From the data on dose per body weight unit (see appendix table A3) of various different insect species, it can be inferred that our recorded LC_{50} dose concentrations were comparatively high in comparison to those sourced from literature. From this comparison, we can also observe that pyrethroids generally exhibited lower LC_{50} values relative to the neonicotinoids (Table 8).

Our calculated LC_{50} values for deltamethrin, cypermethrin (pyrethroids), and thiacloprid (neonicotinoid) were all lower than what can be inferred from recorded field samples (surveyed from insect populations and plant surface samples worldwide) of said insecticides (Table 8; also see appendix table A3). On the other hand, male *B. anynana* treated with imidacloprid (LC_{50} 339.39 ng/µl) and females (LC_{50} 49.97 ng/µl) had higher calculated LC_{50}`s than field samples of imidacloprid from our survey, yet were lower than the recorded field sample by day 8 – 10 in both sexes (Appendix table A3). Thiamethoxam exposed females had a calculated LC_{50} (387.50 ng/ µl) which was also higher than reported field samples (250ng/µl of corn treated with thiamethoxam: Yue *et al*., 2003), but was below this 10 days after exposure (4.58 ng/µl) (Figure 13), inferring that age increases sensitivity. Therefore, not only do males have a shorter lifespan in general in which to mate (due to investment in reproduction), but also males may also be more sensitive to the insecticides at the time of mating (should they be exposed to insecticides) (Krishnan *et al*., 2021a). With the greater question of how timing affects butterfly tolerance to insecticides still un-answered, and called on for further research (Braak *et al*., 2018). Moreover, agricultural stakeholders may also suffer, as if crops are not treated in time, they are more vulnerable to insect feeding damage (Eldefrawi *et al*., 1964; Myers *et al*., 2005), causing substantial loss to the crop yield. Considering that nearly 70% of agricultural insect pests are Lepidoptera (mainly moths) (Braak *et al*., 2018), a delay in crop treatment could give harmful Lepidoptera ample time for additional feeding of unprotected crops. Possibly affect the global food supply, of an already growing human population (section 1.1.1).

In both insecticide classes, LC_{50} values could only be reliably determined in relatively broad timeframes, with pyrethroids being calculated after 1-8 days, and neonicotinoids in 2-9 days (Table 8). A full comparison of this finding proved to be difficult, as our sampled literature (see appendix table A3) had no adult butterfly LC_{50} experiments. Studies like Krishnan *et al* (2021a) were unable to calculate LC_{50} values of neonicotinoids (including imidacloprid and thiamethoxam) and pyrethroids on adult monarch butterflies (*D. plexippus*), within a predetermined timeframe of 1 - 4 days. However, this is a result in itself: in their, and for some of the tested insecticides in our study, the sublethal doses do not immediately show an effect, but eventually the mortality rate is affected as the individuals age, and with age in general comes a higher sensitivity. Additionally other studies on *D. plexippus*, like James (2019), only achieved significant mortality after adults were fed sublethal doses of imidacloprid for 22 days, whilst Krischik *et al* (2015) saw no increase in mortality of both sexes after 29 days of chronic imidacloprid exposure. On the basis of these studies, sublethal doses may actually appear harmless, as matings and egg-laying will have taken place, even when the species displays protandry. However, as argued before in our study, such studies did not take into account potential transgenerational effects, which ultimately may nevertheless affect population health.

4.2 Transgenerational experiment: direct and indirect effects of pyrethroids and neonicotinoids on female *Bicyclus anynana* and their offspring

4.2.1 Direct effects of pyrethroids and neonicotinoids on female *Bicyclus anynana*

The use of insecticides in agriculture is a necessity (Botías *et al*., 2015; Long and Krupke, 2016; Olaya-Arenas *et al.,* 2020), but its use will need to be carefully controlled. Young female butterflies, which soon after eclosion (before mating and egg-laying), come into contact with sublethal doses of insecticides may not die straightaway (see section 1.2), but their overall lifespans may nevertheless be shortened and their reproduction affected (see sections 3.1 and 3.2.2). The latter may manifest itself in terms of numbers (and sizes) of eggs produced, but also in terms of the quality of the eggs and any potential transgenerational effects that may manifest themselves in offspring behaviour and survival (see section 3.2). As such, the combined result of these effects after a brief exposure to

sublethal doses may affect long-term population health and survival, which ultimately will be at the detriment of environmental health (Braak *et al*., 2018; Hahn *et al*., 2017; Myers and Lalonde, 2023). It would therefore be beneficial to agricultural and environmental stakeholders, to know what these transgenerational effects are, and whether widely used insecticides are more harmful than others in terms of population health.

The one major significant lifespan reduction observed in these experiments was from deltamethrin application (by 12.80 days, compared to the no-treatment group and 10.27 days to those only exposed to acetone (see section 3.2.2)). Moreover, thiacloprid reduced the female lifespan by 4.6 days compared to the no-testament group, but not significantly to acetone which was only reduced by 1.7 days (see section 3.2.2).

A small number of females form each of the insecticide groups had died before the completion of the egg laying period, with 47% of deltamethrin females dying (Figure 15). It could be suggested that whilst the number of eggs laid per female, per day, did not differ between groups, the overall amount was reduced compared to the controls (section 3.2.2). Therefore whilst their reproductive output rate was similar to those unexposed, the duration was significantly reduced. This can result in long-term population decline in insects (James, 2019; Müller *et al*., 2019), which could be deleterious to habitats and ecosystems as butterflies provide crucial roles as pollinators, and food at multiple trophic levels (Ghazanfar *et al*., 2016). Further highlighting need for consideration of the long-term effects of insecticides after short-term use (Braak *et al.*, 2018; Müller *et al*., 2019).

To our knowledge, there is no single study that compared all five of our selected insecticides, but we were able to draw systematic comparisons from existing investigations that examined sublethal exposure of pyrethroids and neonicotinoids on monarch butterflies (Krishnan *et al*., 2021a; Krishnan *et al*., 2020). It was found that the butterfly species were most susceptible to pyrethroids (beta-cyfluthrin) and neonicotinoids (including imidacloprid and thiamethoxam) at the egg to pupal stages of development, with minimal changes in longevity observed in adult male and female butterflies (Krischik *et al*., 2015; Krishnan *et al*., 2021a; Krishnan *et al*., 2021b). This would suggest that there are developmental stage-specific sensitivity in the species, likely from differential gene expression (yet undefined in these sources), resulting reduced sensitivity to these insecticides. This demonstrates that the susceptibilities of butterfly species can vary depending on their developmental age and sex, yet reveals that pyrethroids (which were considered ‘reduced risk’ (see section 1.1.2)) can still have adverse ecological effects. Possibly more so than neonicotinoids.

4.2.2 Transgenerational effects - offspring development and hatching success

Embryos of mothers exposed to thiamethoxam took on average longer to complete developing in the egg, resulting in a hatching delay (0.21 days compared to no-treatment and 0.27 days compared to acetone), but saw no reduction in hatching success (see section 3.2.3 - 3.2.4). The exact mechanism for why this effect occurred is unknown, but could be speculated that the presence of the insecticide may have affected development as of the increased metabolic stress required for detoxification (Grünewald and Siefert, 2019). In Lepidoptera, prolonged development or hatching can result in delays to important behaviours later on in life (especially in species that display protandry: where males eclose before females), such as migration timing (in species that do so), or mating behaviour (McDonald and Cole, 1991), however there is currently no research indicating the consequence of delays induced by transgenerational insecticide exposure. Hypothetically, this could occur *in situ* for butterflies like *B. anynana,* if exposed to the same dosage of thiamethoxam, and thus disrupting mating ability. Consequently, declining the number of pollinators in a habitat, and thus the beneficial ecological functions they serve (Hahn *et al*., 2015; Potts *et al*., 2016).

As previously mentioned, female *B. anynana* exposed to deltamethrin saw a greatly reduced total number of eggs overall, of which was a result of many females (47%) dying within the egg-laying period (section 3.2.2). However, offspring of deltamethrin exposed females, saw a significantly reduced hatching success (147 less eggs than the no-treatment group and 156 less than acetone) (section 3.2.3 - 3.2.4). From this it could be concluded that deltamethrin greatly reduces the fitness of each egg laid per female, even though the number laid per female, per day, or size, was no different from the control groups (section 3.2.3- 3.2.4). The consequence of this, is that whilst exposed females can produce similar rates of eggs compared to those not exposed, the majority of these eggs would not hatch, resulting in a population decline for that generation. Severe population declines of Lepidoptera have been recorded in the UK (where both pyrethroids and neonicotinoids are used heavily in agriculture) (Warren, 2021), in agricultural sites heavily treated with systemic insecticides (including neonicotinoids) (Fox, 2013; Sánchez-Bayo and Wyckhuys, 2019). Due to the presence of Lepidoptera in a broad range of habitats, it has been predicted that large declines of Lepidoptera from heterogeneous ecosystems, could result in habitat degradation due to the reduction of ecosystem services (Fox, 2013; Sánchez-Bayo and Wyckhuys, 2019).

Whilst in this investigation we have established that sub-lethal exposure of insecticides can cause deleterious transgenerational effects. There are instances in which persistent sublethal exposure of insecticides on insect populations can lead to increased resistance and fitness of surviving insects and their offspring (i.e. hormesis). Hormesis can occur when sublethal doses of insecticides (i.e. stressor) are exposed to an insect population, causing a hormetic or stimulatory responses in surviving adults, which can increase insecticide resistance in offspring through transgenerational effects (Deans and Hutching, 2022; Melo *et al*., 2022). Such as when Deans and Hutching (2022) exposed *Drosophila suzukii* to widely used insecticides (zeta-cypermethrin, spinetoram, and pyrethrins), they found that males had increased reproductive rates, eclosion success, with their offspring having sustained adverse effect to survival (and thus a level of resistance). Whilst not tested, they suggested that transgenerational effects observed were likely mediated by epigenetic mechanisms, such as methylation/histone modification or resource transfer of RNAs from parent to egg. Comparable to our literature survey, Deans and Hutching (2022) also highlighted that the mechanisms underpinning transgenerational effects were rarely researched (see section 1.2). A review by Rix and Cutler (2022) suggested that hormesis is a response leading to some pest-insect outbreaks and population resurgences, which has the possibility of even stimulating beneficial populations. With increased reproduction most commonly being observed depending on species (Cutler and Guedes, 2017; Deans and Hutching, 2022; Rix and Cutler, 2022).

Even though the transgenerational responses observed in the present study on *B. anynana* were either negative or negligible, this species, and other Lepidoptera, could also exhibit hormesis. We did not follow the offspring through past the egg-hatching stage. Consequently, the repeated sublethal exposure may enhance the overall population health of agricultural-pest Lepidoptera, leading to increased crop damage, resulting in reducing yields. Providing a good example of why it is important to consider a species specific response to an insecticide (as suggested by Braak *et al*., 2018). This poses a threat to insecticide-use in agricultural sites too (Deans and Hutching, 2022), as while effects may be hard to detect in the short-term, it is important to consider the long-term implications to environmental health. As sub-lethal exposure could lead to increased insecticide resistance, leading to higher insecticide doses required to control pest-population in the future (Deans and Hutching, 2022), potentially increasing the harm to non-target insects too (e.g. butterflies).

4.2.3 Behavioural assay

Only 12.5% of offspring from females exposed to pyrethroids were able to successfully reach a host plant within the allotted 60 minute time period (Figure 20). In both cases 6.3% did not move at all during the testing period, showing that 62.5% of the deltamethrin and 75.0% of the cypermethrin individuals attempted to move, with 19% of the deltamethrin and 6.3% of the cypermethrin group leaving the arena (Figure 20). Compared to their acetone and no treatment controls, of whom most larvae successfully found a host plant (Deltamethrin experiment, no-treatment 81.3% success, acetone 62.5% success; Cypermethrin experiment, no-treatment 75% success, acetone 68.8% success) (Figure 20). As for the neonicotinoid groups, thiacloprid and thiamethoxam had significantly lower success than their controls (Thiacloprid experiment, no-treatment 68.8% success, acetone 81.3%, thiacloprid 37.5% success; Thiamethoxam experiment, no-treatment 81.3% success, acetone 62.5%, thiamethoxam 31.3% success), with all individuals from the insecticide groups moving (Figure 20). This would suggest that a majority of pyrethroid and neonicotinoid groups were able to move, but were either disorientated or no longer had a desire to source a host plant. Whilst success did significantly differ between treatment groups in the imidacloprid experiment, this was because the no-treatment control was significantly more successful than the acetone and imidacloprid groups (Figure 20). Our results suggested that maternal imidacloprid exposure did not affect offspring behaviour in comparison to acetone (Imidacloprid experiment, no-treatment 75.0% success, acetone 56.3%, imidacloprid 62.5% success) (Figure 20).

A study by Ferguson (2018; unpublished), who also conducted behaviour assays on *B. anynana* larvae in a single generation, but treated the larvae directly, and not the mothers, by means of topical exposure to deltamethrin (pyrethroid) and thiacloprid (neonicotinoid). It was found that treated larvae were 62% successful in their behavioural arena, which was greater than our successful larvae that were maternally exposed, yet successful individuals travelled at a significantly slower speed. Whilst they gave no suggestion into why their success of treated individuals was lower and slower, it could be speculated that the neurological mode of action the two insecticide classes had (see section 1.1.2) ultimately impaired their movement ability, as seen in bees (Dai *et al*., 2010), and Diptera (Silva *et al*., 2018; Cohnstaedt and Allan., 2011) (see appendix table A1). Furthermore, the difference between our studies may allude that the maternal exposure had a greater impairment than direct exposure in *B. anynana*. Possibly because the insecticide presence in the treated females altered maternal gene expression during oogenesis, leading to physiological and neurological changes in offspring, much like what has been observed in insect growth

regulating insecticides (Lim and Lee., 1982; Perveen and Miyata., 2000; Santorum *et al*., 2021).

Whilst there was evidence of avoidance oviposition behaviour in *P. xylostella* after adults were exposed to permethrin (pyrethroid) (Jallow and Hoy, 2005), our literature survey found no published research on how pyrethroids effect Lepidoptera movement and navigation (single or transgenerational) (chapter 1; also see appendix table A1). There was however evidence of navigation and movement affected by pyrethroids in Diptera, as Cohnstaedt and Allan (2011), found that *C. quinquefasciatus* flew slower, longer, and turned less after deltamethrin and permethrin LD_{25} topical exposure, and flight patterns were interrupted in *A. albimanus*, and *A. aegypti*. However an exact mechanism for these effects were not given. Hymenoptera like *A. ligustica* were also observed to have impaired navigation, learning, fecundity, and feeding behaviour after exposure to sublethal doses of bifenthrin and deltamethrin (Dai *et al*., 2010). Based on the aforementioned examples and our own results, it is clear that pyrethroids have an effect on insect movement and navigation behaviours, yet further study is needed to determine how this occurs transgenerationally, particularly in Lepidoptera.

Our literature survey (chapter 1) also suggested that there was a deficit of studies on offspring behaviour in a transgenerational experiment (especially butterflies). Single generation studies on behaviour found adult *G. molesta* had impaired navigation, poorer flight ability, susceptibility to wind drift when flying, and pheromone communication when exposed to thiacloprid (Navarro-Roldán *et al*., 2019; Navarro-Roldán and Gemeno, 2017). It was suggested movement was affected because thiacloprid acted on the insect's nAChRs and thus impairing normal movement function when flying, and communication was caused by impeded pheromone perception possibly from changes to the antennal lobe interneurons (Navarro-Roldán *et al*., 2019). Whilst in our study pheromone activity was not examined, based on the similarity in the mode of actions our selected neonicotinoids took on an insect's central nervous system (section 1.1.2), it could be speculated the impaired navigation of our neonicotinoid treatment groups were also likely a consequence of affected nAChRs.

Of individuals whose mothers were exposed to neonicotinoids, those in the imidacloprid group travelled on average 1.6 cm shorter than their no-treatment controls and 8.7 cm shorter than their no acetone control. Suggesting that imidacloprid was the one insecticide group that was not unsuccessful at finding food compared to controls, and were more efficient than those exposed to acetone (section 3.2.5). Whilst our literature survey did not corroborate these findings in single generation insect studies, particularly in Diptera (Tasman *et al*., 2021) (chapter 1; also see appendix table A1), there is a lack of investigation on

transgenerational effects to behaviour in Lepidoptera, therefore the addition of further investigation on imidacloprid and other neonicotinoids would better elucidate as to why this occurred.

Larva exposed to thiamethoxam travelled on average 4.25 cm longer than their no-treatment controls and 4.49cm longer than their acetone controls, however individuals were on average nearly 20% larger than the no-treatment controls and nearly 11% bigger than the acetone groups (section 3.2.5). It could be concluded that whilst individuals form the thiamethoxam group were less efficient at finding a host plant, their weight may have influenced their movement ability. Sublethal thiamethoxam has been observed to alter sleeping behaviour, circadian rhythms, and memory impairment in *Drosophila. spp* due to knockdown of the neonicotinoid susceptible nAChRs (Tasman *et al*., 2021). As nAChRs are located in the central nervous system (section 1.1.2), any disruption in their functioning might influence insect neurology, thus locomotion and navigation. Therefore, it is possible that navigation and movement was still impaired in *B. anynana*, because of the effects of thiamethoxam on normal mobility behaviour.

Of the pyrethroids, individuals whose mothers were exposed to cypermethrin took on average 13.37 cm longer than their no treatment control and 7.2 cm longer than their acetone control (section 3.2.5). Suggesting that maternal cypermethrin exposure significantly reduced movement efficiency of offspring. Our literature survey did not find examples of sublethal cypermethrin exposure causing impaired movement efficiency in Lepidoptera offspring or other insect orders (see appendix table A1). Whilst individuals from the deltamethrin group were not observed to have a significantly longer average moment distance, this may have also occurred because of how few successfully were able to reach a host plant (section 3.2.5). From this we can conclude that movement efficiency can be impaired in *B. anynana* after maternal exposure, however this theory would be greatly strengthened by additional evidence from other studies and larger sample sizes.

The consequence of diminished navigation and movement in insects after exposure to insecticides has already been reported across multiple orders, yet the full scope of how this could impact population of insects is still unknown (Braak *et al*., 2018; Dai *et al*., 2010). Any large decline in insect populations (especially those that provide ecological services like many Lepidoptera) (Fox, 2013), could have negative long-term consequences to habitats, and the greater scope of environmental health. What is most alarming is that, as of yet, no research has studied what the transgenerational effects widely used insecticides like pyrethroids and neonicotinoids have on Lepidoptera behaviour (Chapter 1). From our results it is clear that maternal exposure to many neonicotinoids and pyrethroids can impair

movement and navigation, which is critically important for juvenile Lepidoptera, as being able to find host plants, move from over grazed leaves (Cunningham and Zalucki, 2014), move to cover (to avoid predation), and find a suitable pupation site (Connor, 1991), are all vital for survival. Therefore any impairment on these abilities could leave the larvae at risk of predation or starvation, thus decline the population.

Because we do not inspect for transgenerational effects in ecological and insecticide assessments (Braak *et al*., 2018; Brevik *et al*., 2018; Shaw *et al*., 2017), such effects could theoretically already be happening to target/non-target Lepidoptera near agricultural sites. A combination of our recorded sublethal effects with existing drivers of Lepidoptera decline, (such as habitat loss) (Fox, 2013; Krishnan *et al*., 2021a), could increase the magnitude and speed of declines in population over time, along with ecosystem biodiversity.

4.2.4 Overall conclusions and further research

This thesis highlighted the need for multi-generational analysis of the effects insecticides have on Lepidoptera, within or adjacent to agricultural sites (Braak *et al*., 2018; Shaw *et al*., 2017). Whilst the exact direct and indirect mechanisms that cause these effects remain undefined, *B. anynana* had served as a model to suggest that non-target insects, like butterflies and in general (important) pollinators, may not only suffer negative effects to their reproductive output and longevity, but also to their offspring's development, survival, and movement behaviour. Based upon our findings, pyrethroids (deltamethrin most of all) were more deleterious to the direct fitness of the females exposed, but also the offspring's fitness and behaviour (Chapters 3 – 4). Indirect effects to offspring development time were only seen from thiamethoxam (neonicotinoids) exposed females, with offspring from deltamethrin (pyrethroid) and imidacloprid (neonicotinoid) treated females having significantly reduced hatching success. With all pyrethroids and neonicotinoids (except imidacloprid) affecting offspring's fitness and behaviour (Chapters 3 – 4). These results show that different insecticides elicited different degrees of responses from *B. anynana* females and their offspring. This finding holds great significance in regions like the UK, where previous neonicotinoid bans have resulted in greater pyrethroid use (see section 1.1.2), which may consequently exacerbate the already declining pollinating records (Sánchez-Bayo and Wyckhuys, 2021).

Existing research has already determined that sublethal exposure of insects to insecticides, can have a transgenerational effect on the offspring's insecticide resistance, sometimes resulting in advantageous outcomes, such as hormesis (Cutler and Guedes, 2017; Deans and Hutching, 2022; Melo *et al*., 2022; Rix and Cutler, 2022). Yet the mechanisms for such

effects rarely being described (Deans and Hutching, 2022). Further research on the molecular mechanisms underpinning these effects (including where a sublethal doses did not appear to have any effect, indicating a successful defence mechanism), would allow us to provide further depth to the discussion as to why certain individuals, development stages, and traits are affected, and others not, and of course elucidate the variability in effect displayed between the insecticides used. It could be speculated that changes to life-history traits could be related to genes that influence insect detoxification of toxic substances, such as differential expression of cytochrome P450 monooxygenases (P450s) and glutathione S-transferase (GST) gene families (Pavlidi *et al*., 2018; Nauen *et al*., 2021; Nascimento *et al*., 2021). For example the gene CYP6B, allowing *Papilio .spp* (swallowtail butterflies) to overcome some plant defence toxins when feeding (Cohen *et al*., 1992; Nauen *et al*., 2021), or how populations of Cotton Bollworm (*H. armigera*) expressed pyrethroid resistance due to increased CYP337B3 expression (Joußen and Heckel, 2021; Nauen *et al*., 2021).
Finally, the absence of research on transgenerational effects in ecological and insecticide assessments, necessitates multigenerational examinations of insect populations adjacent to agricultural sites. The addition of examining offspring of exposed individuals, to tests on movement would shed light into whether our effects occur in their natural habitat. As without looking at the transgenerational effects of insecticides on non-target insects, we only observe a narrowed-view of the full extent of their effects to environmental health.

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Appendix

Table A 1: Composition of recorded direct and indirect effects of pyrethroids on insect development, fitness, behaviour, hatching success, reproductive output, and survival. Stage: E= egg, L= larva P = pupa, A = adult. Exposure: D= direct exposure, I = indirect exposure.

Species (Order)	Stage of exposure	Pyrethroid	Exposure (D/I)	Effect	Citation
Sitophilus zeamais (Coleoptera)	A	Deltamethrin	(D) Topical	Interference of movement, reproducing, and sexual communication.	Vélez *et al*., 2018
Eriopis connexa (Coleoptera)	A	Lambda-Cyhalothrin	(D) Topical	Reduced longevity after exposure, reduced mating ability of males, and overall reproductive output of females (even in resistant strains)	D'Ávila *et al*., 2018
Eriopis connexa (Coleoptera)	P	Cypermethrin	(D) Topical	High mortality of pupae, and longer oviposition time of those pupae treated.	Fogel *et al*., 2016
Coccinella septempunctata (Coleoptera)	L	Lambda-Cyhalothrin, Cypermethrin	(D) Topical (I) Inherited	Prolonged larval stages, lower adult weight, and reduced offspring survival when exposed.	Afza *et al*., 2023
Coccinella septempunctata (Coleoptera)	L	Deltamethrin	(D) Topical	Having prolonged development time, lower adult weight and predation when exposed.	Skouras *et al*., 2017
Phaedon cochleariae (Coleoptera)	A	Lambda-Cyhalothrin	(D) Ingestion (I) Inherited	Produced offspring with fluctuating levels of antennae asymmetry when exposed to sublethal doses of a pyrethroid, it was speculated this was likely down to a possible reduced maternal transfer of resources into the offspring.	Müller *et al*., 2017
Phaedon cochleariae (Coleoptera)	L	Lambda-Cyhalothrin	(D) Ingestion	Hatching success was marginally higher than control, yet with a lower clutch size in comparison to control. Larvae exposed developed to have lower body mass of adult males and a reduced reproduction of females.	Wolz *et al*., 2022
Leptinotarsa decemlineata (Coleoptera)	L	Deltamethrin	(D) Ingestion	After exposure, females had improved larval and pupal survival compared to controls.	Margus *et al*., 2019
Phaedon cochleariae	L	Lambda-Cyhalothrin	(D) Ingestion	A reduced reproductive output in parents and offspring generation when exposed, and was suggested that there	Müller *et al*., 2019

(Coleoptera)				may have been a heritable epigenetic change involved in the detoxification process. Poor intrasexual communication, aggression behaviour was also observed to be impaired in those exposed.	
Coccinella septempunctata (Coleoptera)	A	Deltamethrin	(D) Topical	Additional and prolonged grooming, indicating an irritated of behaviour to the pyrethroid exposure	Wiles and Jepson, 1994
Aedes aegypti (Diptera)	A	Deltamethrin	(D) Ingestion	A longevity and reproductive output reduction was observed in female mosquito. An increased insecticide resistance in those who survived exposure (P_0).	Martins *et al*., 2012
Culex quinquefasciatus (Diptera)	A	Deltamethrin, Permethrin	(D) Topical	Flew at a slower rate than *Anopheles albimanus*, and *Aedes aegypti* (Who were also treated in same investigation), after exposure, spent more time in flight, and turned less frequently.	Cohnstaedt and Allan, 2011
Anopheles albimanus (Diptera)	A	Deltamethrin, Permethrin	(D) Topical	Greater tendency to fly downwind than controls, and reduced responsiveness.	Cohnstaedt and Allan, 2011
Aedes aegypti (Diptera)	A	Deltamethrin, Permethrin	(D) Topical	Flights tended to be incomplete in comparison to controls.	Cohnstaedt and Allan, 2011
Diamesa zernyi (Diptera)	L	Deltamethrin	(D) Chronic	Prolonged or altered movement patterned after sublethal exposure.	Silva *et al*., 2018
Phytoseiulus persimilis (Mesostigmata)	L	Pyrethrins	(D) Topical	Reduced survival and lower fecundity of adults.	Duso *et al*., 2008
Apis mellifera ligustica (Hymenoptera)	A	Bifenthrin, Deltamethrin	(D) Ingestion	Overall navigation, learning, fecundity, and basic feeding behaviour were impaired to varying degrees.	Dai *et al*., 2010
Telenomus busseolae (Hymenoptera)	A	Deltamethrin, Cyfluthrin	(D) Ingestion	Mother longevity and egg clutch size were significantly reduced compared to their no-treatment control, yet hatching success did not differ.	Bayram *et al*., 2010
Chrysoperla carnea (Neuroptera)	A	Cypermethrin	(D) Ingestion	Oviposition was delayed but had no effect on hatching success or clutch size to those not exposed	Mulligan *et al*., 2010
Cimex lectularius (Hemiptera)	E	Tempid (pyrethroid/ neonicotinoid insecticide)	(D) Topical	A reduction of 34%-73% in egg hatching success, with no impact to later development stages.	Crawley *et al*., 2017

Spodoptera littoralis (Lepidoptera)	L	Deltamethrin	(D) Topical (I) Inherited	Multiple clutches of eggs from the parent generation to have reduced survival and prolonged development time	Massot *et al*., 2021
Danaus plexippus (Lepidoptera)	E	Beta-Cyfluthrin	(D) Topical	High toxicity to eggs, with delayed hatching times. Estimated to cause up to 89% mortality 60m downwind.	Krishnan *et al*., 2021a
Danaus plexippus (Lepidoptera)	L	Beta-Cyfluthrin	(D) Ingestion	High mortality of larvae after exposure. A modelled dose-response analysis predicted that the larval cuticular exposure could have mortality of 100% to 32% at modelled distances of 0, 15, 30, and 60m downwind from the field.	Krishnan *et al*., 2021b
Danaus plexippus (Lepidoptera)	P	Beta-Cyfluthrin	(D) Topical	No adult eclosion after exposure.	Krishnan *et al*., 2021a
Danaus plexippus (Lepidoptera)	L	Bifenthrin, Beta-Cyfluthrin	(D) Ingestion	Highest mortality from bifenthrin as larvae, with Beta-cyfluthrin causing reduced diet consumption and larval growth.	Krueger *et al*., 2021
Heliothis armigera (Lepidoptera)	L	Cypermethrin, Fenvalerate, Deltamethrin, Cyhalothrin	(D) Ingestion	All cases had low egg hatching success and high levels or larval mortality.	Daly *et al*.,1988
Helicoverpa zea (Lepidoptera)	L	Cypermethrin, Permethrin	(D) Topical	Cypermethrin was more toxic than permethrin. High mortality of larvae treated.	Usmani and Knowles, 2001
Spodoptera frugiperda (Lepidoptera)	L	Cypermethrin, Permethrin	(D) Topical	Cypermethrin was more toxic than permethrin. High mortality of larvae treated.	Usmani and Knowles, 2001
Agrotis ipsilon (Lepidoptera)	L	Cypermethrin, Permethrin	(D) Topical	More susceptible to permethrin than to cypermethrin. High mortality of larvae treated.	Usmani and Knowles, 2001
Folsomia candida (Lepidoptera)	L	Etofenprox	(D) Topical	Reduced reproduction over three generations after initial exposure.	Szabó *et al*., 2019
Hadena bicruris (Lepidoptera)	A	Lambda-Cyhalothrin	(D) Ingestion (I) Inherited	resulted in females to have reduced oviposition (as low as one egg overall in some instances), which in turn caused a reduction of eggs and larvae survival of nearly 40% in the treatment group	Hahn *et al*., 2015
Helicoverpa zea (Lepidoptera)	L	Bifenthrin	(D) Ingestion	Reduced sensitivity to bifenthrin after initial sublethal exposure.	Rabelo *et al*., 2020
Spodoptera frugiperda (Lepidoptera)	L	Bifenthrin	(D) Ingestion	Reduced sensitivity to bifenthrin after initial sublethal exposure.	Rabelo *et al*., 2020

Spodoptera eridania (Lepidoptera)	L	Bifenthrin	(D) Ingestion	Reduced sensitivity to bifenthrin after initial sublethal exposure.	Rabelo *et al*., 2020
Spodoptera exigua (Lepidoptera)	L	Bifenthrin	(D) Ingestion	Reduced sensitivity to bifenthrin after initial sublethal exposure.	Rabelo *et al*., 2020
Chloridea virescens (Lepidoptera)	L	Bifenthrin	(D) Ingestion	Reduced sensitivity to bifenthrin after initial sublethal exposure.	Rabelo *et al*., 2020
Cydia pomonella (Lepidoptera)	L	Lambda-Cyhalothrin	(D) Topical	Developed resistance via target site insensitivity.	Soleño *et al*., 2020
Helicoverpa armigera (Lepidoptera)	L	Cypermethrin	(D) Ingestion (I) Inherited	Parents were exposed and offspring had inherited resistance, even to doses tested to be lethal to susceptible strains.	Achaleke and Brévault, 2010
Plutella xylostella (Lepidoptera)	L	Permethrin	(D) Ingestion (I) Inherited	Females exposed chose not to oviposit on sites exposed to the pyrethroid, but offspring on these exposed sites had a significantly lower growth rate, fecundity and higher mortality under laboratory conditions.	Jallow and Hoy, 2005
Bicyclus anynana (Lepidoptera)	L	Deltamethrin	(D) Topical	Field realistic doses caused interference to movement speed and distance, compared to controls.	Ferguson, 2020

Table A 2: Composition of recorded direct and indirect effects of neonicotinoids on insect development, fitness, behaviour, hatching success, reproductive output, and survival. Stage: E= egg, L= larva P = pupa, A = adult. Exposure: D= direct exposure, I = indirect exposure.

Species (Order)	Stage of exposure	Neonicotinoid	Exposure (D/I)	Effect	Citation
Coleomegilla maculate (Coleoptera)	A	Imidacloprid	(D) Ingestion	Sublethal exposure resulted in high mortality in adults.	Krischik *et al*., 2015
Harmonia axyridis (Coleoptera)	A	Imidacloprid	(D) Ingestion	Sublethal exposure resulted in high mortality in adults.	Krischik *et al*., 2015
Hippodamia convergens (Coleoptera)	A	Imidacloprid	(D) Ingestion	Sublethal exposure resulted in high mortality in adults.	Krischik *et al*., 2015
Harmonia axyridis (Coleoptera)	A	Imidacloprid	(D) Topical, Ingestion (I) Inherited	Reduced the longevity, female fecundity, fertility and ovipositing, but offspring larval hatching success was significantly reduced.	Dai *et al*., 2021
Harmonia axyridis (Coleoptera)	A	Thiamethoxam	(I) Inherited	Prolonged pupal development time in the F_1 generation.	Sâmia *et al*., 2019
Coccinella septempunctata (Coleoptera)	A	Imidacloprid	(D) Topical (I) Inherited	LC_5 doses of imidacloprid caused nearly 29% reduced longevity and over 56% reduced fertility. Yet the effects also carried into their offspring, as development time was nearly 1.5 days slower than controls but with a shorter oviposition period of 10-13 days respectively.Offspring also had a reduced fecundity.	Xiao *et al*., 2016
Drosophila.spp (Diptera)	A	Imidacloprid, Clothianidin, Thiamethoxam, Thiacloprid	(D) Topical	Long-term alterations to their behaviour after being exposed to many field-relevant doses. Disruption to sleeping behaviour, circadian rhythms, and impairment to their memory.	Tasman *et al*., 2021
Apis mellifera (Hymenoptera)	A	Thiamethoxam	(D) Chronic	Some field-realistic doses of neonicotinoids had no overall effect on colony survival, yet chronic high-dosages would decreases overwinter survival of colonies.	Wood *et al*., 2019

Osmia bicornis (Hymenoptera)	A	Clothianidin, Thiamethoxam	(D) chronic and dietary exposure (I) Inherited	50% reduction of egg clutch size and markedly a significant male bias of offspring sex ration that did survive.	Sandrock *et al*., 2014
Apis mellifera (Hymenoptera)	A	Imidacloprid	(D) Ingestion	Reduced egg laying ability and movement ability in the queen, with workers having impaired foraging and hygienic activities, therefore resulting in an adverse colony development effects, such as lower brood production and pollen stores needed for food	Wu-Smart and Spivak, 2016
Scaptotrigona aff. Depilis (Hymenoptera)	L	Thiamethoxam	(D) Ingestion	Reduced survival in all exposed, larval development times were accelerated whilst pupal development times were longer.	Rosa *et al*., 2016
Podisus maculiventris (Hemiptera)	A	Imidacloprid	(D) Topical	Adults exposed, had increased fecundity but with no consequence to fertility or offspring survival.	Rix and Cutler, 2020
Euschistus heros (Hemiptera)	A	Imidacloprid	(D) Topical	Reproductive output comparable to untreated individuals, yet saw a significantly shortened longevity.	Santos *et al*., 2016
Podisus maculiventris (Hemiptera)	L (Nymph)	Imidacloprid	(D) Topical (I) Inherited	Nymphs exposed had increased reproduction with negative consequences to their fertility and offspring survival as adults.	Rix and Cutler, 2020
Aphis gossypii (Hemiptera)	A	Nitenpyram	(D) Ingestion (I) Inherited	LC_{10} and LC_{50} doses resulted in reduced parent fecundity and longevity, with their offspring having an increased fecundity compared to control groups. It was speculated that the offspring could have a higher fertility, yet was still to be tested.	Wang *et al*., 2017
Chrysoperla externa (Neuroptera)	A	Thiamethoxam	(D) Ingestion (I) Inherited	Egg fertility and offspring survival in P_0 to F_1 was reduced.	Sâmia *et al*., 2019
Danaus plexippus (Lepidoptera)	L	Imidacloprid	(D) Ingestion	Sublethal exposure resulted in high mortality in larvae. Yet adult exposure did not cause any mortality.	Krischik *et al*., 2015
Vanessa cardui (Lepidoptera)	L	Imidacloprid	(D) Ingestion	Sublethal exposure resulted in high mortality in larvae. Yet adult exposure did not cause any mortality.	Krischik *et al*., 2015
Danaus plexippus (Lepidoptera)	L	Imidacloprid, Clothianidin, Thiamethoxam	(D) Ingestion	Ingested LC_{50} doses of neonicotinoids had arrested larval ecdysis, greatly impacting their development time and survival compared to no-treatment controls.	Krishnan *et al*.,2021b
Phthorimaea operculella (Lepidoptera)	E	Thiacloprid	(D) Topical	Topical exposure to the eggs resulted in reduced larval and adult emergence 14 days after application. Even in	Saour, 2008

				cases where treated larvae were then fed on untreated potato tubers, their survival still decreased.	
Danaus plexippus (Lepidoptera)	E	Imidacloprid, Clothianidin, Thiamethoxam	(D) Topical	All three neonicotinoids had high lethality to egg survival after topical exposure.	Krishnan *et al*., 2021a
Danaus plexippus (Lepidoptera)	L	Imidacloprid, Clothianidin, Thiamethoxam	(D) Topical	Imidacloprid and clothianidin had high toxicity to larvae and was observed to arrest ecdysis.	Krishnan *et al*., 2021b
Danaus plexippus (Lepidoptera)	P	Imidacloprid, Clothianidin, Thiamethoxam	(D) Topical	Pupae that were topically exposed on the spiracles, had 100% eclosion.	Krishnan *et al*., 2021a
Danaus plexippus (Lepidoptera)	A	Imidacloprid, Clothianidin, Thiamethoxam	(D) Ingested	Only imidacloprid caused 5% mortality after consumption.	Krishnan *et al*., 2021a
Helicoverpa armigera (Lepidoptera)	L	Imidacloprid	(D) Ingested (I) Inherited	Survivorship (and increased pupal development) was reduced in parents when exposed to LC_{50} doses of imidacloprid, which resulted in reduced offspring fecundity.	Ahmad *et al*., 2013
Polyommatus Icarus (Lepidoptera)	L	Clothianidin	(D) Ingested	Reduced larval growth for first 9 days after ingesting sublethal doses of clothianidin.	Basley and Goulson, 2018
Helicoverpa zea (Lepidoptera)	L	Imidacloprid	(D) Topical	High susceptibility to imidacloprid causing arrested pupal ecdysis. Adults that did manage to fully develop were observed to have additional unexplained appendages after eclosion to adults.	Krishnan *et al*., 2021b
Galleria mellonella (Lepidoptera)	L	Imidacloprid	(D) Topical	High susceptibility to imidacloprid causing arrested pupal ecdysis.	Krishnan *et al*., 2021b
Vanessa cardui (Lepidoptera)	L	Imidacloprid	(D) Topical	Displayed low sensitivity to imidacloprid after exposure.	Krishnan *et al*., 2021b
Vanessa atalanta (Lepidoptera)	L	Imidacloprid	(D) Topical	Displayed low sensitivity to imidacloprid after exposure.	Krishnan *et al*., 2021b
Danaus plexippus (Lepidoptera)	L	Clothianidin	(D) Ingested	Toxicity of $LC_{10, 50, 90}$ were observed to reduce larval development time, shorter body length and weight.	Pecenka and Lundgren, 2015
Danaus plexippus (Lepidoptera)	L	Imidacloprid	(D) Ingested	Larvae fed on imidacloprid treated leaves developed into adults roughly 5% smaller than their controls.	Kobiela and Snell-Rood, 2020
Pieris rapae (Lepidoptera)	L	Imidacloprid	(D) Ingested (I) Inherited	Extended development time and smaller body sizes in some offspring from exposed mothers, suggesting a maternally effected development after exposure.	Kobiela and Snell-Rood, 2020

Grapholita molesta (Lepidoptera)	A	Thiacloprid	(D) Topical	Disrupted navigation (i.e. slower flight in males, more susceptible to wind drift) after topical exposure.	Navarro-Roldán *et al*., 2019
Agrotis ipsilon (Lepidoptera)	A	Clothianidin	(D) Topical	Endured biphasic effects on pheromone-guided behaviour after exposure	Rabhi *et al*., 2014
Cydia pomonella (Lepidoptera)	A	Thiacloprid	(D) Topical	Altered adult pheromone production and calling behaviour, and therefore having a potential to alter mating behaviour, chemical communication and circadian rhythm.	Navarro-Roldán and Gemeno, 2017
Grapholita molesta (Lepidoptera)	A	Thiacloprid	(D) Topical	Altered adult pheromone production and calling behaviour, and therefore having a potential to alter mating behaviour, chemical communication and circadian rhythm.	Navarro-Roldán and Gemeno, 2017
Lobesia botrana (Lepidoptera)	A	Thiacloprid	(D) Topical	Altered adult pheromone production and calling behaviour, and therefore having a potential to alter mating behaviour, chemical communication and circadian rhythm.	Navarro-Roldán and Gemeno, 2017
Danaus plexippus (Lepidoptera)	A	Clothianidin	(D) Topical	Clothianidin did not affect flight ability or theoretical migration of the species.	Wilcox *et al*., 2021
Chrysodeixis includes (Lepidoptera)	L	Thiamethoxam	(D) Ingested	Thiamethoxam had a deterrent effect after exposure, showing that larvae preferred consuming untreated matter.	Lee and Davis, 2023
Bicyclus anynana (Lepidoptera)	L	Thiacloprid	(D) Topical	Field realistic doses caused interference to movement speed and distance, compared to controls.	Ferguson, 2020

Table A 3: LC_{50} doses of pyrethroids and neonicotinoids in insect models, and converted into (ng/µl)/g of *Bicyclus anynana* bodyweight. Average calculated bodyweight of male *B. anynana* is 0.0182 g (S.D. 0.0073, S.E. 0.0020, N=15) and 0.0573 g (S.D. 0.0147, S.E. 0.0039, N=15) in females. [a]= Field application or field sample dose.

Insecticide	**LC_{50} Dose (ng/µl)**	**Dose concentration (ng/g adult)**	**Species**	**Age**	**Reference**
Deltamethrin	0.0004	0.0111	*Lucilia cuprina*	Adult	Oryon *et al*., 1992
Deltamethrin	0.073	0.057	*Aedes aegypti*	Adult	Sanchez-Arroyo, 2021
Deltamethrin	1		*Plutella xylostella*	Larvae	Agboyi *et al*., 2008
Deltamethrin	2.25		*Heliothis virescens*	Larvae	Sayyed *et al*., 2008
Deltamethrin	4.867		*Drosophila melanogaster*	Adult	Aljedani, 2021
Deltamethrin	6.19		*Spodoptera exigua*	Larvae	Ishtiaq *et al*., 2012
Deltamethrin	12.5		*Field sample from Thrips tabaci populations*	Adult	Foster *et al*., 2010
Deltamethrin	15.084	0.013	*Apis mellifera*	Adult	Sanchez-Arroyo, 2021
a Deltamethrin	19.8		*Crop application dose*	N/A	Martin *et al*., 2003
Deltamethrin	29		*Spodoptera exigua*	Larvae	Ishtiaq and Saleem, 2011
Deltamethrin	85.1		*Callosobruchus maculates*	Adult	Fouad and Abotaleb, 2021
Deltamethrin	100		*Aedes aegypti*	Adult	Alvarez *et al*., 2013
Deltamethrin	170		*Rhynchophorus palmarum*	Adult	Martínez *et al*., 2019
Deltamethrin	345		*Spodoptera litura*	Larvae	Bhatti *et al*., 2013
Deltamethrin	3580		*Spodoptera frugiperda*	Larvae	Vinha *et al*., 2021
a Deltamethrin	500000		*Crop application dose*	N/A	Decourtye *et al*., 2004 (Based on unpublished data)
Cypermethrin	0.00008		*Baetis rhodani*	Nymph	Crowley *et al*., 2021
a Cypermethrin	0.71		*Field sample*	N/A	Sedaghati and Hokmabadi, 2014
Cypermethrin	0.803		*Spodoptera frugiperda*	Larvae	Idrees *et al*., 2022
Cypermethrin	2.038		*Phauda flammans*	Larvae	Huang *et al*., 2019
a Cypermethrin	3.11		*Field sample*	N/A	Singh *et al*., 2015
Cypermethrin	8.57		*Meteorus pulchricornis*	Adult	Sheng *et al*., 2019
Cypermethrin	8.91	4.1	*Plutella xylostella*	Larvae	Abro and Wright, 1989
Cypermethrin	12.83		*Spodoptera litura*	Larvae	Sreelakshmi *et al*., 2017
Cypermethrin	63.33		*Amsacta albistriga*	Larvae	Narayanan *et al*., 2020

Cypermethrin	100		*Spodoptera litura*	Larvae	Karuppaiah *et al*., 2017
Cypermethrin	146		*Spodoptera exigua*	Larvae	Saeed *et al*., 2012
Cypermethrin	210		*Spodoptera litura*	Larvae	Karuppaiah *et al*., 2017
Cypermethrin	220		*Spodoptera litura*	Larvae	Karuppaiah *et al*., 2017
Cypermethrin	277.67		*Helicoverpa armigera*	Larvae	Shinde and Kamtikar, 2011
a Cypermethrin	1000		*Field application dose*	N/A	Singh *et al*., 2015
a Thiacloprid	0.0255		*Field sample*	N/A	Li *et al*., 2022
a Thiacloprid	0.457		*Field sample*	N/A	Li *et al*., 2022
Thiacloprid	2		*Grapholita lobarzewskii*	Larvae	Charmillot *et al*., 2007
Thiacloprid	2.335		*Strategus aloeus*	Adult	Martínez *et al*., 2014
Thiacloprid	2.99		*Bemisia tabaci*	Adult	Saleem *et al*., 2022
a Thiacloprid	3.88		*Field sample*	N/A	Alkassab *et al*., 2020
Thiacloprid	6.6		*Cydia pomonella*	Larvae	Grigg McGuffin, 2011
a Thiacloprid	10		*Field sample*	N/A	Dong *et al*., 2014
Thiacloprid	15.3		*Habrobracon hebetor*	Adult	Fooladi and Ghajarieh, 2015
Thiacloprid	20.49		*Coccinella novemnotata*	Adult	Abdu, 2010
Thiacloprid	75.26		*Trichogramma japonicum*	Adult	Zhao *et al*., 2012
a Thiacloprid	188.6		*Field application dose*	N/A	Mörtl *et al*., 2020
Thiacloprid	293.92		*Tuta absoluta*	Larvae	Taleh *et al*., 2021
Thiacloprid	329.376		*Helicoverpa armigera*	Larvae	Vojoudi and Saber, 2014
a Imidacloprid	0.01		*Field sample*	N/A	Whitehorn *et al*., 2018
a Imidacloprid	0.2		*Field sample*	N/A	Whitehorn *et al*., 2018
a Imidacloprid	0.59		*Field sample*	N/A	Jie *et al*., 2021
a Imidacloprid	2.25		*Field sample*	N/A	Jie *et al*., 2021
Imidacloprid	3	30	*Danaus plexippus*	Larvae	Krishnan., 2021
a Imidacloprid	5		*Field sample*	N/A	Zhang *et al*., 2022
b Imidacloprid	6.7	5.678	*Danaus plexippus*	Larvae	Krishnan, 2021
Imidacloprid	246		*Aphytis melinus*	Adult	Prabhaker *et al*., 2011
a Imidacloprid	250		*Corn crop application dose*	N/A	Yue *et al*., 2003
Imidacloprid	258.75		*Spodoptera litura*	Larvae	Rehan *et al*., 2011
Imidacloprid	286.54		*Spodoptera litura*	Larvae	Ahmed, 2014

a Imidacloprid	500		*Corn crop application dose*	N/A	Yue *et al*., 2003
Imidacloprid	980		*Encarsia formosa*	Adult	Prabhaker *et al*., 2011
Imidacloprid	1930		*Eretmocerus eremicus*	Adult	Prabhaker *et al*., 2011
Imidacloprid	2630		*Gonatocerus ashmeadi*	Adult	Prabhaker *et al*., 2011
Imidacloprid	5180		*Geocoris punctipes*	Adult	Prabhaker *et al*., 2011
Imidacloprid	2780		*Orius insidiosus*	Adult	Prabhaker *et al*., 2011
Imidacloprid	7825.98		*Spodoptera litura*	Larvae	Ahmed, 2014
Imidacloprid	240000		*Leptinotarsa decemlineata*	Larvae	Alyokhin *et al*., 2007
Thiamethoxam	N/A	1.22	*Aphis gossypii*	Adult	Marques *et al*., 2019
Thiamethoxam	0.88		*Epitrix fuscula*	Adult	McLeod *et al*., 2002
Thiamethoxam	4.28		*Apis mellifera*	Adults	Oliveira *et al*., 2014
Thiamethoxam	6.1		*Danaus plexippus*	Larvae	Krishnan, 2021
Thiamethoxam	35		*Danaus plexippus*	Larvae	Krishnan, 2021
Thiamethoxam	24.58		*Blissus occiduus*	Adult	Stamm *et al*., 2011
Thiamethoxam	105		*Aphytis melinus*	Adult	Prabhaker *et al*., 2011
a Thiamethoxam	250		*Field sample*	N/A	Annamalai *et al*., 2018
Thiamethoxam	397		*Encarsia formosa*	Adult	Prabhaker *et al*., 2011
Thiamethoxam	1010		*Eretmocerus eremicus*	Adult	Prabhaker *et al*., 2011
Thiamethoxam	1440		*Gonatocerus ashmeadi*	Adult	Prabhaker *et al*., 2011
Thiamethoxam	1670		*Orius insidiosus*	Adult	Prabhaker *et al*., 2011
Thiamethoxam	2170		*Geocoris punctipes*	Adult	Prabhaker *et al*., 2011
Thiamethoxam	240000		*Leptinotarsa decemlineata*	Larvae	Alyokhin *et al*., 2007
a Thiamethoxam	6200000		*Field application dose*	N/A	Senthil-Nathan, 2013
a Thiamethoxam	7000000		*Field application dose*	N/A	Senthil-Nathan, 2013

Calculated LC_{50}values of male and female *B. anynana*

Table A 4: LC_{50} values of male and female *Bicyclus anynana* exposed to deltamethrin over different time intervals.

Time (day)	Male LC_{50} (ng/µl)	ng/g male	Female LC_{50} (ng/µl)	ng/g female
1	2.68	441.59	N/A	N/A
2	2.28	375.66	N/A	N/A
3	2.31	380.44	N/A	N/A
4	2.32	382.09	3.20	50.86

Table A 5: LC_{50} values of male and female *B.anynana* exposed to cypermethrin over different time intervals.

Time (day)	Male LC_{50} (ng/µl)	ng/g male	Female LC_{50} (ng/µl)	ng/g female
8	4.02	663.13	4.35	227.59
9	1.27	208.68	4.05	211.88
10	0.07	10.77	3.16	165.34

Table A 6: LC_{50} values of male and female *B. anynana* exposed to thiacloprid over different intervals.

Time (day)	Male LC_{50} (ng/µl)	ng/g male	Female LC_{50} (ng/µl)	ng/g female
8	4.90	808.19	N/A	N/A
9	4.26	702.20	5.13	268.32
10	1.33	219.73	4.82	252.57

Table A 7: LC_{50} values of male and female *B. anynana* exposed to imidacloprid over different intervals.

Time (day)	Male LC_{50} (ng/µl)	ng/g male	Female LC_{50} (ng/µl)	ng/g female
1	~1932	318461.55	~1250	65445.03
2	339.30	55928.57	N/A	N/A
3	~237.50	39148.35	N/A	N/A
4	N/A	N/A	49.97	2616.23
8	4.85	800.11	N/A	N/A
10	0.64	105.07	4.92	257.64

Table A 8: LC_{50} values of male and female *B. anynana* exposed to thiamethoxam over different intervals.

Time (day)	Male LC_{50} (ng/µl)	ng/g male	Female LC_{50} (ng/µl)	ng/g female
1	~1062.00	175054.95	~1642	85968.59
2	~320.00	52747.25	~467	24450.26
3	N/A	N/A	387.50	28507.85
4	N/A	N/A	~334.40	23518.32
9	4.65	766.15	4.92	257.43
10	~0.23	43.76	4.58	240.00

Table A 9: Maternal wing measurements of *B. anynana* among each treatment group. Averages were calculated from 15 females per treatment group.

Treatment groups	Average wing length (mm)	S.D.	S.E.
No treatment	20.23	0.63	0.32
Acetone	19.90	0.56	0.29
Cypermethrin	20.43	0.69	0.35
No treatment	20.20	0.50	0.25
Acetone	20.50	0.57	0.30
Deltamethrin	20.62	0.41	0.21
No treatment	20.07	0.57	0.28
Acetone	20.23	0.44	0.22
Thiacloprid	20.43	0.40	0.20
No treatment	20.55	0.34	0.17
Acetone	20.77	0.45	0.23
Imidacloprid	20.30	0.48	0.24
No treatment	20.21	0.48	0.24
Acetone	20.74	0.48	0.24
Thiamethoxam	20.44	0.50	0.25

Table A 10: Variance calculations between wing measurements. TEM = technical error of mean; SEM = standard error of mean; R^2 = Coefficient of determination.

	Average wing length (mm)
TEM	0.64
S.D.	2.06
SEM	0.14
R^2	99.97

Table A 11: Comparative analysis of reproductive output, hatching success, and longevity data between adults (and offspring) exposed to deltamethrin and their respective controls.

	No treatment (control)	Acetone (control)	Deltamethrin
Average development time (days)	5.05	5.67	5.79
S.D.	0.24	0.49	0.62
S.E.	0.08	0.13	0.16
Average egg size (mm^2)	0.709	0.683	0.683
S.D.	0.018	0.021	0.050
S.E.	0.005	0.006	0.013
Average mother longevity (days)	23.33	20.80	10.53
S.D.	4.61	6.19	7.52
S.E.	1.19	1.60	1.94
Average daily output of eggs	45.30	47.50	13.40
S.D.	15.19	20.25	5.90
S.E.	3.92	5.23	1.52
Total number of eggs per treatment groups	680	712	201
S.D.	15.19	20.25	5.90
S.E.	3.92	5.23	1.52
Total number of eggs hatched per treatment group	542	567	101
S.D.	14.98	16.01	3.55
S.E.	3.87	4.14	0.92
Number of eggs hatched per cage	180.67	189.00	33.67
S.D.	12.06	8.72	9.50
S.E.	6.96	5.03	5.49

Table A 12: Comparative analysis of behavioural assays between successful larvae whose mothers were exposed to deltamethrin and their respective controls.

Time (min)				
Treatment group	Mean	Median	S.D.	S.E.
No-treatment	28.43	25.47	9.88	2.74
Acetone	33.55	25.47	9.88	2.74
Deltamethrin	39.10	41.03	11.96	5.35
Distance (cm)				
Treatment group	Mean	Median	S.D.	S.E.
No-treatment	44.39	44.70	2.18	0.60
Acetone	44.61	43.65	2.53	0.80
Deltamethrin	51.80	51.80	6.08	4.30
Weight (g)				
Treatment group	Mean	Median	S.D.	S.E.
No-treatment	0.049	0.046	0.021	0.006
Acetone	0.058	0.060	0.019	0.006
Deltamethrin	0.039	0.039	0.012	0.008

Table A 13: Comparative analysis of behavioural assays between all larvae whose mothers were exposed to deltamethrin and their respective controls.

Time (min)				
Treatment group	Mean	Median	S.D.	S.E.
No-treatment	28.43	25.47	9.88	2.74
Acetone	33.55	25.47	9.88	2.74
Deltamethrin	39.10	41.03	11.96	5.35
Distance (cm)				
Treatment group	Mean	Median	S.D.	S.E.
No-treatment	36.46	43.75	17.18	4.30
Acetone	30.19	43.05	19.65	4.91
Deltamethrin	21.08	10.90	20.47	5.12
Weight (g)				
Treatment group	Mean	Median	S.D.	S.E.
No-treatment	0.052	0.050	0.022	0.006
Acetone	0.059	0.063	0.020	0.005
Deltamethrin	0.051	0.051	0.015	0.004

Table A 14: Comparative analysis of reproductive output, hatching success, and longevity data between adults (and offspring) exposed to cypermethrin and their respective controls.

	No treatment (control)	Acetone (control)	Cypermethrin
Average development time (days)	5.97	6.21	5.79
S.D.	0.24	0.46	0.24
S.E.	0.08	0.12	0.06
Average egg size (mm^2)	0.667	0.656	0.703
S.D.	0.024	0.034	0.038
S.E.	0.006	0.009	0.010
Average mother longevity (days)	21.73	19.33	18.67
S.D.	4.61	7.18	5.05
S.E.	1.19	1.85	1.30
Average daily output of eggs	42.00	32.10	39.60
S.D.	17.29	20.01	19.96
S.E.	4.46	5.17	5.15

Total number of eggs per treatment groups	630	481	594
S.D.	17.29	20.01	19.96
S.E.	4.46	5.17	5.15
Total hatched eggs per treatment group cage	491	367	388
S.D.	17.97	13.29	13.72
S.E.	4.36	3.13	3.07
Number of eggs hatched per cage	163.67	122.33	129.33
S.D.	60.87	42.03	43.02
S.E.	35.14	24.26	24.84

Table A 15: Comparative analysis of behavioural assays between successful larvae whose mothers were exposed to cypermethrin and their respective controls.

Time (min)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	31.20	28.52	8.33	2.512
Acetone	34.90	28.52	13.30	7.68
Cypermethrin	34.90	29.11	13.30	7.68
Distance (cm)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	38.43	44.85	18.27	4.57
Acetone	44.72	44.30	2.35	0.71
Cypermethrin	51.80	51.80	1.13	0.80
Weight (g)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	0.070	0.039	0.014	0.004
Acetone	0.040	0.080	0.018	0.005
Cypermethrin	0.073	0.079	0.017	0.004

Table A 16: Comparative analysis of behavioural assays between all larvae whose mothers were exposed to cypermethrin and their respective controls.

Time (min)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	31.35	30.21	6.965	2.011
Acetone	31.20	30.21	8.713	2.755
Cypermethrin	34.90	29.11	13.297	7.677
Distance (cm)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	38.43	44.85	18.273	4.568
Acetone	34.32	43.95	15.682	4.191
Cypermethrin	14.48	5.75	17.796	4.449
Weight (g)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	0.0702	0.0707	0.019	0.005
Acetone	0.0402	0.0388	0.010	0.003
Cypermethrin	0.0731	0.0788	0.018	0.004

Table A 17: Comparative analysis of reproductive output, hatching success, and longevity data between adults (and offspring) exposed to thiacloprid and their respective controls.

	No treatment (control)	Acetone (control)	Thiacloprid
Average development time (days)	5.79	5.50	5.64
S.D.	1.77	0.54	0.68
S.E.	0.46	0.14	0.18
Average egg size (mm^2)	0.663	0.672	0.678
S.D.	0.026	0.035	0.051
S.E.	0.007	0.009	0.013
Average mother longevity (days)	12.10	9.20	7.50
S.D.	4.99	4.59	4.81
S.E.	1.29	1.18	1.24
Average daily output of eggs	16.90	14.30	17.30
S.D.	4.87	3.58	5.68
S.E.	1.54	0.92	1.47
Total number of eggs per treatment groups	1080	1002	1123
S.D.	31.45	15.00	21.85
S.E.	8.12	3.87	5.64
Total number of hatched eggs per treatment group	484	562	667
S.D.	15.07	11.74	14.09
S.E.	2.13	1.36	1.63
Number of eggs hatched per cage	242.00	187.33	222.33
S.D.	69.30	62.18	38.18
S.E.	49.00	35.90	22.04

Table A 18: Comparative analysis of behavioural assays between successful larvae whose mothers were exposed to thiacloprid and their respective controls.

Time (min)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	26.33	25.41	0.006	0.002
Acetone	25.53	25.53	0.006	0.006
Thiacloprid	26.08	26.08	0.008	0.003
Distance (cm)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	43.39	42.00	3.37	1.02
Acetone	43.08	42.30	2.11	0.56
Thiacloprid	43.63	43.15	2.06	0.84
Weight (g)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	0.053	0.051	0.020	0.006
Acetone	0.031	0.029	0.008	0.002
Thiacloprid	0.027	0.026	0.007	0.003

Table A 19: Comparative analysis of behavioural assays between all larvae whose mothers were exposed to thiacloprid and their respective controls.

Time (min)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	26.33	25.41	0.006	0.002
Acetone	25.53	25.53	0.006	0.002
Thiacloprid	26.08	26.08	0.008	0.003
Distance (cm)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	35.86	41.70	12.26	3.06
Acetone	41.17	42.30	7.41	1.98
Thiacloprid	23.36	19.90	17.49	4.37
Weight (g)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	0.057	0.052	0.021	0.005
Acetone	0.030	0.029	0.008	0.002
Thiacloprid	0.044	0.033	0.028	0.007

Table A 20: Comparative analysis of reproductive output, hatching success, and longevity data between adults (and offspring) exposed to imidacloprid and their respective controls.

	No treatment (control)	Acetone (control)	Imidacloprid
Average development time (days)	5.47	5.69	5.43
S.D.	0.37	0.65	7.63
S.E.	0.17	0.17	1.97
Average egg size (mm^2)	0.655	0.649	0.690
S.D.	0.031	0.175	0.035
S.E.	0.008	0.045	0.009
Average mother longevity (days)	18.40	18.40	16.40
S.D.	4.67	4.37	6.06
S.E.	1.21	1.13	1.56
Average daily output of eggs	73.70	85.20	63.20
S.D.	16.99	26.21	30.43
S.E.	4.39	6.77	7.89
Total number of eggs per treatment groups	1105	1278	948
S.D.	16.99	26.21	30.43
S.E.	4.39	6.77	7.86
Total number of hatched eggs per treatment group	638	629	252
S.D.	11.47	17.13	8.10
S.E.	1.97	3.18	1.51
Number of eggs hatched per cage	212.67	209.67	84.00
S.D.	63.69	28.57	33.05
S.E.	36.77	16.50	19.08

Table A 21: Comparative analysis of behavioural assays between successful larvae whose mothers were exposed to imidacloprid and their respective controls.

Time (min)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	28.02	24.23	0.008	0.002
Acetone	35.54	35.06	0.007	0.002
Imidacloprid	28.24	25.39	0.007	0.002
Distance (cm)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	48.38	48.10	4.07	1.18
Acetone	57.10	52.85	15.91	5.03
Imidacloprid	46.80	47.80	3.08	0.98
Weight (g)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	0.061	0.055	0.021	0.006
Acetone	0.055	0.056	0.011	0.004
Imidacloprid	0.063	0.056	0.022	0.007

Table A 22: Comparative analysis of behavioural assays between all larvae whose mothers were exposed to imidacloprid and their respective controls.

Time (min)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	28.02	24.23	0.008	0.002
Acetone	35.54	35.06	0.007	0.002
Imidacloprid	28.24	25.39	0.007	0.002
Distance (cm)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	40.70	46.45	15.28	3.82
Acetone	39.15	44.00	26.25	7.02
Imidacloprid	35.31	43.20	16.70	4.17
Weight (g)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	0.066	0.066	0.021	0.005
Acetone	0.059	0.057	0.019	0.005
Imidacloprid	0.065	0.070	0.021	0.005

Table A 23: Comparative analysis of reproductive output, hatching success, and longevity data between adults (and offspring) exposed to thiamethoxam and their respective controls.

	No treatment (control)	Acetone (control)	Thiamethoxam
Average development time (days)	5.71	5.65	5.92
S.D.	0.41	0.44	0.32
S.E.	0.13	0.11	0.08
Average egg size (mm^2)	0.675	0.667	0.671
S.D.	0.032	0.041	0.030
S.E.	0.008	0.012	0.008
Average mother longevity (days)	14.93	16.07	15.00
S.D.	6.16	5.05	5.46
S.E.	1.59	1.30	1.41
Average daily output of eggs	49.30	69.10	58.70
S.D.	22.52	27.33	27.66
S.E.	5.81	7.06	7.14
Total number of eggs per treatment groups	740	1037	880
S.D.	22.52	27.33	27.66
S.E.	5.81	7.06	7.14
Total number of hatched eggs per treatment group	596	770	589
S.D.	23.35	27.11	19.90
S.E.	6.03	7.0	5.14
Number of eggs hatched per cage	198.67	256.67	196.33
S.D.	79.53	15.95	15.04
S.E.	45.91	9.21	8.69

Table A 24: Comparative analysis of behavioural assays between successful larvae whose mothers were exposed to thiamethoxam and their respective controls.

Time (min)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	28.47	29.36	9.084	2.519
Acetone	35.39	29.36	7.237	2.559
Thiamethoxam	35.44	34.55	7.703	3.445
Distance (cm)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	46.43	44.5	3.618	1.004
Acetone	46.19	45.2	2.350	0.743
Thiamethoxam	50.68	47.6	6.097	2.727
Weight (g)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	0.056	0.044	0.030	0.008
Acetone	0.061	0.061	0.014	0.005
Thiamethoxam	0.067	0.067	0.027	0.012

Table A 25: Comparative analysis of behavioural assays between all larvae whose mothers were exposed to thiamethoxam and their respective controls.

Time (min)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	28.47	29.36	9.08	2.52
Acetone	35.39	29.36	7.24	2.56
Thiamethoxam	35.44	34.55	7.70	3.44
Distance (cm)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	38.29	44.40	17.82	4.45
Acetone	32.11	44.25	19.45	4.86
Thiamethoxam	26.51	20.05	20.44	5.11
Weight (g)				
Treatment group	**mean**	**Median**	**S.D.**	**S.E.**
No-Treat	0.056	0.047	0.028	0.007
Acetone	0.064	0.064	0.021	0.813
Thiamethoxam	0.087	0.064	0.021	0.059