Influence of Insecticides on the Partner and Host Finding of Parasitoid Wasps

DISSERTATION ZUR ERLANGUNG DES DOKTORGRADES DER NATURWISSENSCHAFTEN (DR. RER. NAT.) DER FAKULTÄT FÜR BIOLOGIE UND VORKLINISCHE MEDIZIN DER UNIVERSITÄT REGENSBURG

vorgelegt von

Nils Hauke Schöfer

aus

Witten, Deutschland

im Jahre

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Abstract

Understanding how the utilisation of pesticides in contemporary agriculture affects the wider biosphere is an important aspect of conservation ecology. A main focus of this area of research are pollinators and agriculturally significant species like the Western Honeybee (*Apis mellifera*). But these species cannot be used as a perfect proxy for the entire insect clade with the varying life histories of even disparate groups in the family *Hymenoptera*. As such, other species, that also play an important role in agricultural systems, are less well studied, even though they may be more exposed to pesticide usage than pollinators, due to their biology. Of these groups, the parasitoid wasps stand out as both their usefulness as natural predators of pest species and their intimate relationship with their hosts, and were studied in their susceptibility to various insecticides, ranging in their novelty.

In chapter 3, the jewel wasp *Nasonia vitripennis*, which parasitises flesh and blow fly larvae, was found to be quite susceptible to the four insecticides acetamiprid, dimethoate, flupyradifurone and sulfoxaflor. Three main parameters impacting the fitness of the wasps, their ability to find partners, to mate, and to locate hosts – each of which are dependent on the proper functioning of the olfactory system – were shown to be affected by sublethal doses of all four insecticides, though the effects varied in strength depending on which insecticide was used. In chapter 4, similar effects were seen with *Lariophagus distinguendus*, a parasitoid of numerous beetle pests, though here it was surprising that while both species are generally equivalent in size, *L. distinguendus* was far more susceptible to the four insecticides, resulting in sublethal doses as low as 0.021ng.

Likewise, the partner finding ability, both in contact and over distance, and host finding ability of *Leptopilina heterotoma* (chapter 5), were affected negatively by sublethal doses of the four insecticides. Additionally, it was found that dimethoate mixed into the feeding medium of *L. heterotoma*'s host, *Drosophila melanogaster*, still affected the all three parameters, displaying an avenue of uptake exclusive to parasitoids. Altogether, the results of this thesis contributes further to the impact of agricultural chemicals on beneficial insects.

Works arising from this thesis

The chapters in this thesis are comprised of three manuscripts (chapters 3-5). As of the time of submission, one of them is published, one is submitted and awaiting publication, and one is soon to be submitted.

A. **Schöfer, N**., Ackermann, J., Hoheneder, J., Hofferberth, J., & Ruther, J. (2023). Sublethal effects of four insecticides targeting cholinergic neurons on partner and host finding in the parasitic wasp *Nasonia vitripennis*. *Environmental Toxicology and Chemistry*, *42*(11), 2400-2411. (chapter 3)

B. **Schöfer, N**., Ratschmann, G., & Ruther, J. (2024) Effects of sub-nanogram doses of acetamiprid, dimethoate, flupyradifurone, and sulfoxaflor on courtship, mating, and olfactory host finding of the parasitic wasp *Lariophagus distinguendus*. *Entomologia Experimentalis et Applicata*. (chapter 4)

C. **Schöfer, N.**, Saxinger, N., Braumandl, K., & Ruther, J (In Press). Four Neurotoxic Insecticides Impair Partner and Host Finding in the Parasitoid *Leptopilina heterotoma* and Bioactive Doses Can Be Taken Up Via the Host. *Journal of Chemical Ecology.* (chapter 5)

Table of contents

1.Introduction

Pesticides are chemicals used to protect agricultural products, by controlling organisms of various taxa that would damage the plants or crops directly. Use of natural pesticides has been a part of agricultural practises of many civilizations throughout history (Smith & Secoy, 1975) and was vital in securing a stable supply of food and other resources. With the advent of the industrial revolution, and modern medical breakthroughs, human population growth increased rapidly and went from more than 1 billion humans at the start of the 19th century to more than 6 billion at the end of the 20th(Lutz & Qiang, 2002). In order to secure food for humanity at such as scale, more was land converted to agricultural land (Meyer & Turner, 1992). At the same time, agricultural methods were continuously refined to increasing the yield per hectare. During the 20th century, the development of synthetic pesticides allowed for more specific control of pest species to increase agricultural yield. Synthetic pesticides inhibit the proper function of enzymes, hormones or entire cells to either disrupt development, or more commonly kill the offending pests.

One of the taxonomic groups that hosts a large number of pest species is the class Insecta, hence why the development and deployment of synthetic insecticides has been a matter of major economic importance. Since the 1950s, new products have been formulated and applied yearly (Maino et al., 2023). While the mode of actions of these substances are diverse, most function by blocking or overstimulating the transmission of signals within the insect nervous system. This leads to a loss of motor control and ultimately death (Radcliffe et al., 2009). As many aspects of insect nervous systems are highly conserved within the class, insecticides are often broad spectrum, meaning they affect multiple families of insects indiscriminately and cause declines in various species besides the target (Epstein et al., 2000). Such effects on non-target organisms and other negative effects as environmental pollutants are main reasons why the widespread use of synthetic insecticides is controversial. Infamously, dichloro-diphenyl-trichloroethane (Commonly abbreviated to DDT), was one of the first modern insecticides which became popular due to its efficacy (Jarman & Ballschmiter, 2012). However, through a process known as bioaccumulation, whereby concentrations of chemicals increase within higher trophic levels (Thomann, 1989), high levels of DDT within bird populations caused their eggshells to thin (Bitman et al., 1970), leading to population declines in many species. This led to population declines in many species.

These detrimental effects on natural ecosystems ultimately led to use of DDT being outlawed, except for disease vector control.

Since DDT, numerous classes of insecticides have been patented and registered for global use. Novel insecticides are designed to be more specific to insects and to hold low risk for humans, though effects on non-target insect species can still be a significant environmental impact. Due to global insect biodiversity losses recorded in recent years, marked by decreases in biomass and increased extinction rates (Hallmann et al., 2017; Raven & Wagner, 2021), more focus has been given to the ecosystem services that insects provide. As pollinators, decomposers or natural enemies of pests, the importance of a healthy diverse insect ecosystem stands at odds to the increased use of synthetic insecticides. Even newer insecticides have been shown to be damaging to the environment and a driver of insect diversity decline (Azpiazu et al., 2019; Strobl et al., 2021), and older formulations continue to be banned from markets in an effort to lessen negative human impact on the environment.

One of the most crucial pollinator species is the western honey bee, *Apis mellifera,* which is often the main focus for legislations restricting the use of pesticides (Authority, 2016; EU Commision, 2013). Similarly, a significant amount of research has been concentrated on environmental factors affecting health of *A. mellifera* colonies, due to the myriad of ecosystem services they provide (Belzunces et al., 2012; Campbell et al., 2016). One service *A. mellifera* do not fulfil is that of a natural enemy of insect pests. Natural enemies are predators, parasitoids or parasites which, through their consumption of pests, act as biological controls on local populations (Gamliel et al., 2000). A taxa that has gain recent prominence in their role as natural enemies of several agricultural pests, are the parasitoid wasps. Owing to their parasitic nature, and some wasps strong specialisation on a narrow set of host species, parasitoid wasps populations are linked to population of their hosts. This makes them highly vulnerable to the effects of insecticide usage. Additionally, parasitic wasps are often employed in Integrated Pest Management (IPM), a management method that focusses on making use of chemical, mechanical and biological control agents to control pest populations (Radcliffe et al., 2009).

To highlight the potential lethal and sublethal effects that different insecticides can have on beneficial insects like the parasitoid wasps, as well as accentuating differences in

effect between classes of insecticides and wasp species, multiple active ingredients were tested on wasp species from various genera. The individual compounds and wasp species are discussed subsequently.

1.1 Insecticides

1.1.1 Acetamiprid
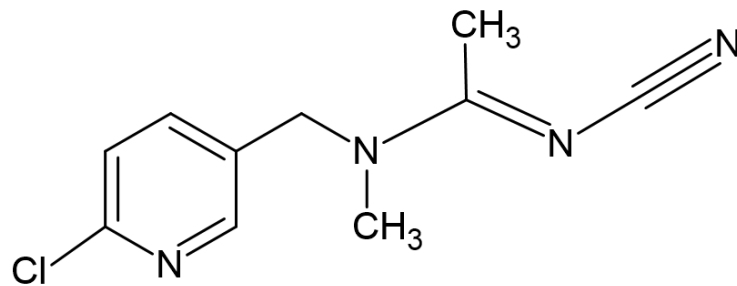
Figure 1.1. Structural formula of acetamiprid

Acetamiprid is a member of the neonicotinoid class of insecticides, popularly considered the most widely used insecticide in use against sucking insects (Klingelhöfer et al., 2022). The name derives from similarities in the chemical structure of nicotine and each of the neonicotinoids, such as the pyridine ring present in nicotine, imidacloprid and acetamiprid. Correspondingly, neonicotinoids also share an affinity for nicotinic acetylcholine receptors (nAChRs) and work as acetyl choline receptor agonists, binding to the diverse receptor subunits, resulting in overstimulation of the nerves and finally death (Brown et al., 2006). These agonists also bind more readily to insect nAChRs in comparison to mammalian nAChRs and so pose lower health risks if mammals are exposed to them (Tomizawa, 2004). Additionally, neonicotinoids act systemically, meaning that they are usually applied to the seed of the crop, and are then taken up and expressed in every part of the crop plant (Elbert et al., 2008). Imidacloprid was the first active ingredient of the group to be developed and sold in 1985 (Elbert et al., 1998), with more following throughout the 1990s and 2000s, including acetamiprid, which was registered in Japan in 1995 by Nippon Soda Co., Ltd. (Yamada, 1997).

The neonicotinoids gained infamy in recent decades when Imidacloprid and others were implicated in being a contributing factor to declining honeybee colony health (Decourtye & Devillers, 2010; Dively et al., 2015; Pohorecka et al., 2012a). Due to their ubiquitous use, it was feared that without stricter regulation bee colonies would start to

collapse, affecting the security of pollination services. As a response, the EU first restricted (EU Commision, 2013) and in 2018 banned the use of some neonicotinoids, restricting them to only greenhouse use (European Commission, 2018). There have also been partial restrictions and bans have also been implemented in the USA and Canada. As a reaction to these restrictions as yet unrestricted neonicotinoids were sold as alternatives, since active ingredients such as acetamiprid were not considered a high risk to humans or environment (Jerez et al., 2022). Moreover, acetamiprid is only a fraction as toxic in comparison to substances like imidacloprid when topically applied to *A. mellifera* thorax (Iwasa et al., 2004).

1.1.2 Dimethoate
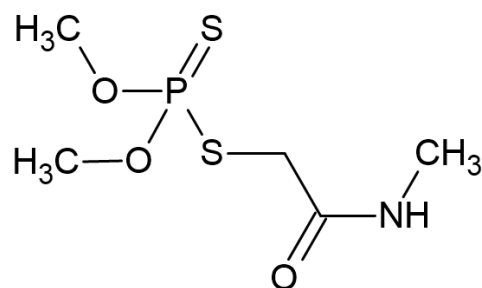
Figure 1.2. Structural formula of dimethoate

In contrast to the other active ingredients analysed in this thesis, dimethoate is a much older substance. Developed and brought to market in the 1950s, dimethoate is a member of the organophosphate class of compounds (Organization, 1989). The organophosphates are a diverse group that do not exclusively contain pesticides, but the substances that are utilized as vector control agents and function as acetylcholinesterase inhibitors (Aroniadou-Anderjaska et al., 2023). Inhibiting the proper operation of acetylcholinesterase prevents the neurotransmitter acetylcholine from being hydrolysed. This results in the overstimulation of nicotinic receptors, ultimately leading to paralysis and death (Engenheiro et al., 2005).

As the organophosphate pesticides have been deployed since the 1930s, multiple compounds have been studied for their risk to both environmental and human health. Multiple substances have been studied for their destructive effects on the pheromone communication of non-target insects (Delpuech, Froment, et al., 1998; Delpuech, Gareau, et al., 1998) and ecotoxicity in aquatic systems contaminated by pesticide runoff (Huang et al., 2020). Therefore, the use of many substances has been restricted

and organophosphates generally have been phased out, and replaced by newer class of insecticides (Hites, 2021).

Dimethoate as a substance is used as both a contact and systemic insecticide, often as a broad-spectrum control agent of various insect families, and has been used as crop protection for fruit orchards, wheat fields and vegetables (World Health Organization, 1989). Previous research on the substance has found that dimethoate is highly soluble in water, increasing its potential of leach into the surrounding environment (Broz_nić et al., 2021), as well as having comparably high contact toxicity in numerous bee species (Uhl et al., 2016). Dimethoate also was not reapproved for usage within the EU in 2019, though its use can still be allowed on the national level and it is still being utilized outside the EU (Broz_nić et al., 2021). such it is still valuable to study the effects that dimethoate as a representative of the organophosphates can have on pheromone communication. Additionally, its function as a acetylcholinesterase inhibitor in contrast to the other three tested compounds being nAChR agonists helps display potential differences in the way parasitoid wasps process the different compounds.

1.1.3 Flupyradifurone
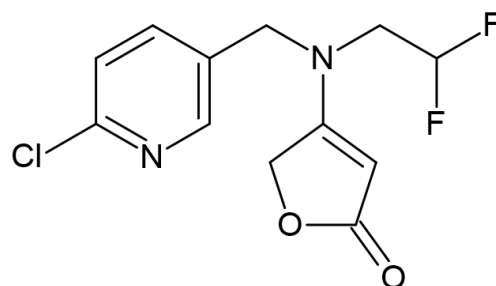
Figure 1.3. Structural formula of flupyradifurone

Flupyradifurone is the first pesticide in the butenolide class of pesticides, and was developed by Bayer CropScience. In contrast to dimethoate, flupyradifurone is a relatively novel pesticide, having been introduced to global markets in 2014 (Nauen et al., 2015). Like acetamiprid and sulfoxaflor, flupyradifurone is a nAChr agonist and has a high selectivity for postsynaptic nAChRs in insects (Jeschke et al., 2015), and thus poses lower risks for human exposure. The commercial formulations of flupyradifurone are applied to crops by foliar, drench, drip or seed treatment, depending on which pests are being protected against (Nauen et al., 2015). In the field flupyradifurone is used mainly to control damaging populations of aphids and

whiteflies. It has especially been promoted as an alternative to neonicotionoids in areas where pests have developed resistances to neonicotinoids (Tosi & Nieh, 2019).

It is able act as an alternative to neonicotinoids due to the fact that flupyradifurone has chemical similarities to the neonicotinoids, but the butenolide structure acts as an alternative pharmacophore (Jeschke et al., 2015). The compounds ecotoxicological profile, a measure of how toxic a substance is to model species of different taxa, also deemed the substance as have low ecotoxic effects, including showing a high tolerance for flupyradifurone in *A. mellifera* (Nauen et al., 2015). However, in subsequent years, a multitude of studies have analysed the effects of flupyradifurone in more detail, finding that flupyradifurone does increase oxidative stress and induce cell death in *A. mellifera* (Chakrabarti et al., 2020).

Other studies have since described lethal and sublethal effects of flupyradifurone on other non-target organisms. Studies on beneficial insects demonstrated that flupyradifurone affected pheromone profiles of solitary bees (Boff & Ayasse, 2023), reduced the survival rate and longevity of parasitoid wasps (Gao et al., 2023), and increased mortality in beetles used in IPM (Siviter & Muth, 2020). Nonetheless, despite concerns of such negative effects on the environment being raised, flupyradifurone passed peer review within the EU (Products et al., 2022), and remains unrestricted in other parts of the world.

1.1.4 Sulfoxaflor

Figure 1.4. Structural formula of sulfoxaflor
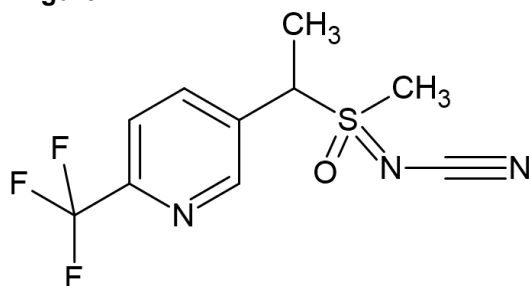
Similarly to flupyradifurone, sulfoxaflor is the first active ingredient in the novel class of insecticides, the sulfoximines. It was developed by Dow AgroSciences and first registered commercially in 2013 (Sparks et al., 2013). The sulfoximines share some structural similarities to neonicotinoids, Therefore some don't consider the sulfoxamines as a distinct class. One distinguishing feature that is used to separate the two groups is the absence of an amine nitrogen within the sulfoximines (Watson et

al., 2011), whereas the commercial neonicotinoids all contain at least one. As with acetamiprid and flupyradifurone, sulfoxaflor functions as an nAChR agonist, though it has a lower affinity for binding sites than tested neonicotioids, or even other sulfoxamines (Sparks et al., 2013). However, sulfoxaflor was more toxic to insects than other sulfoxamines, showing that other factors than simple binding site affinity are responsible for its efficacy as an insecticide (Watson et al., 2011).

Sulfoxaflor is selective for insect neuron receptors and binds more strongly, and like flupyradifurone is supported as an alternative to neonicotinoids for resistant populations (Bacci et al., 2018). The main applications for sulfoxaflor are sap-feeding aphids, whiteflies and true bugs, but it is also effective against a wide range of species (Bacci et al., 2018). In Europe, sulfoxaflor is bought for foliar spraying on cereals, cotton, greenhouse and field vegetables and other crops (European Food Safety Authority et al., 2019). In cotton especially, sulfoxaflor has seen success in controlling infestations of the tarnished plant bug (Siebert et al., 2012). Sulfoxaflor is also mentioned as being effective for IPM, as it requires fewer repeat applications and so is less likely to leach into the environment (Bacci et al., 2018).

Since its release, research has detailed a variety of negative influences that sulfoxaflor can have on non-target organisms. In bumblebees exposed to sulfoxaflor, reproductive success was lowered (Siviter et al., 2018), and parasitoid wasps were unable to parasitize host eggs properly (Jiang, Liu, Zhang, et al., 2019). In contrast, there were also results that showed no negative impact on a non-target organism (Siviter et al., 2019), showing there is still ambiguity in the impact of sulfoxaflor on non-targets. In 2022, following pressure from member states the EU restricted the outside use of sulfoxaflor (EU Commision, 2022), though exceptions within the EU exist and sulfoxaflor is still registered in many countries across the world.

1.2 Parasitic Wasps

1.2.1 *Nasonia vitripennis*

The haplodiploid parasitoid wasp *Nasonia vitripennis* is the most studied parasitoid wasp and is often used as a model organism for the clade. Part of the family Pteromalidae, *N. vitripennis* has been researched since the 1960s, though at that point it was the only species described in the genera (Raychoudhury et al., 2010). While

research on *N. vitripennis* focussed mainly on genetics in the past, the ease with which they can be reared in a laboratory setting has also made extensive behavioural research possible (Ruther et al., 2010; Schurmann et al., 2009).

The life cycle of *N. virtripennis* begins as one of multiple eggs developing with the pupae of their primary hosts, blowflies (*Calliphoridae*) and flesh flies (*Sarcophagidae*). *N. vitripennis* are gregarious and the number of eggs laid per host varies and is determined by the quality of the host (Whiting, 1967). Eggs hatch within the host pupae, after which the wasp larvae attached themselves to the host and devour it's bodily fluids (Whiting, 1967). Development time till emergence can vary depending on factors like temperature and age of the mother but at 25°C takes 14 days (Saunders, 1965). Adult males emerge earlier than females and compete for access to females in their natal host patch (A. M. Moynihan & D. M. Shuker, 2011), with females emerging shortly after. As adults, male and female *N. vitripennis* are easy to distinguish, females having fully developed wings that when relaxed reach past the end of the abdomen and males having shortened wings (Whiting, 1967). The underdeveloped wings render the males incapable of flight, meaning that males' only chance to mate is on their natal host patch.

Mating between *N. vitripennis* is mediated by pheromones. Females do not utilize a volatile sex pheromone, which is not necessary, as host patches are small (Steiner et al., 2006). At close range, males come into contact with either short trials of female pheromones or females themselves, these contact sex pheromones cause arrestment in the male, which then follow the trial to the female (Steiner et al., 2006). When the males come into contact with a female, males climb onto the back of the female and begin the mating ritual by nodding their head and stroking their mouth against the antennae of the female while concurrently vibrating their wings in short pulses (Ruther et al., 2007). If the female is receptive and the male produces enough male sex pheromone, the female will open its genital opening and the male deposits his semen (Ruther et al., 2009).

After mating, females will fly and leave the host patch, generally only flying short distances to find a novel host patch. Females orient themselves after the smell of hosts, even using their memory of the smells of their natal host patch to distinguish host odours (Schurmann et al., 2009). When females encounter suitable hosts, they assess the host quality and regulate their oviposition to lay an appropriate number of eggs

(Whiting, 1967). Being haplodiploid, fertilized *N. vitripennis* eggs develop into females, while unfertilized eggs become males (Steiner & Ruther, 2009), continuing the life cycle.

The pheromone communication of *N. vitripennis* has been studied extensively, to the point that the male pheromone has been characterised and can be produced artificially (Ruther et al., 2008). Additionally, previous research by (Tappert et al., 2017) demonstrated that the neonicotinoid imidacloprid disrupted pheromone recognition, mating behaviour and host orientation. These results provide preliminary rationale for the research presented in this thesis and has already established methodology suitable for testing pheromone communication disruption.

1.2.2 *Lariophagus distinguendus*

Lariophagus distinguendus is a haplodiploid parasitoid wasp of the family *Pteromalidae* and is a generalist, parasitizing a multitude of species of true weevils (*Curculionidae*) (Adarkwah et al., 2012). As many of the species *L. distinguendus* parasitises are themselves synanthropic species and are found on all inhabited continents, *L. distinguendus* also has a cosmopolitan distribution (Niedermayer et al., 2016). Due to its role as natural enemy of some very damaging pest species, even in the early days of research its potential as a biocontrol agent was considered (Niedermayer et al., 2016).

Unlike *N. vitripennis*, *L. distinguendus* are not gregarious and females lay a solitary egg per host (Hase, 1924), though superparasitism has been known to occur. While *L. distinguendus* can parasitise different species, the viability of the offspring does depend on the host (Steidle et al., 2006). Furthermore, while *L. distinguendus* can lay eggs on hosts in both the final two larval instars and the pupal stage, host age can also have an effect on fecundity, sex ratio and longevity (Bellows, 1985a). Regardless, while the time scale of development may differ, the basic life cycle remains the same. After the eggs have been laid in the host cavity, the wasp larvae feeds on the host, pupates and then emerges after 21 days at 25°C, though temperature can have a strong influence on development time (Ryoo et al., 1991). At emergence, *L. distinguendus* display protoandry, though female emergence is only delayed by a couple of hours (Ryoo et al., 1991). Both adult males and females have wings capable

of flight, but females can be distinguished as being larger (2-3mm) than males (1,1-2mm) and having a sharper abdomen (Ruschka, 1921).

Mating takes place at the natal host patch, shortly after both males and females have hatched. When males encounter a female sex pheromone over short distances the males start beating their wing in rapid burst, in a behaviour called wing fanning (Ruther et al., 2000). Males follow the scent to the female and climb onto her back, all the while displaying wing fanning behaviour, as the quality of wing fanning can determine the willingness of the female to mate (Benelli, Bonsignori, et al., 2013). As with *N. vitripennis* the male then strokes the male sex pheromone over the antennae of the female, and if she is receptive, she opens her genital cavity, into which the male deposits his sperm (Van den Assem, 1969). Female *L. distinguendus* only mate once and leave their natal patch soon after. Using larval faeces for orientation the females are able to travel over long distances (Steidle & Schöller, 1997) to locate suitable host patches. After having located a potential host patch, females are well adapted to determine which grains contain hosts and which do not (Steidle, 1998).

L. distinguendus' use as a biological control agent and its concurrent usage in IPM schemes have skewed recent research to focus more on increasing the potency of *L. distinguendus*, either by developing methodologies to ease rearing and deployment (Niedermayer & Steidle, 2013), or by exploring new pest species that *L. distinguendus* can potentially parasitise (Steidle et al., 2006). Critically, a research gap has evolved in the influences that pesticides may have on the pheromone communication of *L. distinguendus,* an important topic that this thesis makes an effort to close.

1.2.3 *Leptopilina heterotoma*

The model system *Leptopilina hetertoma* is a member of the *Figitidae* family, and is an endoparasitoid of clyclorraphous flies (Quicray et al., 2023). Males and females are differentiated simply by observing the antennae, as females have 13 segments and males have 15 (Quicray et al., 2023). As parasitoids of the *Drosophila* genera, *L. heterotoma* are tied closely to the evolution of their hosts and research on their complex interactions have centered on the role of genetic factors (Delpuech et al., 1994; Fleury et al., 2004; Wertheim, 2022).

L. heterotoma females generally lay a single egg per host, but adaptive superparasitism is not uncommon (Viser, 1993). Eggs are laid in later larval instars, hatch before the host pupates and feed on the hosts hemolymph. Feeding continues after host pupation, with the larvae exiting the host pupae and transforming from an endo- to an ectoparasitoid (Quicray et al., 2023). The wasp larvae then pupates with the host pupae, and after a total of 21 days at 25°C male adults emerge, with the female adults emerging 1-2 days later (Fauvergue et al., 1999). Unlike the other two species tested here, the majority of the mating is undertaken away from the natal host patch. Males and females both disperse shortly after emergence, and as such require volatile sex pheromones to find potential partners over long distances (Fauvergue et al., 1999). For this purpose, *L. heterotoma* courtship is mediated by former chemical defence compounds called iridoids (Böttinger et al., 2021), more specifically (-)-Iridomyrmecin. Using these compounds, males are able to follow the scent of conspecific females. When a male and a female come into close contact the male exhibits a similar wing fanning behaviour to *L. distinguendus* males, in reaction to higher concentration of female (-)-iridomyrmecin and to a lesser extent cuticular hydrocarbons (Weiss et al., 2015). Males will then climb on top of the female and rub their antennae on the female's antennae, waiting for the female to accept the male and allow him to ejaculate into her genital pouch (Quicray et al., 2023).

Host finding in *L. heterotoma* is dependant upon the learning capability of the egg-laying female. Females associate the odours of their natal host patch with a microhabitat suitable for potential hosts, and are able to orient themselves faster with growing experience (Papaj & Vet, 1990). Female *L. heterotoma* also discriminate between unparasitised and parasitised hosts using conspecific olfactory cues, though this also comes with egg-laying experience (Henneman et al., 1994).

Substantial research has been undertaken on the pheromone communication of *L. heterotoma*, and there are multiple studies analysing effects that pesticides can have on motor function or behaviour (Delpuech et al., 2015; Delpuech et al., 2005). This thesis seeks to expand on this knowledge, additionally allowing a more direct comparison between three diverse parasitoid wasp species.

2. Research Objectives

Pesticides, specifically insecticides have been repeatedly shown to inhibit the proper function of insects' olfactory system and are contributing to global insect biodiversity loss. Older compounds, like acetamiprid and dimethoate are phased out, but are replaced with newer compounds, like flupyradifurone and sulfoxaflor. To highlight that the lethal and sublethal effects on non-target organisms like parasitic wasps are the rule and not the exception requires comparable methodologies between multiple non-target species and multiple substances. To achieve this, 3 main tasks are needed:

Test sublethal exposure of insecticides on *Nasonia vitripennis* (Nv)

- Toxicity tests of the four active ingredients (acetamiprid, dimethoate, flupyradifurone, sulfoxaflor) to determine acute toxicity and sublethal levels.
- Pheromone experiments: reaction of virgin female Nv to synthetic male Nv pheromone after exposure to sublethal doses
- Courtship experiments: virgin Nv mating trials where the male, female or both participants have been exposed to sublethal doses
- Host-finding experiments: reaction of gravid female Nv to potential hosts after exposure to sublethal doses.

Test sublethal exposure of insecticides on *Lariophagus distinguendus* (Ld)

- Toxicity tests of the four active ingredients (acetamiprid, dimethoate, flupyradifurone, sulfoxaflor) to determine acute toxicity and sublethal levels.
- Pheromone experiments: reaction of virgin male Ld to female Ld "dummies" after exposure to sublethal doses
- Courtship experiments: virgin Ld mating trials where the male female or both participants have been exposed to sublethal doses
- Host-finding experiments: reaction of gravid female Ld to host odour after exposure to sublethal doses.

Test sublethal exposure of insecticides on *Leptopilina heterotoma* (Lh)

- Toxicity tests of the four active ingredients (acetamiprid, dimethoate, flupyradifurone, sulfoxaflor) to determine acute toxicity and sublethal levels.

- Contact pheromone experiments: reaction of virgin male Lh to female Lh extract in close contact after exposure to sublethal doses
- Distance pheromone experiments: reaction of virgin male Lh to female Lh extract over distance after exposure to sublethal doses
- Host-finding experiments: reaction of gravid female Lh to host odour after exposure to sublethal doses.
- Development of a multi trophic model system and assessment of the potential of taking up sublethal doses of dimethoate via trophic transfer

3. Sublethal Effects of Four Insecticides Targeting Cholinergic Neurons on Partner and Host Finding in the Parasitic Wasp *Nasonia vitripennis*

N. Schöfer, J. Ackermann, J. Hoheneder, J. Hofferberth and J. Ruther

Abstract: Lethal and sublethal effects of pesticides on nontarget organisms are one of the causes of the current decline of many insect species. However, research in the past decades has focused primarily on pollinators, although other beneficial nontarget organisms such as parasitic wasps may also be affected. We studied the sublethal effects of the four insecticides acetamiprid, dimethoate, flupyradifurone, and sulfoxaflor on pheromone-mediated sexual communication and olfactory host finding of the parasitic wasp Nasonia vitripennis. All agents target cholinergic neurons, which are involved in the processing of chemical information by insects. We applied insecticide doses topically and tested the response of treated wasps to sex pheromones and host-associated chemical cues. In addition, we investigated the mating rate of insecticide-treated wasps. The pheromone response of females surviving insecticide treatment was disrupted by acetamiprid (≥0.63 ng), dimethoate (≥0.105 ng), and flupyradifurone (≥21 ng), whereas sulfoxaflor had no significant effects at the tested doses. Olfactory host finding was affected by all insecticides (acetamiprid ≥1.05 ng, dimethoate ≥0.105 ng, flupyradifurone ≥5.25 ng, sulfoxaflor ≥0.52 ng). Remarkably, females treated with ≥0.21 ng dimethoate even avoided host odour. The mating rate of treated *N. vitripennis* couples was decreased by acetamiprid (6.3 ng), flupyradifurone (≥2.63 ng), and sulfoxaflor (2.63 ng), whereas dimethoate showed only minor effects. Finally, we determined the amount of artificial nectar consumed by *N. vitripennis* females within 48 h. Considering this amount (~2 µL) and the maximum concentrations of the insecticides reported in nectar, tested doses can be considered field-realistic. Our results suggest that exposure of parasitic wasps to field-realistic doses of insecticides targeting the cholinergic system reduces their effectiveness as natural enemies by impairing the olfactory sense.

3.1 Introduction

As modern agriculture has developed to supply a world of eight billion people, multiple agrochemicals have been established to improve crop yields. Pesticides, chemicals designed to control animals and plants that damage crops, are widely used but also controversial because many substances have been shown to be harmful to natural ecosystems (Sánchez-Bayo, 2021; Uhl & Brühl, 2019). In particular, insecticides such as neonicotinoids are considered a cause of the massive pollinator declines in western Europe (Pistorius et al., 2010). In addition to their intended lethal effects on target organisms, negative sublethal effects on nontarget organisms have been demonstrated for many neonicotinoids (Brandt et al., 2016; Di Prisco et al., 2013; Strobl et al., 2021; Tappert et al., 2017). As a consequence, the use of the neonicotinoids imidacloprid, thiamethoxam, and clothianidin was banned in 2018 in the European Union (European Commission, 2018). However, these neonicotinoids continue to be used in many countries outside Europe (Anand et al., 2021; Mahai et al., 2021) and some members of this class of insecticides such as acetamiprid are still registered in the European Union (Jerez et al., 2022). After the ban of the aforementioned neonicotinoids in the European Union, newly developed insecticides such as flupyradifurone and sulfoxaflor are increasingly used by industrial agriculture. These compounds have a similar mechanism of action as the neonicotinoids by inhibiting the synaptic function of neurons as agonists of nicotinergic acetylcholine receptors (nAChRs) and trigger their uncontrolled continuous stimulation (Nauen et al., 2015; Watson et al., 2011). The compounds have a higher affinity for the nAChR in arthropods than for those of mammals, lowering their health risk for humans (Nauen et al., 2015; Watson et al., 2011; Yamada, 1997). However, recent studies on bees suggest that flupyradifurone (Siviter & Muth, 2022; Tan et al., 2017; Tosi & Nieh, 2019; Tosi et al., 2021) and sulfoxaflor (Cartereau et al., 2022; Siviter et al., 2018) may also cause negative sublethal effects, such as compromising the learning ability and performance of these pollinators, whereas other studies did not find any adverse effects of sulfoxaflor (Siviter et al., 2019; G. Tamburini et al., 2021). Another class of insecticides also targeting cholinergic neurons is the organophosphates. A member of this class is the dithiophosphate derivative dimethoate. This compound, while no longer approved in the European Union, is still in use in the United States and in many

developing markets. Dimethoate, unlike neonicotinoids, flupyradifurone, and sulfoxaflor, is not an nAChR agonist but inhibits the enzyme acetylcholinesterase.

Sublethal effects of insecticides on beneficial nontarget organisms such as pollinators, predators, and parasitic wasps have been intensively studied for >40 years (Reviewed by Desneux et al., 2007; Haynes, 1988). In past years, however, the focus of this research has been on neonicotinoids and bees. Parasitic wasps develop in or on different stages of other arthropods and therefore play an important role as natural enemies maintaining ecological balance (Wang et al., 2019). Bred and released en masse, they can be used for biological pest control (Harush et al., 2021; Waage & Hassell, 1982; Wang et al., 2019). Given that parasitic wasps spend parts of their life cycle feeding on their hosts, including many pest species (Mackauer et al., 1997), they are exposed to the same pesticides as their hosts but have various additional avenues for uptake. Parasitic wasps use floral and extrafloral nectar as well as honeydew as a source of carbohydrates (Dulaurent et al., 2011; Jervis et al., 1993; Lee et al., 2006; Wackers et al., 2008; Wanner et al., 2006) and thus have similar exposure pathways as pollinators. In addition, some parasitic wasps have been shown to consume guttation water (Urbaneja-Bernat et al., 2020) that can contain a considerable amount of pesticides (Hrynko et al., 2021; Reetz et al., 2016; Schmolke et al., 2018). Finally, bioactive amounts of pesticides can be taken up by parasitic wasps simply by tarsal contact with treated plants (Prabhaker et al., 2011). Therefore, it is not surprising that parasitic wasps are also affected by sublethal doses of insecticides (Cook et al., 2016; Desneux et al., 2007; Kang et al., 2018; Kremer & King, 2019; Pisa et al., 2015; Tappert et al., 2017; Teder & Knapp, 2019).

Like most insects, the olfactory sense of parasitic wasps aids them in orienting themselves in complex environments and locating both food resources and hosts (Wang et al., 2003; Zhang et al., 1998). In addition, olfaction plays a major role in pheromone-mediated mate finding, in identifying a mate at close range, and during courtship (Mair & Ruther, 2019; Ruther, 2013). Given that nAChRs are involved in the processing of olfactory stimuli (Dupuis et al., 2012; Rabhi et al., 2016), it is reasonable to assume that sublethal doses of insecticides targeting cholinergic neurons also affect

the sense of smell in insects. Consistent with this hypothesis, insecticides targeting nAChRs have been shown to interfere with chemical orientation of parasitic wasps. Males of the egg parasitoid Trichogramma brassicae exposed to the organophosphate chlorpyriphos were less arrested by the female sex pheromone (Delpuech, Froment, et al., 1998; Delpuech, Gareau, et al., 1998). Females of the ichneumonid wasp Microplitis croceipes feeding on extrafloral nectar contaminated with imidacloprid were impaired in responding to herbivore-induced volatiles released from infested host plants (Stapel et al., 2000). Imidacloprid has also been shown to disrupt olfactory host and mate finding in *Nasonia vitripennis*, a frequently used model organism for the study of parasitic wasp biology (Tappert et al., 2017). Females surviving insecticide treatment no longer responded to the male sex pheromone, and both sexes exhibited altered courtship behavior, resulting in decreased mating rate. In addition, treated females were less successful in olfactory host finding. It is unknown, however, whether other insecticides targeting the cholinergic system of insects have similar effects in *N. vitripennis*.

Nasonia vitripennis is a cosmopolitan parasitoid that parasitises pupae of numerous cyclorraphous flies (Whiting, 1967). Host species of *N. vitripennis* include many flesh flies and blowflies, which not only feed on carrion and other decaying materials but also are pollinators in the adult stage and therefore integral to farmland ecosystems (Currah & Ockendon, 1983; Rader et al., 2013; Rader et al., 2009). Given the ecology of their hosts living adjacent to farmland ecosystems and the problem of pesticides leaching into the wider environment, *N. vitripennis* as a parasitoid is at risk of exposure to sublethal doses of pesticides in agricultural ecosystems and serves not only as a model organism but as a real example of an affected species (Garrido-Bautista et al., 2020). Mate finding in *N. vitripennis* is mediated by a male-derived, substrate-borne sex pheromone that attracts females as long as they are unmated (Lenschow et al., 2018; Ruther et al., 2010). The pheromone is synthesized in the rectal vesicle of males (Abdel-Latief et al., 2008) and consists of (4R,5S)- and (4R,5R)-5-hydroxy-4-decanolide and the synergistic minor component 4-methylquinazoline (Ruther et al., 2007; Ruther et al., 2008). Close-range mate recognition is mediated by female-derived cuticular hydrocarbons that trigger stereotypic male courtship behavior (Steiner et al., 2006). During courtship, males elicit female receptivity by using an oral

aphrodisiac pheromone of unknown chemical structure (van den Assem et al., 1980). After mating, females search for suitable hosts to lay their eggs. They find these hosts through olfactory stimuli, which the hosts passively emit (Frederickx et al., 2014; Steiner & Ruther, 2009).

In the present study, we investigated the sublethal effects of acetamiprid, dimethoate, flupyradifurone, and sulfoxaflor on pheromone-mediated sexual communication and olfactory host finding in *N. vitripennis*. We applied the insecticides topically to the wasps and performed bioassays to test the response of females to the synthetic male sex pheromone and host odour, respectively. We also examined the mating rates of insecticide-treated couples. Finally, we quantified the amount of a sugar solution ingested by *N. vitripennis* females to discuss bioassay results in the context of insecticide concentrations reported in contaminated nectar.

3.2 Materials and Methods

In Germany, experiments with insects do not require Institution Animal Care and Use Committee approval.

3.2.1 Insects

Wasps of the species *N. vitripennis* were of the strain Phero1 and were originally collected from a bird's nest near Hamburg, Germany (Steiner et al., 2006). Wasps were reared on freeze-killed pupae of the fly species *Lucilia caesar* that were obtained as larvae from a commercial supplier (b.t.b.e. Insektenzucht). Two days after pupation, fly pupae were frozen at −20 °C and used when needed. On Monday, Wednesday, and Friday, wasps of the previous generation of *N. vitripennis* were transposed onto new hosts to copulate and lay eggs for the next generation. Before use, hosts were thawed and dried for at least 2 h in a drying cabinet at 30 °C to prevent the formation of mold.

To make sure that the wasps used in the experiments were virgin and naive, parasitised hosts were dissected and parasitoid pupae removed from their hosts 24–48 h before their estimated emergence dates (generation time at 25 °C and 50% relative humidity is 14–15 days). Fully melanized wasp pupae were isolated from the hosts and transferred singly to 1.5-mL Eppendorf microcentrifuge tube. Tubes were monitored every morning for the appearance of newly emerged wasps. These wasps were

defined as being 0 days old. Wasps were used for the experiment at an age of 1 to 2 days.

3.2.2 Insecticides

Analytical standards of the tested insecticides, acetamiprid (≥98.0% purity), dimethoate (≥98.0% purity), and flupyradifurone (≥98.0% purity), were sourced from Sigma-Aldrich. All tested pesticides were of PESTANAL®-grade purity. Sulfoxaflor (99.23% purity) was obtained from Dr. Ehrenstorfer. Insecticides were dissolved in acetone (ROTISOLV®, ≥99.8% purity), purchased from Carl Roth.

3.2.3 Toxicity tests

To have the best possible control over the insecticide dose taken up by the tiny insects, we did not feed the wasps with the active substances but chose topical application of acetone solutions (Tappert et al., 2017). Pure acetone was applied as control, which had no negative effects on the studied parameters in a closely related species (Jatsch & Ruther, 2021). A microinjector (Nanoliter 2010; World Precision Instruments) was used to apply 210 nL of different dilutions (Supporting Information, Table S3.1) made from 1-mg/mL stock solutions to the abdominal tips of ice-cooled wasps. Groups of eight wasps (n = 3 replicates/dose/sex for acetamiprid and dimethoate, n = 4 replicates/dose/sex for flupyradifurone and sulfoxaflor) were treated with the insecticides or with pure acetone (control). Mortality was assessed 72 h after application, and dose–mortality curves were generated for each insecticide using the Quest Graph™ LD50 Calculator (AAT Bioquest, 2023). The functional equations of the resulting sigmoidal curves (Figure 3.1) were used to calculate the doses at which 50% of the treated wasps died (i.e., median lethal dose [LD50]). For the bioassays, three or four doses were chosen for each insecticide that caused mortalities ≤30% in our toxicity

tests. As for sulfoxaflor, one additional dose was tested that caused a higher mortality (39%; Table 3.1).

$$Y = 0 + \frac{1-0}{1+\left(\frac{X}{12838.272}\right)^{0.8049}} \qquad Y = 0 + \frac{1-0}{1+\left(\frac{X}{2220.5662}\right)^{1.4341}} \qquad Y = 0 + \frac{1-0}{1+\left(\frac{X}{102248.6228}\right)^{0.8549}} \qquad Y = 0 + \frac{1-0}{1+\left(\frac{X}{14895.9023}\right)^{1.0359}}$$
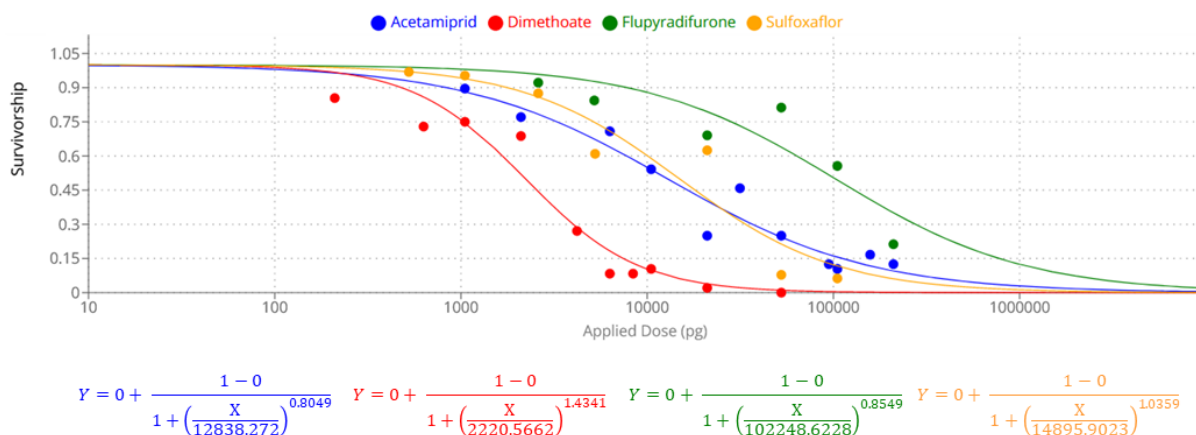
Figure 3.1 Dose–mortality curves after 72 h of *Nasonia vitripennis* treated topically with different doses of acetamiprid, dimethoate, flupyradifurone, and sulfoxaflor dissolved in acetone. Dots represent the mean of six groups (three of either sex, acetamiprid, and dimethoate) or eight groups (four of either sex, flupyradifurone and sulfoxaflor) of eight wasps each.

Table 3.1 Median lethal dose (LD50) values (evaluated after 72 h) and calculated from the functions of the respective sigmoidal curves using the online tool Quest Graph LD50 Calculator and doses of the four insecticides tested in the bioassays with *Nasonia vitripennis.*

	LD$_{50}$ (ng/Wasp)	Tested Sublethal Doses (ng/Wasp)			
Acetamiprid	12.6	0 (96%)	0.63 (n.t.)	1.05 (90%)	2.1 (77%)	6.3 (71%)
Dimethoate	2.2	0 (94%)	0.105 (n.t.)	0.21 (85%)	0.63 (73%)	1.05 (75%)
Flupyradifurone	102	0 (98%)	2.63 (92%)	5.25 (84%)	21 (70%)	-
Sulfoxaflor	14.9	0 (100%)	0.525 (97%)	1.05 (95%)	2.63 (88%)	5.25 (61%)

[a] Sublethal doses, that is, those below the no-observed-effect level (see Supporting Information, Table S3.1). Values in parentheses represent the percentages of surviving wasps in the toxicity tests. n.t. = doses were tested in the bioassays but not in the toxicity tests.

3.2.4 Effects on pheromone communication

The effect of insecticides on the pheromone response of *N. vitripennis* females was tested by isolating and treating virgin female *N. vitripennis* with different doses of the four insecticides or acetone (control; $n = 20$ per treatment); 24 h after the treatment, females were tested in a dual-choice olfactometer, as described in previous studies (Ruther et al., 2014; Tappert et al., 2017). Briefly, 1 µL of the synthetic sex pheromone dissolved in dichloromethane (200 ng/µL (4*R*,5*S*)-5-hydroxy-4-decanolide, 100 ng/µL (4*R*,5*R*)-5-hydroxy-4-decanolide, and 3 ng 4-methylquinazoline, synthesized as described previously (Ruther et al., 2016; Ruther et al., 2007; Ruther et al., 2008), was applied to a disk of filter paper. Control paper disks were treated with the same amount of pure dichloromethane. After evaporation of the solvent, test and control disks were put into the test and control cavity of the olfactometer, and treated females were released individually into its center. The time females spent in either cavity of the olfactometer was recorded using The Observer XT 15 software (Noldus Information Technology). The olfactometer was rotated 90° after every observation to avoid bias due to external influences.

3.2.4 Effects on mating rate

To test the influence of the four insecticides on mating rate, virgin male and female *N. vitripennis* were isolated and separated into three groups ($n = 20$/dose/group), depending on whether the male, the female, or both partners had been treated with insecticide doses or pure acetone (control), respectively. Couples of treated wasps were then placed in a 2-cm-diameter plexiglass arena and observed for 5 min using a stereomicroscope. Successful copulations during the observation time were recorded and converted into a percentage value for each dose and group constellation for further analysis.

3.2.5 Effects on host finding

To test whether the four insecticides influence the ability of females to locate hosts by olfactory cues, newly emerged females (<1 day old) were mated and subsequently treated with an insecticide dose or the pure solvent ($n = 20$/dose/treatment). Treated females were held for 24 h and then tested in a T-olfactometer (Figure 3.2). This olfactometer was divided into four zones: (a) the start zone, where the microcentrifuge tube with the female was attached at the beginning of each observation; (b) the neutral

zone, which included a corridor to the tested zones and a buffer between them; (c) the control zone, to which an empty microcentrifuge tube was connected; and (d) the host zone, on the opposite side of the control zone, to which a microcentrifuge tube with five *Lucilia caesar* pupae was connected. Polyamide gauze (mesh width 125 µm) was installed between microcentrifuge tubes of test and control zones, allowing host volatiles to diffuse into the olfactometer but excluding visual cues. Females were observed for 5 min after the microcentrifuge tube with the female had been attached to the olfactometer. The time the females spent in test and control zone was recorded using The Observer XT 15 software.
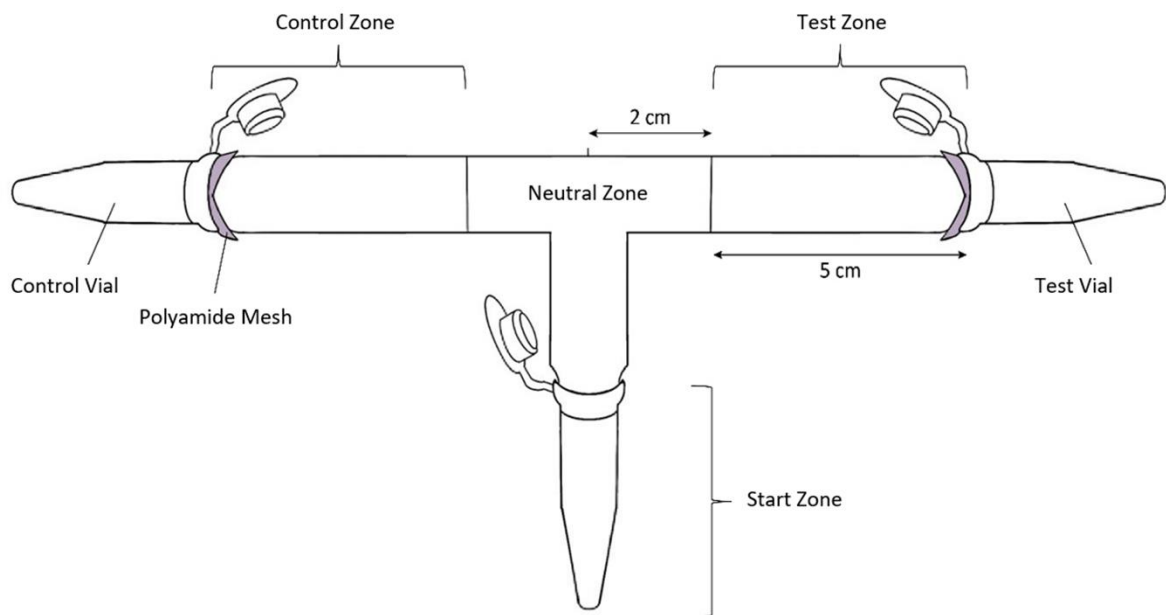
Figure 3.2 Schematic of the T-olfactometer used in the host-finding experiments (for details, see text).

3.2.6 Quantification of the amount of artificial nectar consumed by *N. vitripennis*

Feeding experiment

A likely major source through which parasitic wasps ingest insecticides is the consumption of contaminated nectar. To obtain a realistic basis for discussion of the ecological relevance of the bioassay results, the amount of an artificial nectar (30% glucose solution in water) consumed by *N. vitripennis* females within 48 h was quantified by gas chromatography/mass spectrometry (GC/MS). Samples (10 µL each) of a 30% (m/v) solution of α-D-glucose (Sigma-Aldrich) were pipetted into 1.5-mL microcentrifuge tubes, and one *N. vitripennis* female per tube was added ($n = 15$). Females were allowed to feed on the artificial nectar for 48 h. To ensure that the wasps had expended some of their energy resources and to increase their motivation to feed,

females were mated before the experiment and given the opportunity to lay eggs for 2 days. Lids of the tubes were perforated with a needle for aeration. Control tubes (n = 15) were prepared the same way without adding a wasp.

Sample preparation

After 48 h, wasps were removed, and the residues of the glucose solution were redissolved in 20 µL of water containing 3 mg of D-(+)-mannose as an internal standard. The sugar solutions were transferred to 1.5-mL GC vials, and microcentrifuge tubes were rinsed with another 20 µL of distilled water which were added to the respective samples. Sugar solutions were lyophilized overnight, and sugar residues were derivatized for GC/MS analysis by a two-step derivatization method (Yi et al., 2014). In the first step, sugars were oximized by adding 200 µL of a solution of methoxamine hydrochloride (5% dissolved in pyridine; Sigma-Aldrich) and incubating the vials for 30 min at 75 °C. This step reduced the number of possible sugar isomers to two per sugar (*cis-* and *trans*-oximes), facilitating the analysis by GC/MS. In the second step, 50 µL of each sample were transferred to new GC vials, and the oximes were silylated by adding 40 µL of N,O-bis(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich) and incubating the vials for another 30 min at 75 °C. The vials were allowed to cool, 900 µL of dichloromethane were added, and the samples were diluted 1 to 5 with the same solvent prior to GC/MS analysis.

GC/MS analysis

The GC/MS analysis was performed on a Shimadzu QP2010 GC/MS system operated in electron ionization mode at 70 eV and equipped with a 60-m BPX-5 capillary column (inner diameter 0.25 mm, film thickness 0.25 µm). Helium was used as carrier gas at a linear velocity of 40 cm/s. Samples were injected in split mode (1:25) at 300 °C using an AOC20i autosampler. The temperature program started at 70 °C, was increased at 20 °C/min to 160 °C, then at 4 °C/min to 180 °C, and finally at 10 °C/min to 300 °C (held for 10 min). Glucose amounts were determined by relating the total peak area of the two glucose peaks to the total peak area of the two mannose peaks (internal standard).

3.2.7 Statistical analyses

All statistical analyses were performed using R (R Development Core Team, 2023). If data did not meet the assumptions for parametric testing, nonparametric tests were

used. Survival rates of insecticide-treated wasps were compared with the respective controls using Fisher's exact test. Pheromone and host finding data were analyzed within each dose using a Wilcoxon matched-pairs test. Copulation rates of insecticide-treated and control couples were analyzed across all doses using a Fisher's exact test. If this test showed a significant result ($p < 0.05$), pairwise comparisons were done between the control dose and each insecticide dose. The glucose residues in the centrifuge tubes of the fed females and the control group were each averaged and analyzed using a t test. Box plots and bar plots in the results were plotted using the PAST 4.03 software (Hammer et al., 2001).

3.3 Results

3.3.1 Toxicity tests

The dose–mortality curves revealed clear differences in the acute toxicity (Figure 3.1 and Table 3.1) of the four insecticides. Dimethoate showed the highest toxicity (LD50 = 2.2 ng/wasp), while flupyradifurone was the least toxic of the four (LD50 = 102 ng/wasp). Acetamiprid (LD50 = 12.6 ng/wasp) and sulfoxaflor (LD50 = 14.9 ng/wasp) had similar, intermediate toxicities. A sublethal dose is defined as a dose inducing no statistically significant mortality in the experimental population (Desneux et al., 2007). Accordingly, the no-observed-effect levels (NOELs) for acetamiprid, dimethoate, flupyradifurone, and sulfoxaflor were determined as 1.05, 0.21, 2.63, and 1.05 ng, respectively (Table 3.1; Supporting Information, Table S3.1). Doses below these NOELs were considered sublethal.

3.3.2 Effects on pheromone communication

Virgin control females treated with pure acetone spent significantly more time in the pheromone cavity than in the untreated control cavity in all experiments (Figure 3.3a-d). In contrast, females treated with any dose of acetamiprid or dimethoate no longer preferred the pheromone cavity (Figure 3.3a,b). Both insecticides, however, showed a relatively high degree of variability, as depicted by numerous outliers. Flupyradifurone disrupted the pheromone preference of virgin females only at the highest tested dose (Figure 3.3c), whereas sulfoxaflor doses had no significant effect on the pheromone response of virgin females at tested levels (Figure 3.3d).
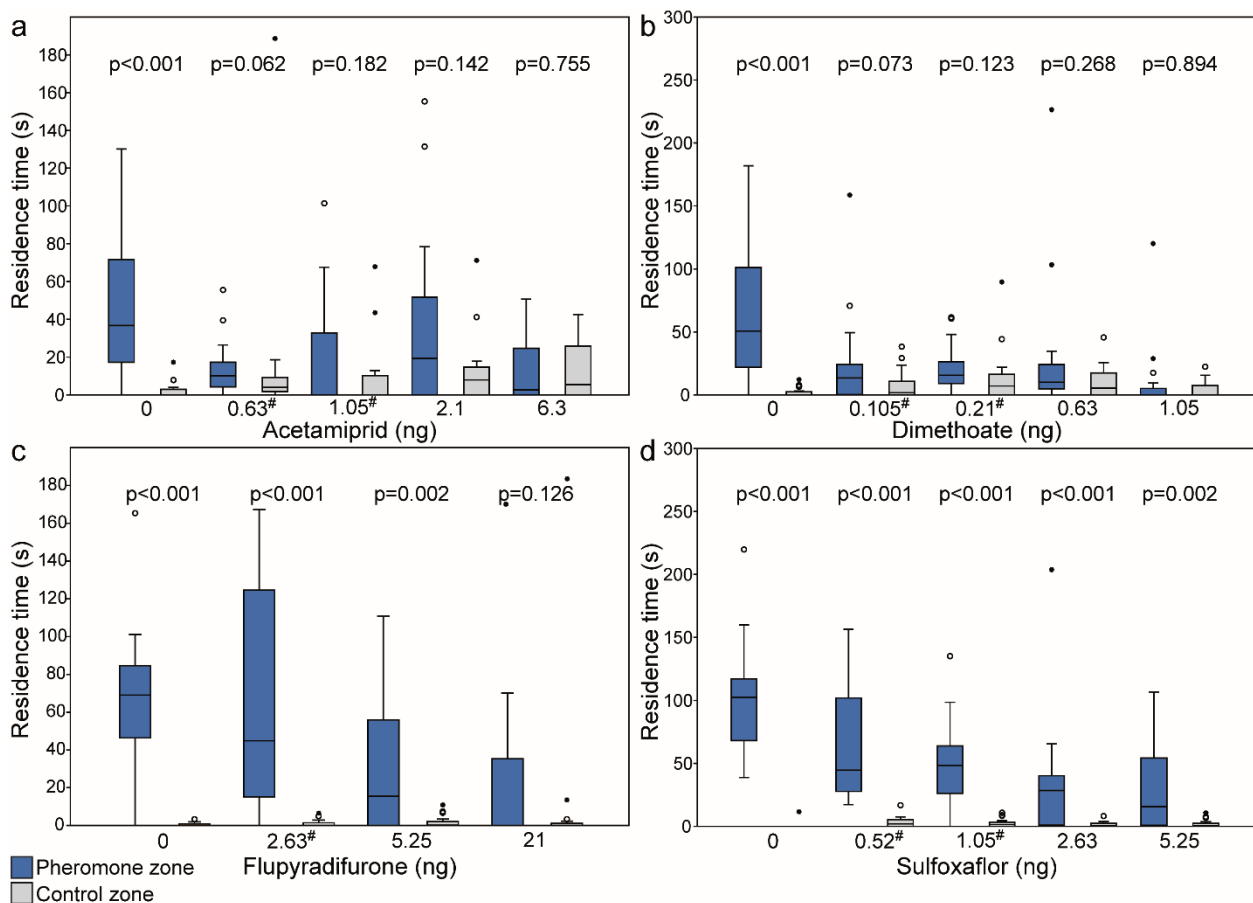
Figure 3.3 Effect of different doses of the four insecticides on the response of virgin *Nasonia vitripennis* females to the synthetic male sex pheromone. Females were treated with either pure acetone (0 ng, control) or doses of (a) acetamiprid, (b) dimethoate, (c) flupyradifurone, or (d) sulfoxaflor. #Sublethal doses. One day after application the females were tested in a two-choice olfactometer. Shown are the residence times of females in the pheromone cavity and the solvent-treated control cavity of the olfactometer. Box-and-whisker plots show median (horizontal line), 25% to 75% quartiles (box), maximum/minimum range (whiskers), and outliers (° means >1.5 × and * means >3 × box height). Statistical analysis for each treatment by Wilcoxon matched pairs test ($n = 20$).

3.3.3 Effects on the mating rate

Tested doses of any of the four insecticides reduced the mating rate of treated couples significantly in at least one of the tested concentrations/constellations (Figure 3.4 a-d). When only males were treated, all four substances significantly reduced the mating rate of couples at the highest tested doses (Figure 3.4a-d). When only the females were treated, only flupyradifurone and sulfoxaflor had significant effects at the highest tested dose (Figure 3.4c,d). When both partners were treated, acetamiprid and sulfoxaflor decreased mating rates of treated couples significantly at the highest tested

doses (Figure 3.4a,c). Flupyradifurone caused significant effects at any of the tested doses (Figure 3.4c), whereas dimethoate had no significant effects at the tested dose range (Figure 3.4b).
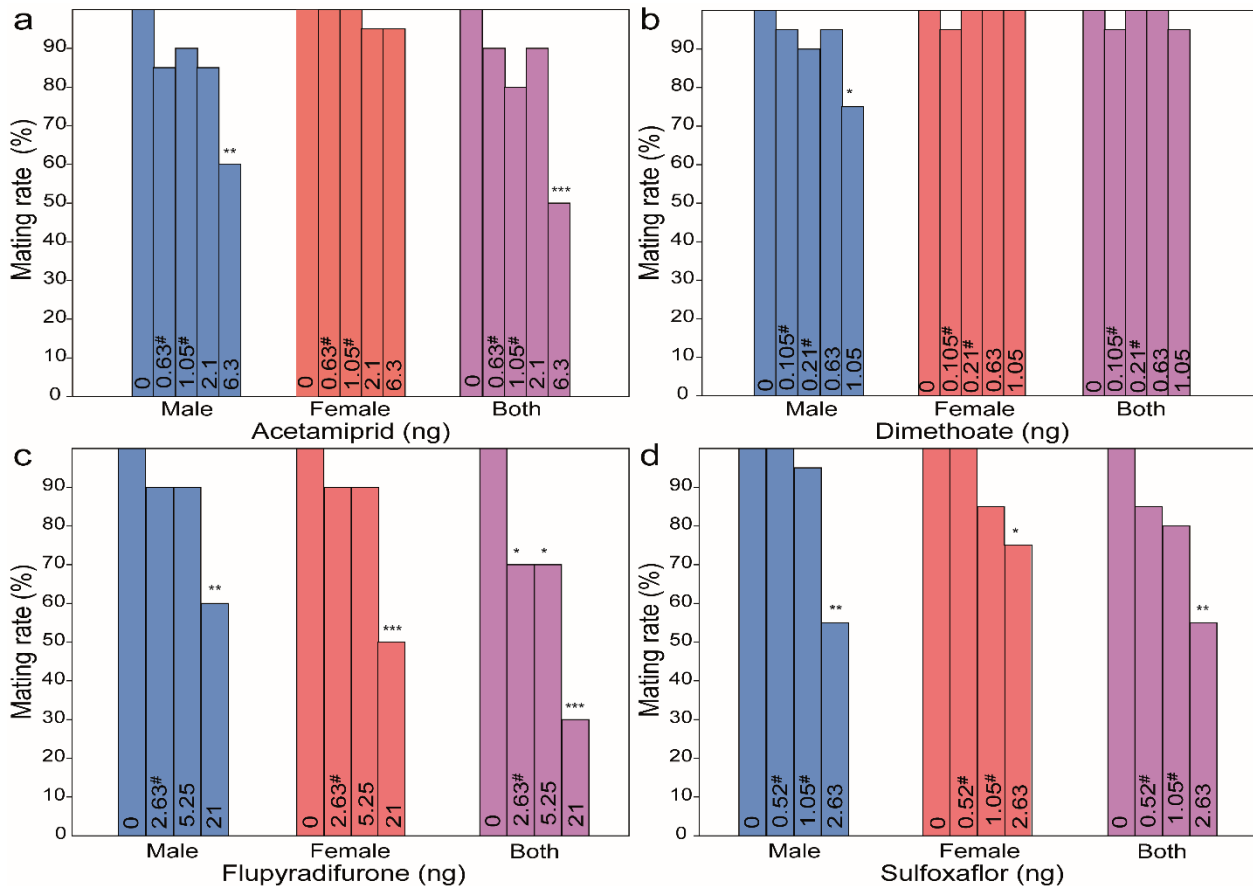
Figure 3.4 Effect of different doses of the four insecticides on the mating rates of virgin *Nasonia vitripennis*. Shown are the mating rates of *N. vitripennis* couples, where either the male partner, the female partner, or both partners were treated with either pure acetone (0, control) or a dose (doses in nanograms given in the bars) of (**a**) acetamiprid, (**b**) dimethoate, (**c**) flupyradifurone, or (**d**) sulfoxaflor. [#]Sublethal doses. Significant differences between the individual sublethal doses and the control are marked by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 20$, Fisher's exact test).

3.3.4 Effects on host finding

In all experiments, mated females treated with pure acetone spent significantly more time in the host zone of the T-olfactometer than in the untreated control zone (Figure 3.5a-d). This preference for host odour, however, was absent in females treated with any dose of the four insecticides except for the lowest doses of acetamiprid

and flupyradifurone, respectively. Remarkably, females treated with doses ≥0.21 ng of dimethoate even avoided the host zone significantly (Figure 3.5b).
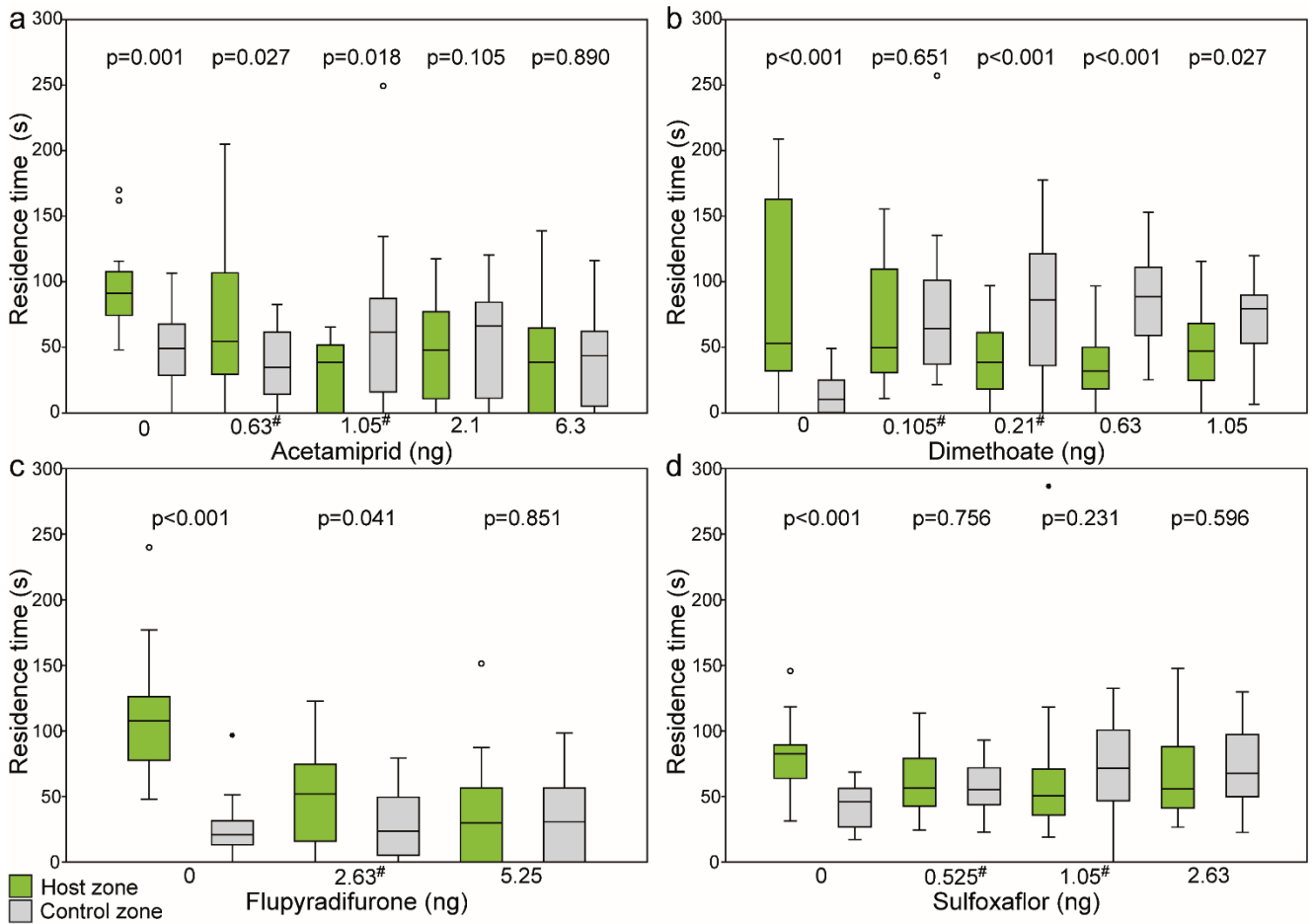
Figure 3.5 Effect of different doses of the four insecticides on the response of mated female *Nasonia vitripennis* to host odour (pupae of *Lucilia caesar*). Mated females were treated with pure acetone (0, control) or a dose of (**a**) acetamiprid, (**b**) dimethoate, (**c**) flupyradifurone, or (**d**) sulfoxaflor. #Sublethal doses. One day after application, females were tested in a two-choice T-olfactometer. Shown are the residence times of females in the host zone and the untreated control zone of the olfactometer. Box-and-whisker plots show median (horizontal line), 25% to 75% quartiles (box), maximum/minimum range (whiskers), and outliers (° means >1.5 × and * means >3 × box height). Statistical analysis for each treatment by Wilcoxon matched pairs test (*n* = 20).

3.3.5 Quantification of the amount of artificial nectar consumed by *N. vitripennis*

Mean glucose residues in the microcentrifuge tubes after 48 h feeding by *N. vitripennis* females were significantly lower (mean ± SE 1.94 ± 0.11 mg) than in the control tubes (2.37 ± 0.09 mg; *t* test, *p* = 0.0048). Thus, female wasps consumed 18% of the total amount supplied to them within 48 h, which corresponds to 0.43 mg.

Considering the sample volume of 10 µL applied in the feeding experiment, females consumed approximately 1.8 µL of the artificial nectar.

3.4 Discussion

In the present study, we extend our knowledge of the adverse effects of pesticides on beneficial nontarget organisms by showing that sublethal doses of four insecticides targeting the insect cholinergic system impair important traits related to olfaction in the parasitic wasp *N. vitripennis*. Thus, not only imidacloprid (Tappert et al., 2017; Whitehorn et al., 2015), one of the neonicotinoids banned in the European Union, but also the still-approved neonicotinoid acetamiprid and the next-generation nAChR agonists flupyradifurone and sulfoxaflor, as well as the acetylcholinesterase inhibitor dimethoate, interfere with the chemical sense-mediated performance of this model organism. Mate and host finding are crucial prerequisites for the reproductive success of parasitic wasps. Females that are unable to locate their hosts for oviposition are also unable to produce any offspring. Those that remain unmated, even if they could find hosts, are forced to produce all-male offspring as a result of haplodiploid sex determination in hymenopterans (Beukeboom & van de Zande, 2010). Given that *N. vitripennis* and many other parasitoids prefer to produce female-biased offspring because of local mate competition (Quicke, 1997), female virginity is another fitness-relevant factor in these species that might be caused by sublethal insecticide doses. Therefore, our results suggest that doses of the four insecticides, at which the vast majority of the exposed wasps survive, compromise the important ecosystem function these insects have as natural enemies as well as their applicability in the context of biocontrol. Although this suggestion is supported by previous studies that have also found sublethal effects of these compounds in parasitic wasps (Costa et al., 2023; Gao et al., 2023; Mohammed & Karut, 2021), further experiments under more realistic semifield conditions are needed for a definitive evaluation.

The sublethal effects of the four insecticides varied with the ecological context in which they were investigated and depended on the dose tested, with the lowest bioactive doses correlating largely with the acute toxicity (LD50) of the respective compounds. Dimethoate had the highest acute toxicity for *N. vitripennis* and disrupted pheromone communication and host finding even at doses as low as 0.1 ng/wasp. The mating frequency was only affected at a 10-fold higher dose (1.05 ng/wasp) of dimethoate

when applied to males. However, this result could have occurred by chance because the effect was weak (Figure 3.4b) and no longer present when both males and females were treated. Flupyradifurone exhibited the lowest acute toxicity, and consequently, significantly higher doses were necessary to influence both the pheromone response (21 ng) and olfactory host finding (5.25 ng), while it affected mating rate at a sublethal dose of 2.63 ng, which was comparable with the other agents. Acetamiprid impaired the pheromone response of *N. vitripennis* females at subnanogram levels, while nanogram doses were necessary to affect the mating rate and to disrupt olfactory host finding. Sulfoxaflor had no effects on the pheromone response at the tested dose range but affected olfactory host finding even at a sublethal dose of 0.525 ng. This suggests that pheromones and host volatiles are processed differently and that behavioral reactions are differentially influenced by insecticide residues in *N. vitripennis* females. This idea is supported by the fact that dimethoate disrupted the pheromone response, while the preference for host odour even turned into avoidance at doses ≥0.21 ng. Interestingly, chlorpyrifos, another organophosphate insecticide, disrupted the male pheromone response in the egg parasitoid *T. brassicae* (Delpuech, Froment, et al., 1998; Delpuech, Gareau, et al., 1998), while it even increased the response of females to host-associated kairomones in the *Drosophila*-parasitoids *Leptopilina heterotoma* and *L. boulardi* (Delpuech et al., 2005; Rafalimanana et al., 2002). Pheromones in insects are often perceived and processed by specialized components of the olfactory system (pheromone receptor proteins, pheromone-binding proteins, macroglomerular complex in the antennal lobe) that differ from those for general, food-associated odourants (Renou, 2014). The mechanisms underlying the differing sublethal effects of insecticides on the response to pheromones and host-associated volatiles, respectively, deserve further research in the future. Our results, however, underline that possible sublethal effects on the perception of chemical stimuli need to be investigated in different contexts.

An important question arising from our results is whether the tested insecticide doses are field-realistic and ecologically relevant. Again, further research is needed because the uptake pathways of insecticides in parasitic wasps have not been fully explored. In the present study, we deliberately chose topical application (as an acetone solution) for the administration of the active ingredients because this allows more control of the absorbed doses. Uptake of bioactive doses via the cuticle or tarsi after exposure to

insecticide aerosols or contact with treated surfaces is a possible route by which bioactive amounts of insecticides can enter parasitic wasps (Delpuech et al., 2005; Prabhaker et al., 2011; Salerno et al., 2002). Ingestion of insecticides via the consumption of contaminated floral and extrafloral nectar, however, is probably the primary route by which parasitic wasps in natural habitats may be exposed to insecticides (Jervis et al., 1993; Rose et al., 2006; Stapel et al., 2000) Supporting information, Table S3.2). Our data show that females with partly depleted energy resources consumed approximately 2 µL of an artificial nectar within 48 h. Based on this amount, literature data on nectar contamination with the insecticides we studied (Supporting information, Table S3.2) indicate that the highest concentrations reported for the four active ingredients are in the range of, or slightly below, concentrations that would cause sublethal effects in *N. vitripennis*. In addition, it must be considered that some active substances have a stronger effect when taken up orally than when applied topically (Tosi et al., 2022) and that even relatively closely related species may exhibit drastic differences in their susceptibility to insecticides (Hayward et al., 2019). Hence, other parasitic wasps may be impaired in their olfactory capabilities by even lower doses of the insecticides. Furthermore, at least neonicotinoids can have cumulative effects (Huang et al., 2021; Sanchez-Bayo & Tennekes, 2020; van der Sluijs et al., 2013). Thus, multiple ingestions of very low doses could ultimately lead to measurable sublethal effects. Insecticides are often used in multicomponent blends (Yu & Ting, 2019), and the simultaneous use of pesticides from other classes (fungicides, herbicides; (Schuhmann et al., 2022)) may result in exposure of parasitic wasps to multiple active substances, which may cause additive or synergistic adverse effects (Tosi et al., 2022; Y. H. Wang et al., 2020; Willow et al., 2019). Guttation water from plants grown from coated seeds may contain significant amounts of insecticides (e.g., up to ~100 ng/µL thiacloprid, 150 ng/µL thiamethoxam, and up to 200 ng/µL imidacloprid; (Girolami et al., 2009)), although no literature data are available for the insecticides tested in the present study. It has been shown, however, that parasitic wasps use guttation water as a food source (Urbaneja-Bernat et al., 2020) and that guttation water from arable wild weeds may also contain systemic pesticides in significant amounts if they grow near treated crops (Mörtl et al., 2019). Honeydew, the sugary excretion of aphids and other Hemiptera, is also commonly used by parasitic wasps as a carbohydrate source and may contain biologically active levels of insecticides (Calvo-Agudo et al., 2019; Calvo-Agudo et al., 2022). Because all nAChR

agonists tested in the present study are widely used against hemipteran pests such as aphids, whiteflies, and thrips (Elbert et al., 2008; Nauen et al., 2015; Zhu et al., 2011), it can be expected that these substances may enter beneficial insects also via honeydew, although data on residue levels are not yet available. The question of whether parasitic wasps, because of their particular lifestyle, can also take up bioactive doses of pesticides when developing in or host-feeding on contaminated hosts and whether the ingested amounts are sufficient to affect olfactory responses is still largely unresolved and requires further research. This aspect could become an increasing problem if parasitic wasps parasitise resistant hosts that are able to survive despite insecticide ingestion (Umoru & Powell, 2002).

Many features of the olfactory system are largely conserved in insects (Hansson & Stensmyr, 2011), and therefore it is unlikely that the sublethal effects demonstrated in the present study are unique for *N. vitripennis* or parasitic wasps, respectively. Because of the special importance of olfaction to insect performance, additional species from other insect guilds need to be studied to determine whether insecticides targeting nAChRs also affect the mating and foraging behaviour of these species and in this way contribute, largely unnoticed, to the current decline of many insect species. Our study supports the claim that sublethal effects should be given more consideration in the approval process of new pesticides (Siviter et al., 2023). Because of the ease of breeding, the availability of well-established and easily performed bioassays, and the extensive knowledge available on chemical communication (Mair & Ruther, 2019), the *Nasonia* model system offers excellent conditions for future use in such registration procedures.

3.5 Supporting Information

Table S3.1 Results of the toxicity tests with *Nasonia vitripennis* treated with 210 nl each of acetone solutions of (a) acetamiprid, (b) dimethoate, (c) flupyradifurone, and (d) sulfoxaflor. Given is the number of live/dead wasps (evaluated 72 h after the treatment). Statistical analysis by Fisher's exact test, p-values refer to the comparison between pesticide doses and the respective controls (0 ng, pure acetone). Yellow entries indicate no-observed-effect-levels (NOEL). Doses ≤ NOEL are considered as sublethal doses.

a) Acetamiprid

Dose (ng)	210	157.5	105	94.5	52.5	31.5	21	10.5	6.3	2.1	1.05	0
Rep. 1	2/6	0/8	3/5	1/7	6/2	5/3	3/5	6/2	5/3	6/2	8/0	7/1
Rep. 2	3/5	0/8	1/7	0/8	0/8	5/3	1/7	3/5	6/2	6/2	7/1	7/1
Rep. 3	0/8	2/6	0/8	0/8	2/6	4/4	2/6	4/4	6/2	7/1	8/0	8/0
Rep. 4	0/8	2/6	0/8	0/8	2/6	1/7	3/5	4/4	6/2	7/1	6(2	8/0
Rep. 5	1/7	2/6	1/7	2/6	2/6	4/4	1/7	5/3	6/2	5/3	6/2	8/0
Rep. 6	0/8	2/6	0/8	3/5	0/8	3/5	2/6	4/4	5/3	6/2	8/0	8/0
total	6/42	8/40	5/43	6/42	12/36	22/26	12/23	26/22	34/14	37/11	43/5	46/2
p-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0,002	0,014	0,435	

b) Dimethoate

Dose (ng)	52.5	21	10.5	8.4	6.3	4.2	2.1	1.05	0.63	0.21	0
Rep. 1	0/8	0/8	0/8	0/8	1/7	2/6	4/4	5/3	4/4	4/4	8/0
Rep. 2	0/8	0/8	1/7	1/7	0/8	3/5	8/0	4/4	4/4	5/5	8/0
Rep. 3	0/8	0/8	0/8	1/7	0/8	0/8	4/4	7/1	7/1	8/0	6/2
Rep. 4	0/8	1/7	1/7	1/7	1/7	2/6	7/1	7/1	6/2	8/0	7/1
Rep. 5	0/8	0/8	1/7	0/8	0/8	1/7	6/2	6/2	7/1	8/0	8/0
Rep. 6	0/8	0/8	2	1/7	2	5/3	4/4	7/1	7/1	8/0	8/0
total	0/48	1/47	5/43	4/44	4/44	13/35	33/15	36/12	35/13	41/7	45/3
p-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0,003	0,022	0,012	0,317	

c) Flupyradifurone

Dose (ng)	210	105	52.5	21	5.2	2.63	0
Rep. 1	2/6	4/4	7/1	3/5	7/1	8/0	7/1
Rep. 2	3/5	3/5	8/0	4/4	4/4	7/1	8/0
Rep. 3	1/7	7/1	5/3	6/2	8/0	8/0	8/0
Rep. 4	2/6	6/2	5/3	3/5	8/0	7/1	8/0
Rep. 5	0/8	4/4	7/1	7/1	8/0	8/0	8/0
Rep. 6	4/4	6/2	8/0	8/0	6/2	7/1	8/0
Rep. 7	1/7	4/4	6/2	7/1	7/1	7/1	8/0
Rep. 8	1/7	2/6	6/2	7/1	6/2	7/1	8/0
total	14/50	36/28	52/12	45/19	54/10	59/5	63/1
p-value	<0.001	<0.001	<0.001	<0.001	0,009	0,208	

d) Sulfoxaflor

Dose (ng)	105	52.5	21	5.25	2.6	1.05	0.525	0
Rep. 1	0/8	0/8	8	2	8	7	7	8
Rep. 2	0/8	0/8	8	4	8	7	7	8
Rep. 3	0/8	0/8	1	4	8	7	8	8
Rep. 4	0/8	0/8	3	4	8	8	8	8
Rep. 5	0/8	0/8	4	6	6	8	8	8
Rep. 6	2/6	2/6	5	6	6	8	8	8
Rep. 7	1/7	2/6	5	8	6	8	8	8
Rep. 8	1/7	1/7	6	5	6	8	8	8
total	**4/60**	**5/59**	**40/24**	**39/25**	**56/8**	**61/3**	**62/2**	**64/0**
p-value	<0.001	<0.001	<0.001	<0.001	0,006	0,244	0,496	

Table S3.2 Literature data on the contamination of floral and extrafloral[a] nectar with the four insecticides tested in this study. Values refer to an assumed consumption of 2 µl ($\approx$ 2 mg) by *N. vitripennis.* For comparison, the lowest sublethal doses are given having shown significant effects in this study in at least one of the bioassays.

	Amount in nectar (ng/2mg $\approx$ ng/2µl)	First significant effects in this study	Reference
Acetamiprid	0.0001 - 0.0152	0.63	(Zioga et al., 2020)
	0.14		(Heller et al., 2020)
	0.012		(Azpiazu et al., 2019)
	0.02-0.36		(Capela et al., 2022)
	0.024 / 0.194[a]		(Zhou, Milne, et al., 2022)
	up to 0.026		(Pohorecka et al., 2012a, 2012b)
	up to 0.012		(Demares et al., 2022)
Flupyradifurone	0.52	2.63	(Campbell et al., 2016)
	8.0		(Siviter & Muth, 2022)
	0.4-3.0 / up to 31.8[a]		(EPA, 2015)
	up to 0.004		(Bishop et al., 2020)
Dimethoate	0.2 - 45.9	0.105	(Zioga et al., 2020)
Sulfoxaflor	0.014 - 0.028	0.525	(Jiang et al., 2020)
	0.01 – 0.094		(Siviter et al., 2019)
	0.4		(Zhou, Chen, et al., 2022)
	0.06 – 2.0		(EPA, 2016)

[a]extrafloral nectar

3.6 Author Contributions

Nils Schöfer: Methodology; Investigation; Formal analysis; Visualization; Writing—original draft, Writing-review & editing.

Julian Ackermann, Julian Hoheneder: Investigation; Formal analysis.

John Hofferberth: Resources; Writing-review & editing.

Joachim Ruther: Conceptualization; Funding acquisition; Methodology; Project administration; Supervision; Writing-review & editing.

4. Effects of sub-nanogram doses of acetamiprid, dimethoate, flupyradifurone, and sulfoxaflor on courtship, mating, and olfactory host finding of the parasitic wasp *Lariophagus distinguendus*

N. Schöfer, G. Ratschmann and J. Ruther

Abstract: Continued use and development of new insecticides are seen as a necessary part of modern agriculture, but insecticides, even at sublethal levels, can also affect beneficial non-target species, such as pollinators, predators and parasitic wasps. Here, we studied sublethal effects of the four insecticides, acetamiprid, dimethoate, flupyradifurone and sulfoxaflor, all targeting cholinergic neurons in insects, on sexual communication and olfactory host finding in the parasitic wasp *Lariophagus distinguendus* Förster (Hymenoptera: *Pteromalidae*). We found that all four insecticides reduced, at least at one of the tested sublethal doses, pheromone-mediated courtship behaviour (wing fanning) in males and the mating frequency of treated wasps. Treated females no longer preferred host-associated kairomones. This suggests that the olfactory sense of the wasps is hampered by sublethal insecticide doses. The lowest bioactive doses tested in the bioassays were 0.021 ng for sulfoxaflor and 0.105 ng for acetamiprid, dimethoate and flupyradifurone. These low amounts are field realistic and might be taken up by the wasps, e.g., by feeding contaminated plant nectar. Exposure to these compounds likely compromise the important ecosystem service provided by parasitic wasps as natural enemies in terrestrial ecosystems.

4.1 Introduction

Insect pests are a global threat to agriculture and thus to humanity's food supply. In industrialised agriculture, insect pests have long been controlled with different classes of insecticides, including insect growth regulators, designed to inhibit their growth, reproduction and/or spread, often by attacking the pests' nervous system (Nauen et al., 2015; Tomizawa et al., 2003; Van Scoy et al., 2016; Watson et al., 2011). These insecticides are easy to use and can be either sprayed in crops or applied to the crop substrate aiming at systemic activity. Due to their non-specificity as well as adverse effects at sublethal levels (Demirci & Gungordu, 2020; Huang et al., 2022; Stapel et al., 2000), insecticides are thought, among other factors, to contribute to the massive decline of insect biodiversity worldwide (Gibbs et al., 2009; Gilburn et al., 2015). There is, however, increasing evidence that very low doses of chemical stressors such as insecticides may also have stimulatory (hormetic) effects on insects (Cutler et al., 2022; Guedes et al., 2022). Many of the active compounds in commercial insecticides are neurotoxins. Some of the most widely used insecticides such as the neonicotinoids overstimulate the nervous system by attacking nicotinic acetylcholine receptors (nAcChR). The use of these insecticides is considered safe for crops, posing less risk to mammals and humans due to their lower affinity for mammalian nAcChR (Nauen et al., 2015; Van Scoy et al., 2016). However, they still exert pressure on wild non-target insect populations that are even remotely associated with farmland ecosystems (Hallmann et al., 2017; Pistorius et al., 2010; Watson et al., 2011; Yamada, 1997). In addition to being lethal for agricultural insect pests, several insecticides have adverse lethal and effects on non-target organisms (Fischer et al., 1997; Tosi & Nieh, 2019). The honeybee, in particular, has become an important model organism for studying these insecticide side effects (Colin et al., 2004; Tosi & Nieh, 2019).

Adverse effects on bees were also the reason that three members of the intensively used insecticide class of neonicotinoids, imidacloprid, thiamethoxam and clothianidin, were banned within the European Union (EU) (European Commission, 2018). Outside the EU, these substances are still widely used, with large markets, for instance in India and China (Anand et al., 2021; G. H. Wang et al., 2020). Following this ban, alternative insecticides became more popular within the EU, including other neonicotinoids such as acetamiprid (Jerez et al., 2022). While novel insecticides such as the butenolide flupyradifurone and the sulfoximine sulfoxaflor have a lower acute toxicity than

imidacloprid (Nauen et al., 2015; Watson et al., 2011), they are still effective as control agents against pests. Another mode of action of pesticides found in the organophosphates is the inhibition of the enzyme acetylcholine esterase (O'brien, 1963). Inhibition of this enzyme prevents degradation of the natural receptor ligand acetylcholine, which leads to overstimulation of the receptor and eventually to paralysis and death in invertebrates. A member of this class is dimethoate, which is no longer approved in the EU but still in use in the USA and many developing markets (Center for Disease Control and Prevention, 2017).

In the past decades, many insect taxa including pests (Guedes et al., 2016), pathogen vectors (Agathokleous et al., 2023), and beneficial non-target insects (Desneux et al., 2007) have been studied with respect to sublethal effects of insecticides. Apart from pollinators, parasitic wasps were of particular interest in studies on beneficial non-target species in the past (Desneux et al., 2007; Jiang, Liu, Huang, et al., 2019; Jiang, Liu, Zhang, et al., 2019; Kremer & King, 2019; Tappert et al., 2017; Teder & Knapp, 2019), because of their important function as biological control agents of insect pests (Niedermayer et al., 2016). Parasitic wasps have a close relationship with their hosts, developing within or attached to their host species (Godfray, 2010; Niedermayer et al., 2016), many of which are pest insects. As such, these wasps provide a valuable ecosystem service (Bengtsson, 2015). Parasitic wasps are exposed to various insecticides via their use of floral and extrafloral nectar and honeydew as carbohydrate sources (Bailey et al., 2009; Bugg et al., 1989; Idris & Grafius, 1995; König et al., 2015; Wackers et al., 2008).

Parasitic wasps, like pollinators, use their olfactory sense for orientation. In particular, they rely on pheromones, kairomones and synomones to find sexual partners and hosts for egg-laying, respectively (Ruther et al., 2009; Schurmann et al., 2009; Steidle & Schöller, 1997; Zhang et al., 1998). As nAcChR are involved in the processing of chemical information in insects (Dupuis et al., 2012; Jones & Sattelle, 2010), previous research on parasitic wasps has shown that insecticides targeting the cholinergic system have sublethal effects on the response of parasitoids to semiochemicals (Jiang, Liu, Zhang, et al., 2019; Schöfer et al., 2023; Tappert et al., 2017; Teder & Knapp, 2019). In the pteromalid wasp *Nasonia vitripennis* Walker (Hymenoptera: *Pteromalidae*), the neonicotinoid imidacloprid affected the wasps' ability to find and recognize mates, the copulation rate and their ability to find hosts using host-

associated volatiles (Tappert et al., 2017). Similar effects were found in a recent study investigating the effects of acetamiprid, dimethoate, flupyradifurone and sulfoxaflor on sexual communication, mating and host finding in *N. vitripennis* (Schöfer et al., 2023). It must be assumed that sublethal effects, such as reported for *N. vitripennis*, have a negative impact on the fitness of wild parasitic wasp populations. As parts of the olfactory system are highly conserved within insects (Couto et al., 2017; Krieger et al., 2003), effects seen within *N. vitripennis* can reasonably be expected to occur also in other parasitic wasps, though the strength of the effects may vary. If the results are transferable to other species, this would certainly affect the efficacy of parasitic wasps as biological control agents. Before broader conclusions can be drawn, however, additional species with well-developed bioassay methods need to be studied with respect to their susceptibility to sublethal insecticide doses. In the present study, we investigate sublethal effects of four insecticides on *Lariophagus distinguendus* Förster (Hymenoptera: *Pteromalidae*), like *N. vitripennis* a member of the subfamily Pteromalinae (Graham, 1969).

L. distinguendus is a solitary parasitoid wasp that lays its eggs on the late larval to pupal stages of stored-product infesting beetles (Niedermayer et al., 2016). Females find seeds infested by grain weevils, their main hosts, by volatile odourants in the larval faeces (Steidle & Schöller, 1997) and typically lay a single egg per infested seed, though superparasitism may occasionally occur (Bellows, 1985b). The hatched larvae feed on the hosts and, depending on the temperature, develop within 14-31 days to the adult stage (Ryoo et al., 1991). Males emerge earlier than females and mating occurs primarily at their natal host patch (Niedermayer et al., 2016). *L. distinguendus* has shown great potential in controlling populations of many beetle species harmful to grain crops and has been used in this context as part of integrated pest management (Adarkwah et al., 2012; Hansen et al., 2013; Harush et al., 2021; Iturralde-Garcia et al., 2020).

In this paper, we investigate the sublethal effects of acetamiprid, dimethoate, flupyradifurone and sulfoxaflor applied topically as an acetone solution to the wasps, on the males' response to the female sex pheromone, the mating frequency and the females' olfactory host finding ability.

4.2 Materials and Methods

4.2.1 Insects

A culture of *L. distinguendus* was obtained from a commercial supplier (Biologische Beratung Prozell & Schöller, Berlin, Germany) and reared at 25°C, 50% r.h. and a 16-8h light/dark cycle on larvae and pre-pupae of the granary weevil, *Sitophilus granarius* L. (Coleoptera: *Curculionidae*). Hosts were reared in 400-ml screw-top glass jars containing 200 ml of wheat, *Triticum aestivum* L., grain that had been moistened with 7 ml of distilled water. 25 ml of newly emerged, mixed sex weevils were added to each glass jar and allowed to mate and lay eggs for one week. Subsequently, weevils were removed by sieving and transferred to new breeding jars. On the first Monday of each month, weevils were replaced by those from the next generation. To rear *L. distinguendus*, ca. 20 g of 4-5-week-old weevil-infested grains were filled into Petri dishes, and ca. 50-100 adult *L. distinguendus* were added. Females were allowed to parasitise weevil larvae until they died. At rearing conditions, the next wasp generation emerged after approximately 21 days. To obtain wasps of defined age and mating status for experimentation, weevil-infested grains parasitised by *L. distinguendus* were individually placed in Eppendorf tubes shortly before their expected emergence. Tubes were examined daily for newly emerged wasps that were then used for the experiments.

4.2.2 Insecticides

Analytical standards of the tested insecticides acetamiprid (≥98.0% purity), dimethoate (≥98.0% purity), flupyradifurone (≥98.0% purity) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Sulfoxaflor (99.23% purity) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Insecticides were dissolved in acetone (ROTISOLV®, ≥ 99.8% purity, Carl Roth GmbH, Karlsruhe, Germany).

4.2.3 Toxicity tests

Testing sublethal effects of substances requires an accurate assessment of the substances' acute toxicity. For this purpose, dilution series (Table S4.1 in the supporting information) of acetamiprid, dimethoate, flupyradifurone and sulfoxaflor were prepared in acetone, starting with a stock solution of 1 mg/ml. 210 nl of these dilutions were then applied to the abdomens of ice-cooled wasps using a microinjector

(Nanoliter 2010, World Precision Instruments, USA). Topical application was chosen instead of oral uptake to enable exact dosage control (Tappert et al., 2017). The applied volume of acetone has been shown to have no adverse effects in another pteromalid wasp of comparable size (Jatsch & Ruther, 2021). Groups of 8 wasps (n = 3 replicates per dose/sex) were treated with ever-decreasing insecticide doses and with pure acetone (control). After 72 h, the proportion of surviving wasps was determined for each insecticide concentration. Survival data were used to fit probit sigmoid dose-response curves for the four insecticides (Figure 4.1) using MedCal (version 22.016) scientific software (MedCal Software Ltd., Ostend, Belgium) and to calculate those doses (with 95% confidence intervals) from the respective models at which 50% of the treated wasps died (LD50). For the bioassays, 2-3 doses were tested for each insecticide that were lethal for less than 30% of the treated wasps (Table 4.1). For control, wasps treated with pure acetone were used in the bioassays.

4.2.4 Effects on pheromone communication

Male *L. distinguendus* show a characteristic wing-fanning behaviour when perceiving the odour of a female, and this behaviour is an integral part of the mating ritual (Benelli et al., 2014; Ruther et al., 2000). Assessing whether the four insecticides inhibit this response, males (n=20 for each experiment) were treated with a dose of the active ingredient or with pure acetone (control) and placed one day later into a round mating arena (2 cm diameter, 5 mm height) containing a dead female (dummy). Dummies were prepared by freezing females at -20° C shortly after emergence (maximum age 1 d). Previous studies demonstrated that males react strongly to these dummies and exhibit wing-fanning in response to the female contact pheromone consisting of cuticular hydrocarbons (Kühbandner et al., 2012; Steiner et al., 2005). Preliminary experiments revealed that untreated males and control males treated with acetone show no significant difference in their reactions (results not shown). Males were exposed to the female dummies for 5 min with the number of wing-fanning bouts being recorded using The Observer XT 15 Software (Noldus Information Technology, Wageningen, The Netherlands). Dummies were used only once, and the arena was cleaned with ethanol after each bioassay.

4.2.5 Effects on mating frequency

Male and female *L. distinguendus* exhibit ritualized courtship behaviour that involves the female contact sex pheromone arresting males near the female (Ruther et al., 2000) and a male oral pheromone of unknown chemical structure that males spread over the female antennae to elicit receptivity (König et al., 2015). For this series of experiments, wasp pairs were assigned to three groups. In the first group, only the males were treated one day prior to the bioassay with a sublethal dose of the insecticides. In the second group, only the females and in the third group both partners were treated (n=20 for each treatment). For the bioassay, the pair was placed into a courtship arena of the same dimensions as the one used in the pheromone experiments, with the female always being placed first. Once the male was added, the arena was closed with a cover slip, and the pair was observed for 5 min using The Observer XT 15 Software. If the female accepted the male and allowed copulation, the trial was recorded as a success. For control, the respective mates were treated with pure acetone.

4.2.6 Effects on host finding

After mating, female *L. distinguendus* search for hosts by using volatiles emitted from larval host faeces (Steidle & Schöller, 1997; Steiner et al., 2007). To test sublethal effects of the four insecticides on this process, mated females were treated with a sublethal insecticide dose (control: acetone, n=20 for each treatment). After the treatment, females were kept overnight in Eppendorf tubes with five weevil-infested wheat grains. The lids of the tubes were punctured with a fine needle to supply the wasps with air. This pre-treatment was performed to increase the responsiveness of females to the odour of host faeces, as they have been shown to learn odours associatively when rewarded with oviposition success (Steidle, 1998). The next day, the response of females to the odour of host faeces was tested in a four-chamber olfactometer (Ruther et al., 2000; Steidle & Schöller, 1997). This olfactometer consisted of a plastic cylinder (19 cm diameter, 4 cm height) divided by vertical plates into four equal chambers. The cylinder was covered by a walking arena of metal mesh that prevented visual orientation to the samples presented in the chambers but allowed diffusion of volatiles from the samples through the mesh into the walking arena. The walking arena was covered by a glass plate to prevent the wasps from escaping. A

cardboard cylinder (21 cm diameter, 9 cm height) was placed over the arena to exclude external visual stimuli, and the olfactometer was illuminated from above by a desk lamp (60 W). In one of the chambers, a glass dish was presented that contained 150 mg of *S. granarius* larval faeces, while the opposite chamber (control) contained an empty glass dish. The two remaining chambers of the olfactometer were left empty. A female (n=20 per treatment) was then placed in a central cavity of the arena (starting point) and allowed to move freely around the arena for 5 min. The residence time of females in the sectors above the sample and the control chamber, respectively, was recorded using the Observer XT 15 software. The olfactometer was rotated clockwise by 90° after each observation to avoid biased results due to potential side preferences.

4.2.7 Quantification of the amount of artificial nectar consumed by *L. distinguendus*

Contaminated carbohydrate sources such as plant nectar is a likely natural source of exposure for parasitic wasps. To evaluate whether the concentrations of insecticides reported in the literature justify the claim that *L. distinguendus* may take up bioactive doses via this avenue, it is necessary to know the volume of nectar taken up by the wasps. Therefore, a feeding experiment with artificial nectar (30% glucose in water) was performed as recently described for *N. vitripennis* (Schöfer et al., 2023). Briefly, females (n=6) were exposed for 48 h in Eppendorf tubes to 10 µl of the glucose solution. After this period, females were removed, and the remaining glucose was quantified after derivatization by gas chromatography/mass spectrometry. Control tubes (n=6) were treated equally without adding a wasp. The difference between the two treatments gives an estimate of the glucose solution consumed by the wasps. For more details regarding the bioassay, the sugar derivatization method and the GC/MS parameters see (Schöfer et al., 2023).

4.2.8 Statistical analyses

All statistical analyses were performed using R 4.0.1 (R Development Core Team, 2023). Data from the pheromone experiment was analysed with a Kruskal-Wallis test and subsequent Dunn's tests for comparisons between each sublethal dose and the respective acetone control utilising the kruskal.test (base R package) and dunn.test (dunn.test package) functions. The survival rates of insecticide-treated and control wasps, copulation rates from the courtship experiments, as well as the number of

males not showing any wing fanning behaviour in the pheromone experiments were analysed with Fisher's exact test. To visualise the data, PAST 4.03 software (Hammer et al., 2001) was used to plot bar charts and boxplots.

4.3 Results

4.3.1 Toxicity tests

The dose-mortality curves disclosed the insecticides differing strongly in their acute toxicity (Figure 4.1, Table 4.1). Dimethoate (model fit: Chi^2=159.77, df=1, p<0.001) exhibited the lowest toxicity (LD50 = 2.1 ng), Acetamiprid (model fit: Chi^2=220.68, df=1, p<0.001, LD50 = 0.77 ng) and flupyradifurone (model fit: Chi^2=274.27, df=1, p<0.001, LD50 = 0.80 ng) displayed intermediate toxicities, while sulfoxaflor (model fit: Chi^2=354.82, df=1, p<0.001) was most toxic to *L. distinguendus* (LD50 = 0.30 ng). The differences in acute toxicity of the four insecticides led to the use of different sublethal doses in the bioassays depending on the active ingredient (Table 4.1). Sublethal doses are defined as inducing no statistically significant mortality in an experimental population (Desneux et al., 2007). Accord to this definition, the no-observed-effect levels (NOEL) for acetamiprid, dimethoate, flupyradifurone and sulfoxaflor were determined as 0.21, 0.63, 0.21 and 0.105 ng, respectively (Table 4.1; Table S4.1 in the supporting information). Except for the highest doses of acetamiprid and flupyradifurone, the doses tested in this study can be considered as being sublethal (Table 4.1, Table S4.1 in the supporting information).

Table 4.1 LD50-values (evaluated after 72 h) and 95% confidence intervals (CI) obtained from the toxicity tests by fitting probit sigmoid dose-response curves using MedCal (version 22.016) scientific software (figure 4.1) and doses of the four insecticides tested in the bioassays with *Lariophagus distinguendus*. Values in brackets represent the percentages of surviving wasps in the toxicity tests. Sublethal doses, i.e., those below the no-observed-effect-level (NOEL, see Table S4.1 in the supporting information) are indicated by an octothorpe (#). Doses indicated with (n.t.) were tested in some of the bioassays but not in the toxicity tests.

	LD_{50} (ng/wasp)	95% CI	Tested sublethal doses (ng/wasp)			
Acetamiprid	0.77	0.63-0.91	0 (96%)	0.105# (94%)	0.21# (83%)	0.42 (n.t.)
Dimethoate	2.1	1.8-2.5	0 (90%)	0.105# (88%)	0.21# (96%)	0.63# (92%)
Flupyradifurone	0.80	0.70-0.90	0 (96%)	0.105# (n.t.)	0.21# (83%)	0.42 (77%)
Sulfoxaflor	0.30	0.26-0.36	0 (92%)	0.021# (94%)	0.063# (92%)	0.105# (83%)
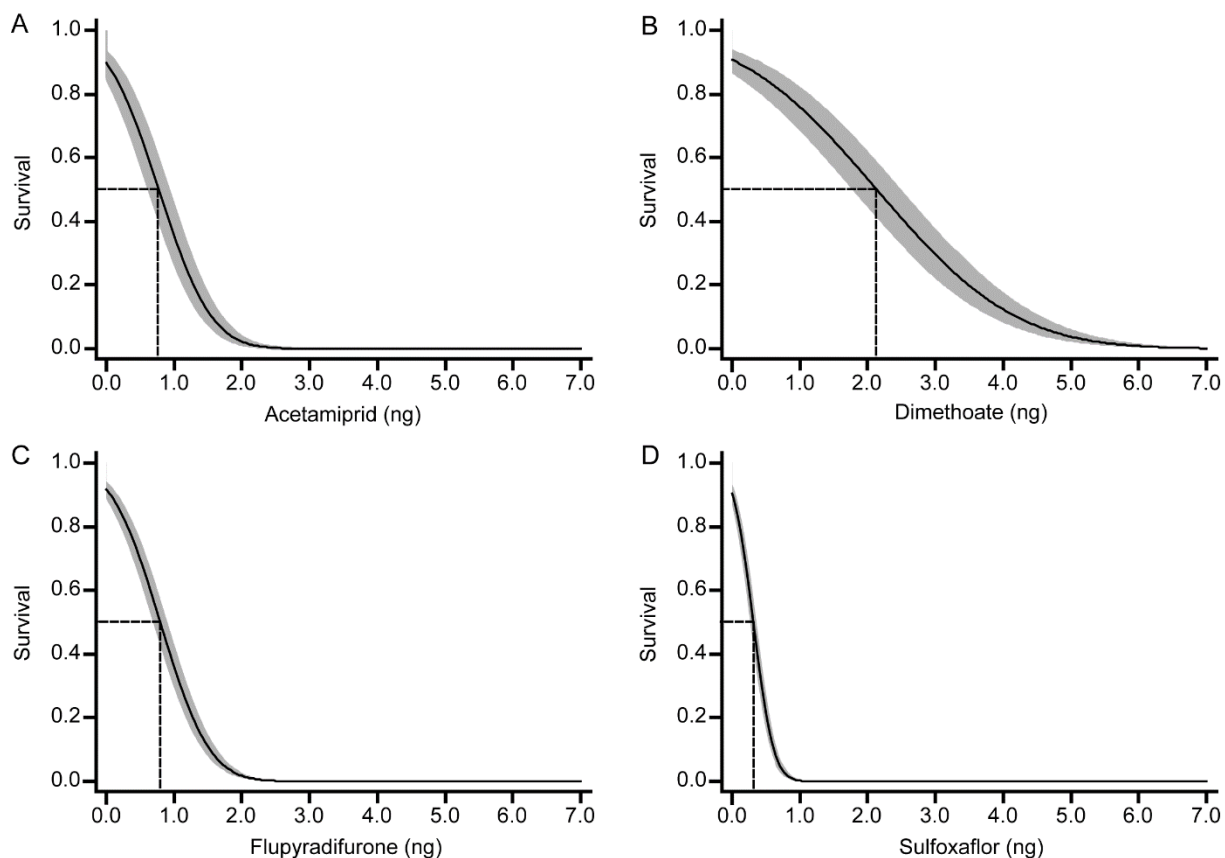
Figure 4.1 Dose-mortality curves after 72 h of *Lariophagus distinguendus* treated topically with different doses of acetamiprid, dimethoate, flupyradifurone and sulfoxaflor dissolved in acetone. The 95% confidence interval is indicated in grey. The dashed lines indicate the LD50.

4.3.2 Effects on pheromone communication

In all experiments, acetone-treated control males exhibited extensive wing fanning behaviour towards female dummies (Figure 4.2 a-d). Except for the lowest dose of dimethoate, each dose of all four active ingredients reduced the number of wing fanning bouts in insecticide-treated males. Sulfoxaflor affected male wing fanning at doses as low as ≥ 0.021ng, while acetamiprid (0.21ng), flupyradifurone (≥ 0.105ng) and dimethoate (≥ 0.21ng) showed effects only at the higher of the tested doses, but still in the sub-nanogram range. With all substances, there were also males that no longer showed any wing fanning behaviour at all. When compared to the control males (100% response), however, the number of these non-responders was significantly increased only for the two higher doses of flupyradifurone (Fishers exact test: 0.21ng, 75% response, p=0.0471; 0.42ng, 75% response, p=0.0471).
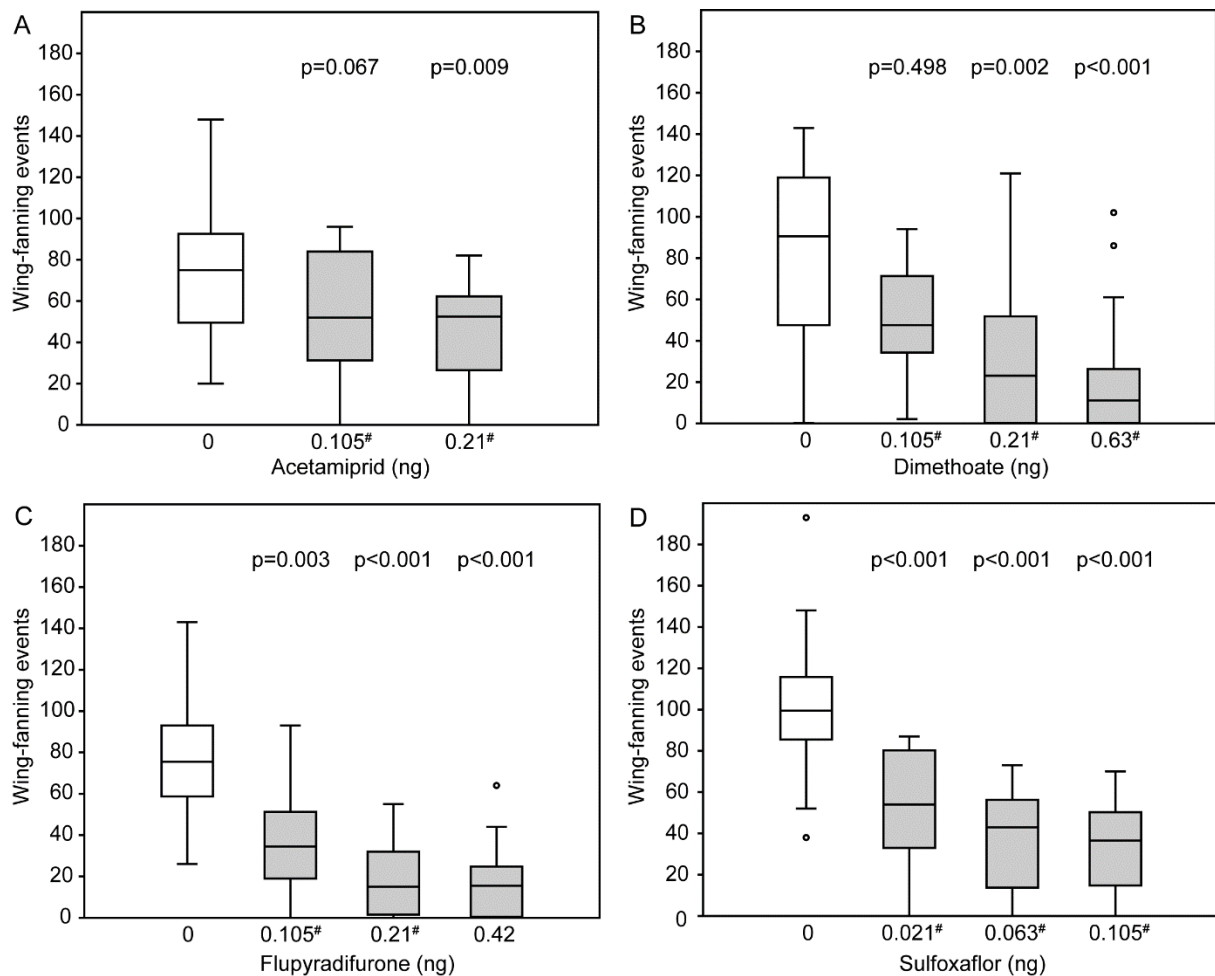
Figure 4.2 Effect of sublethal doses of the four insecticides on the wing fanning response of virgin *Lariophagus distinguendus* males to freeze-killed females (dummies). Males were either treated with pure acetone (0 ng, control) or a sublethal dose of (a) acetamiprid, (b) dimethoate, (c) flupyradifurone, or (d) sulfoxaflor. Given is the number of wing fanning bouts during a 5 min observation time. Box-and-whisker plots show median (horizontal line), 25–75% quartiles (box), maximum/minimum range (whiskers) and outliers (° means > 1.5 × box height). Statistical comparison between treatments and controls by a Kruskal-Wallis test and subsequent Dunn's tests (n=20). Sublethal doses are indicated by an octothorpe (#).

4.3.3 Effects on the mating frequency

Male and female *L. distinguendus* exhibit ritualized courtship behaviour that involves the female contact sex pheromone arresting males near the female (Ruther et al., 2000) and a male oral pheromone of unknown chemical structure that males spread over the female antennae to elicit receptivity (König et al., 2015). For this series of experiments, wasp pairs were assigned to three groups. In the first group, only the males were treated one day prior to the bioassay with a sublethal dose of the

insecticides. In the second group, only the females and in the third group both partners were treated (n=20 for each treatment). For the bioassay, the pair was placed into a courtship arena of the same dimensions as the one used in the pheromone experiments, with the female always being placed first. Once the male was added, the arena was closed with a cover slip, and the pair was observed for 5 min using The Observer XT 15 Software. If the female accepted the male and allowed copulation, the trial was recorded as a success. For control, the respective mates were treated with pure acetone.

Figure 4.3 Effect of sublethal doses of the four insecticides on the mating frequencies of virgin *Lariophagus distinguendus*. Shown are the mating frequencies of pairs, in which either the male, the female or both partners were treated with pure acetone (0, control) or a sublethal dose (given in ng in the columns) of (a) acetamiprid, (b) dimethoate, (c) flupyradifurone, or (d) sulfoxaflor. Significant differences between the sublethal doses and the control analysed by Fisher's exact test are marked by asterisks (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, n=20). Sublethal doses are indicated with an octothorpe (#).

4.3.4 Effects on host finding

In all control experiments, acetone-treated females showed a significant preference for the host zone of the olfactometer (Figure 4.4a-d). When treated with any of the sublethal doses of acetamiprid ($\geq$0.105 ng), females no longer preferred the host odour (Figure 4.4a). In contrast, dimethoate and sulfoxaflor disrupted the preference for host odour only at the highest tested doses (dimethoate: 0.63 ng, sulfoxaflor: 0.105 ng, Figure 4.4b and d). Interestingly, females treated with 0.105 ng of flupyradifurone spent similar amounts of time in the host and control zones, while those treated with higher doses (0.21-0.42 ng) again showed a slight but significant preference for host odour (Figure 4.4c).
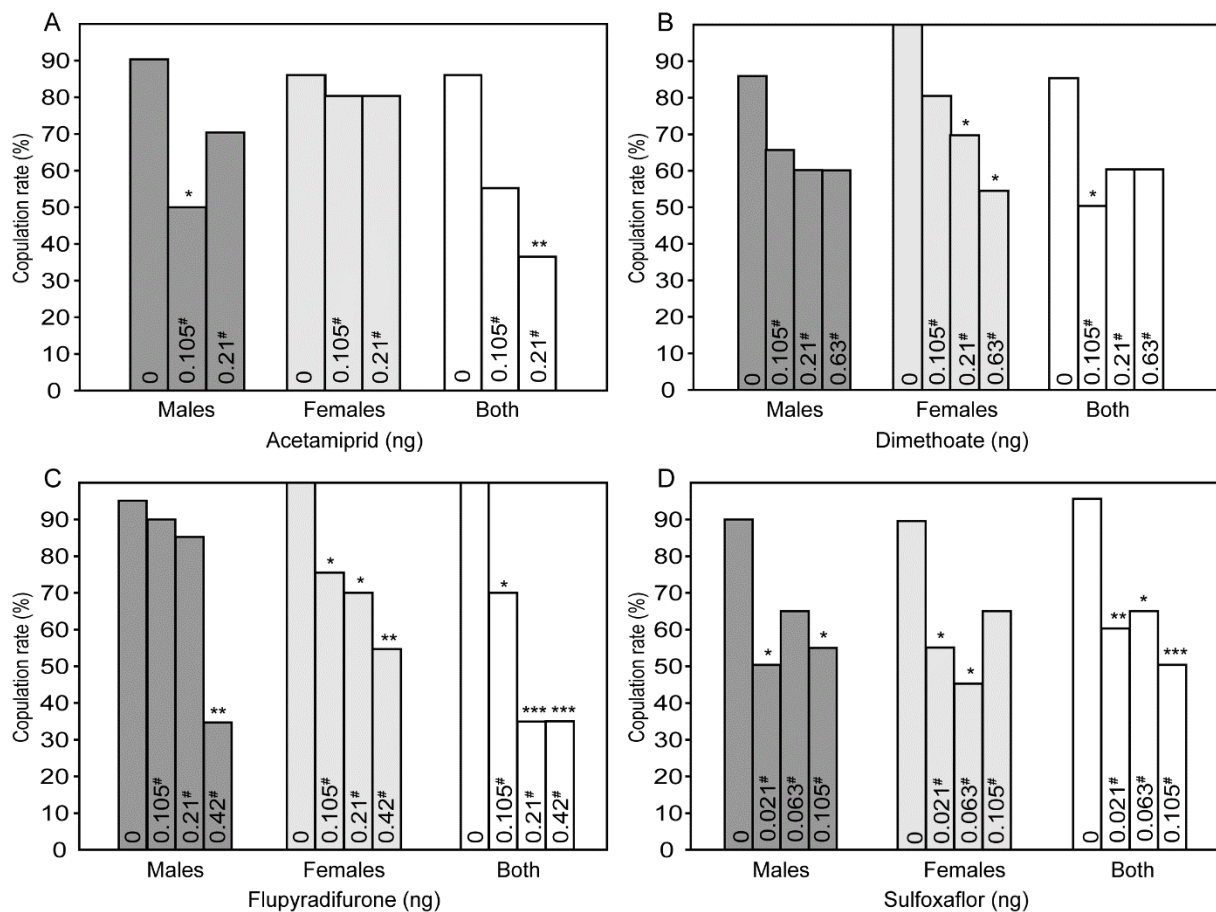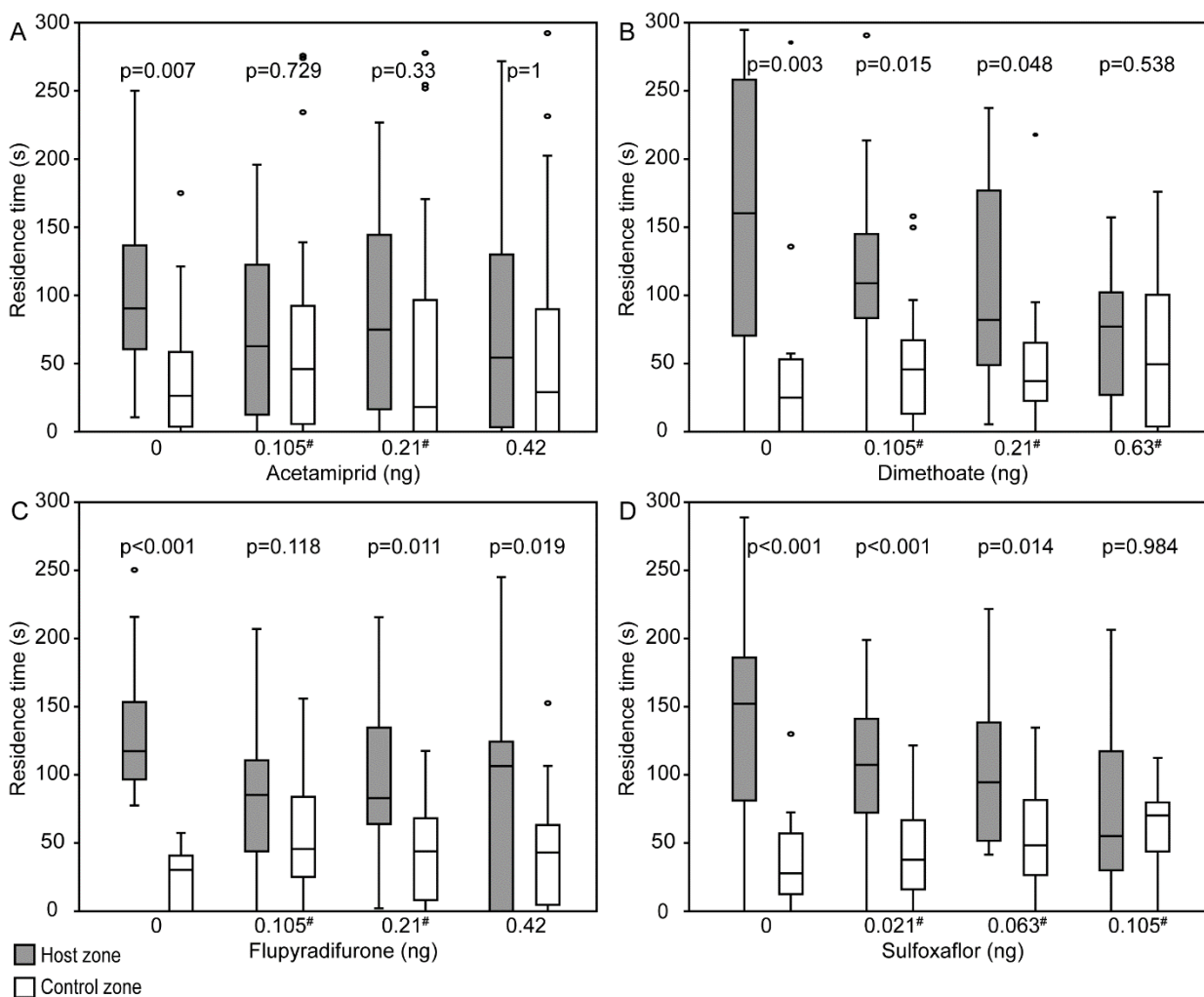

Figure 4.4 Effect of sublethal doses of the four insecticides on the response of mated *Lariophagus distinguendus* females to host odour (150 mg larval faeces of *Sitophilus granarius*). Females were either treated with pure acetone (0 ng, control) or a dose of (a) acetamiprid, (b) dimethoate, (c) flupyradifurone, or (d) sulfoxaflor and tested in a four-chamber olfactometer one day after application. Shown are the residence times of females in the host zone and the untreated control zone of the olfactometer. Box-

and-whisker plots show median (horizontal line), 25–75% quartiles (box), maximum/minimum range (whiskers) and outliers (° means > 1.5 × and * means > 3 × box height). Statistical analysis for each treatment by Wilcoxon matched pairs test (n = 20). Sublethal doses are indicated by an octothorpe (#).

4.3.5 Quantification of the amount of artificial nectar consumed by *L. distinguendus*

After the 48-h feeding period, 3.57 ± 0.0.16 mg (mean ± SE) glucose was recovered from the control Eppendorf tubes while in those containing *L. distinguendus* females significantly lower amounts were found (2.85 ± 0.17 mg) (Mann-Whitney U-test: p=0.0306). Hence, female *L. distinguendus* consumed within 48 h 20% of the total amount supplied to them (0.72) mg. With a sample volume of 10 µl used in the feeding experiment, females ingested approximately 2 µl of the artificial nectar.

4.4 Discussion

The results of this study expand our understanding of the adverse effects of insecticides on beneficial species by showing that the four insecticides acetamiprid, dimethoate, flupyradifurone and sulfoxaflor interfere with the function of the olfactory system of *L. distinguendus.* Treatment of the wasps with sublethal doses of the four active substances resulted in wasps struggling to recognise mating partners and hosts by their odours. A comparison of the results of the present study with *N. vitripennis*, for which similar experiments were recently conducted with the same four insecticides (Schöfer et al., 2023) revealed that, except for dimethoate, *L. distinguendus* is much more sensitive to the insecticides than *N. vitripennis* (Table 4.2). The LD50 of acetamiprid, flupyradifurone and sulfoxaflor is 16-, 50-, and 128-fold lower for *L. distinguendus.* This difference in acute toxicity was associated with the fact that much lower doses of the three insecticides were sufficient to affect pheromone-mediated mate finding, mating frequency and olfactory host finding in *L. distinguendus.* For example, doses as low as 21 picograms of sulfoxaflor reduced both the wing fanning response of males to the female sex pheromone and the mating frequency in *L. distinguendus*, whereas in *N. vitripennis* a 125-fold higher dose was required to affect the mating frequency, and the pheromone response of females to the male sex pheromone was not affected at all even at a 250-fold higher dose. Similar stark differences between the two species were observed for flupyradifurone and, less pronounced, for acetamiprid. Since both species are closely related (subfamily

Pteromalinae) and have a similar size (2-3 mm), this difference in sensitivity is surprising and confirms previous findings in bees. The leafcutter bee *Megachile rotunda* Fabricius (Hymenoptera: *Megachilidae*) has been shown to be >2500-fold and 170-fold more sensitive (acute toxicity) to the neonicotinoid thiachloprid and flupyradifurone, respectively, than other managed bee pollinators. This difference is due to *M. rotunda* lacking a P450 enzyme involved in the detoxification of xenobiotics (Hayward et al., 2019). Whether this is also the cause of the difference in sensitivity of *N. vitripennis* and *L. distinguendus* requires further investigation. In any case, the results with *M. rotunda* and those of the present study show that results obtained with one model organism in toxicological studies are not necessarily transferable to others, even closely related ones. This must be taken into account in future approval procedures for new active substances.

Wing-fanning plays an important role in interspecific communication of many parasitic wasps (Benelli et al., 2012; Benelli, Bonsignori, et al., 2013). In *L. distinguendus*, this behaviour is elicited by female cuticular hydrocarbons which are processed by the males' olfactory system (Benelli, Giunti, et al., 2013; Ruther et al., 2000). When compared to acetone-treated control males, each insecticide reduced the number of wing-fanning bouts in reaction to female dummies. The proportion of treated males showing no longer wing fanning at all was significantly increased only for the two highest flupyradifurone doses suggesting that these inactive males were no longer able to perceive the female cuticular hydrocarbons. In treated males which exhibited wing fanning but to a lesser extent than the control males, motor skill impairment may have contributed to the reduced wing fanning response in addition to olfactory impairment, since low doses of insecticides targeting nAChR may also influence motor function in insects (Eiri & Nieh, 2012; Williamson et al., 2014). Either way, given that the frequency of wing fanning in *L. distinguendus* differs between successful and unsuccessful males (Benelli, Bonsignori, et al., 2013), it is reasonable to assume that insecticide-induced impairment of male wing fanning leads to a reduction in their mating success. Another decisive stimulus for successful mating in *L. distinguendus* is the male oral aphrodisiac pheromone (König et al., 2015). Females that are no longer able to perceive this chemical signal due to insecticide treatment should remain unreceptive. Consistent with these considerations, treating males, females, or both partners with insecticides reduced the mating frequency in our experiments to levels as low as 35% for

flupyradifurone. A recent study demonstrated that sublethal doses of flupyradifurone modified the cuticular hydrocarbon profile in an oligolectic bee (Boff & Ayasse, 2023). In our study, treatment of females with flupyradifurone reduced the mating frequency at any of the tested doses. Given the importance of cuticular hydrocarbons in the courtship behaviour of *L. distinguendus* (Kühbandner et al., 2012; Steiner et al., 2005) and the fact that slight modifications of the profiles can disrupt the male response (Kühbandner et al., 2013), it will be interesting to investigate whether a flupyradifurone-induced change of the female cuticular hydrocarbon profile has contributed to the reduction of the mating frequency and whether other insecticides cause similar effects. A recent study (Muller et al., 2017) investigating sublethal effects of the pyrethroid lambda-cyhalothrin on a leaf beetle suggests that sublethal effects of insecticides on the CHC profile of insects are not restricted to flupyradifurone.

In the mating experiments, it is noticeable that some findings seem to be contradictory or inconsistent. For example, it is difficult to understand why an insecticide has an effect when only one sex was treated, but this effect is lost when both partners were treated. Also, effects of a low or medium dose that are lost at a higher dose seem difficult to understand at first glance. However, such biphasic dose-response relationships are not uncommon in biology, toxicology and medicine (Calabrese, 2013; Cutler et al., 2022; Guedes et al., 2022). An alternative explanation is the fact that the mating frequency was not 100% in all control experiments. Hence, only trends were observed in some experiments that might translate into statistically significant differences at higher sample sizes.

Except for parthenogenic insects, mating is a prerequisite to produce offspring. Therefore, it is tempting to assume that the effects of failed mating are less severe in *L. distinguendus* than in other insects, because, as a haplodiploid species, females are able to produce male offspring from unfertilized eggs. However, *L. distinguendus* is a quasi-gregarious parasitoid with a mating system characterized by local mating competition, and the sex ratio is typically skewed towards females when sufficient high-quality hosts are available (Simbolotti et al., 1987; Werren & Simbolotti, 1989). Hence, virginity caused by exposure to sublethal insecticide residues is likely associated with fitness costs for *L. distinguendus* females.

Table 4.2 Comparison between the susceptibility of *L. distinguendus (*this study) and *N. vitripennis (*Schöfer et al., 2023) to acetamiprid (Ace), dimethoate (Dim), flupyradifurone (Flu) and sulfoxaflor (Sul). Given are the doses (ng) that killed 50% of treated wasps after topical application (LD50) and the lowest sublethal doses that caused significant effects in bioassays studying pheromone response, mating frequency and olfactory host finding. The rightmost column shows the factor by which *L. distinguendus* is more sensitive (>1) or less sensitive (<1) to each parameter than *N. vitripennis.*

		N. vitripennis	*L. distinguendus*	factor
LD50	Ace	12.6	0.77	16
	Dim	2.2	2.1	1.05
	Flu	102	0.80	128
	Sul	14.9	0.30	50
Pheromone response	Ace	0.63	0.105	6
	Dim	0.105	0.21	0.5
	Flu	21	0.105	200
	Sul	>5.25	0.021	>250
Mating frequency	Ace	6.3	0.105	60
	Dim	1.05	0.105	10
	Flu	21	0.105	200
	Sul	2.63	0.021	125
Host finding	Ace	1.05	0.105	10
	Dim	0.105	0.63	0.17
	Flu	5.25	0.105	50
	Sul	0.525	0.105	5

Treatment of *L. distinguendus* females with all four insecticides also hampered their ability to orientate toward volatiles present in the larval faeces of their hosts. The ability to use chemical cues from hosts and host-associated materials is key to the reproductive success of most parasitic wasps (Steidle & van Loon, 2002). Previous studies with *Microplitis croceipes* Cresson (Hymenoptera: *Braconidae*) (Stapel et al., 2000) and *N. vitripennis* (Tappert et al., 2017) revealed similar effects with the neonicotinoid imidacloprid. In *Aphidius ervi* Haliday (Hymenoptera: *Braconidae*), the

carbamyltriazole triazamate disrupted olfactory host finding while lambda-cyhalothrin, the organophosphate chlorpyrifos and the carbamate pirimicarb showed no effects (Desneux, Pham-Delegue, et al., 2004). Likewise, no effects were observed for the pyrethroid deltamethrin in *Aphidius matricariae* Haliday (Hymenoptera: *Braconidae*) and *Diaeretiella rapae* M'Intosh (Hymenoptera: *Braconidae*) (Desneux, Wajnberg, et al., 2004). Treatment of the *Drosophila*-parasitoid *Leptopilina heterotoma* Thomson (Hymenoptera: *Figitidae*) with a LD20 dose of chlorpyrifos and deltamethrin even improved odour-guided host finding (Delpuech et al., 2005). This indicates that the effects of insecticides on the host finding process of parasitic wasps can be variable and need to be investigated with different species before more general conclusions on a given substance can be drawn.

The results of our previous study investigating the effects of the four insecticides tested here on *N. vitripennis* (Schöfer et al., 2023) suggested that host and mate finding are differently affected. Sulfoxaflor interfered with females' host finding at all doses tested but had no effect on their response to the male sex pheromone. Dimethoate, on the other hand, disrupted pheromone orientation of *N. vitripennis* females, while the preference for host-associated volatiles even turned into avoidance. Similar effects were found in the present study for acetamiprid which had no detectable effects on the mating frequency, i.e., on the females' perception of the male aphrodisiac pheromone, but interfered with host finding at all doses tested. Future studies are needed to unravel the neurophysiological mechanisms underlying the differential effects of sublethal insecticide doses on sexual communication or host finding in parasitic wasps.

In toxicological studies, it is important to discuss whether the results are ecologically relevant. One might argue that *L. distinguendus* is not an ideal model system to study sublethal effects of insecticides in parasitic wasps, since this species is typically found in grain warehouses where it parasitises larvae of beetles infesting grain and stored products (Niedermayer et al., 2016). The use of fumigants such as methyl bromide and insecticides such as spinosad, methoprene, or chlorpyrifos is common practice in many countries to protect stored grain from pests (Daglish et al., 2018) . Therefore, *L. distinguendus* is likely exposed to insecticidal substances in its natural habitat, but, to our knowledge, none of the four insecticides tested here is currently used in stored product environments. Little is known about the occurrence of *L. distinguendus* outside human grain and food stores, but according to (Graham, 1969), it is also found in open

country, where it might get into contact with contaminated floral and extrafloral nectar, honeydew, or guttation water all of which have been shown to be consumed by parasitic wasps (Idris & Grafius, 1995; Jervis et al., 1993; Rose et al., 2006; Urbaneja-Bernat et al., 2020; Wanner et al., 2006). All these fluids may contain pesticide residues and can thus function as carriers of these substance to parasitic wasps (Davis et al., 1988; Schmolke et al., 2018; Zhou, Milne, et al., 2022). The doses for topical application used in the present study were chosen according to their acute toxicity (causing <30% mortality) and most of them were in fact sublethal. Topical application is an unnatural way to expose insects to pesticides, but it is standard in toxicological studies and allows more precise control of the dose than offering contaminated food sources. Assuming that topical application and oral uptake of the insecticides have comparable effects and considering the volume of sugar solution ingested by *L. distinguendus* (approximately 2 µl) and the reported residues of the four insecticides in nectar (Table S4.2 in the supporting information), *L. distinguendus* would take up doses in the field that are sufficient to cause adverse effects on mate and host finding. Nonetheless, studies under more realistic conditions are necessary that consider also other routes of uptake such as contact with treated plants or via the host to fully assess the extent of exposure of parasitic wasps to insecticides in the field.

The results obtained in the present study as well as earlier work (Desneux et al., 2007; Jiang, Liu, Huang, et al., 2019; Jiang, Liu, Zhang, et al., 2019; Kremer & King, 2019; Schöfer et al., 2023; Tappert et al., 2017; Teder & Knapp, 2019; Willow et al., 2019) strongly suggest that the ecosystem service provided by parasitic wasps as natural enemies and biocontrol agents is compromised when these are exposed to sublethal residues of insecticides, and there is ample evidence that other insects are similarly affected (Desneux et al., 2007; Guedes et al., 2016; Haynes, 1988; Muller, 2018). Hopefully, these findings will accelerate the shift toward more sustainable crop protection with even more prudent use of pesticides to ensure species-rich and functioning ecosystems in the future.

4.5 Supporting Information

Table S4.1 Results of the toxicity tests with *Lariophagus distinguendus* treated with 210 nl each of diluted acetone stock solutions (1 mg/ml) of (a) acetamiprid, (b) dimethoate, (c) flupyradifurone and (d) sulfoxaflor. Given is the number of live/dead wasps (evaluated 72 h after the treatment). Statistical analysis by Fisher's exact test, p-values refer to the comparison between pesticide doses and the respective controls (0 ng, pure acetone). Yellow entries indicate no-observed-effect-levels (NOEL). Doses ≤ NOEL are considered as sublethal doses.

a) Acetamiprid

Dilution	1:33.33	1:100	1:200	1:333,33	1:1000	1:2000	-
Dose (ng)	6.3	2.1	1.05	0.63	0.21	0.105	0
Rep. 1	0/8	1/7	3/5	4/4	7/1	8/0	8/0
Rep. 2	0/8	1/7	4/4	4/4	7/1	8/0	8/0
Rep. 3	0/8	0/8	0/8	1/7	5/3	7/1	8/0
Rep. 4	0/8	0/8	1/7	4/4	6/2	8/0	7/1
Rep. 5	0/8	1/7	6/2	2/6	7/1	6/2	7/1
Rep. 6	0/8	0/8	0/8	3/5	8/0	8/0	8/0
total	**0/48**	**3/45**	**14/34**	**18/30**	**40/8**	**45/3**	**46/2**
p-value	<0.001	<0.001	<0.001	<0.001	0.090	1.00	

b) Dimethoate

Dilution	1:10	1:33.33	1:100	1:200	1:333,33	1:1000	1:2000	-
Dose (ng)	21	6.3	2.1	1.05	0.63	0.21	0.105	0
Rep. 1	0/8	0/8	0/8	0/8	7/1	8/0	8/0	7/1
Rep. 2	0/8	0/8	7/1	5/3	8/0	8/0	8/0	8/0
Rep. 3	0/8	1/7	1/7	7/1	7/1	7/1	5/3	7/1
Rep. 4	0/8	0/8	4/4	8/0	8/0	8/0	8/0	8/0
Rep. 5	0/8	0/8	3/5	4/4	8/0	8/0	6/2	7/1
Rep. 6	0/8	0/8	6/2	7/1	6/2	7/1	7/1	6/2
total	**0/64**	**1/63**	**21/27**	**31/17**	**44/4**	**46/2**	**42/6**	**43/5**
p-value	<0.001	<0.001	<0.001	0.007	1.00	1.00	1.00	

c) Flupyradifurone

Dilution	1:40	1:100	1:200	1:333,33	1:500	1:1000	-
Dose (ng)	5.25	2.1	1.05	0.63	0.42	0.21	0
Rep. 1	0/8	0/8	1/8	3/5	6/2	6/2	8/0
Rep. 2	0/8	1/7	3/5	6/2	6/2	7/1	8/0
Rep. 3	0/8	0/8	2/6	4/4	6/2	8/0	7/1
Rep. 4	0/8	0/8	2/6	4/4	7/1	6/2	8/0
Rep. 5	0/8	1/7	0/8	6/2	6/2	6/2	8/0
Rep. 6	0/8	0/8	3/5	6/2	6/2	7/1	7/1
total	**0/64**	**2/62**	**11/37**	**29/19**	**37/11**	**40/8**	**46/2**
p-value	<0.001	<0.001	<0.001	<0.001	0.014	0.091	

d) Sulfoxaflor

Dilution	1:33.33	1:100	1:200	1:333,33	1:1000	1:2000	1:3333.33	1:10000	-
Dose (ng)	6.3	2.1	1.05	0.63	0.21	0.105	0.063	0.021	0
Rep. 1	0/8	0/8	0/8	0/8	2/6	7/1	7/1	8/0	7/1
Rep. 2	0/8	0/8	0/8	2/6	5/3	8/0	7/1	8/0	8/0
Rep. 3	0/8	0/8	0/8	0/8	0/8	6/2	8/0	7/1	7/1
Rep. 4	0/8	0/8	0/8	2/6	4/4	7/1	7/1	7/1	8/0
Rep. 5	0/8	0/8	0/8	2/6	5/3	6/2	8/0	8/0	6/2
Rep. 6	0/8	0/8	0/8	1/7	5/3	6/2	7/1	7/1	8/0
total	**0/64**	**0/64**	**0/64**	**7/41**	**21/27**	**40/8**	**44/4**	**45/3**	**44/4**
p-value	<0.001	<0.001	<0.001	<0.001	<0.001	0.355	1.00	1.00	

Table S4.2 Literature data on the contamination of floral and extrafloral nectar with the four insecticides tested in this study. Values refer to an assumed consumption of 2 µl ($\approx$ 2 mg) by *Lariophagus distinguendus.* For comparison, the lowest sublethal doses are given having shown significant effects in this study in at least one of the bioassays.

	Amount in nectar (ng/2mg $\approx$ ng/2µl)	First significant effects in this study	Reference
Acetamiprid	0.0001 - 0.0152	0.105	(Zioga et al., 2020)
	0.14		(Heller et al., 2020)
	0.012		(Azpiazu et al., 2019)
	0.02-0.36		(Capela et al., 2022)
	0.024 / 0.194[a]		(Zhou, Milne, et al., 2022)
	up to 0.026		(Pohorecka et al., 2012a)
	up to 0.012		(Demares et al., 2022)
Dimethoate	0.2 - 45.9	0.105	(Zioga et al., 2020)
Flupyradifurone	0.52	0.105	(Campbell et al., 2016)
	8.0		(Siviter & Muth, 2022)
	0.4-3.0 / up to 31.8[a]		(EPA, 2015)
	up to 0.004		(Bishop et al., 2020)
Sulfoxaflor	0.014 - 0.028	0.021	(Jiang et al., 2020)
	0.01 – 0.094		(Siviter et al., 2019)
	0.4		(Zhou, Chen, et al., 2022)
	0.06 – 2.0		(EPA, 2016)

[a]extrafloral nectar

4.6 Author Contributions

Nils Schöfer: Writing – original draft, review and editing; Methodology; Investigation; Formal analysis; Visualization

Gabriel Ratschmann: Writing – review and editing; Investigation; Formal analysis

Joachim Ruther: Conceptualization; Funding acquisition; Methodology; Project administration; Supervision; Writing-review & editing.

5. Four Neurotoxic Insecticides Impair Partner and Host Finding in the Parasitoid *Leptopilina heterotoma* and Bioactive Doses Can Be Taken Up Via the Host

N. Schöfer, N. Saxinger, K. Braumandl and J. Ruther

Abstract: In modern agriculture, control of insect species that damage agricultural crops is mainly regulated by using chemical agents. Since these chemical agents are broad spectrum insecticides, they not only kill their targets, but also can have lethal and sublethal effects on other insect taxa, some of which are actually beneficial. Here, four insecticides, acetamiprid, dimethoate, flupyradifurone and sulfoxaflor, were applied topically on *Leptopilina heterotoma*, a parasitoid wasp, to test both their lethality and potential sublethal effects on males abilities to find partners and females ability to orient towards hosts. 1 day After application with an insecticide, males were presented with female pheromone extract either in a dish arena (contact) or a T-olfactometer (distance), and females were presented with host substrate with minimum 5 host larvae (host-finding). Sublethal doses of acetamiprid (0.42ng), dimethoate (0.21ng), flupyradifurone (≥6.3ng) decreased the wing-fanning responses of the males in contact. In the distance experiments, effects were seen at low levels of acetamiprid (0.21ng), dimethoate (0.105ng), fluypyradifurone (6.3ng) and sulfoxaflor (0.21ng). Females were unaffected by acetamiprid, but low levels of dimethoate (0.105ng) and flupyradifurone (6.3ng) as well as higher levels of sulfoxaflor (0.63ng) lessened the reaction of females to the host odour. Additionally, males and females were raised on host fed with dimethoate contaminated medium to test a potential route of insecticide uptake. While wasps raised in a control environment (medium mixed with 1ml 10% acetone/h2o) reacted normally to either female extract or host odour, those raised on dimethoate were inhibited significantly in all three parameters tested. As well as demonstrating that even subnanogram doses can affect the partner and host finding capabilities of *L. heterotoma*, which can significantly impact population dynamics in wild populations, contaminated host were shown to also be a way parasitoid wasps can uniquely come into contact with doses of agricultural chemicals.

5.1 Introduction

Controlling insect pests is an important method of securing food resources internationally but has become more controversial in recent years as the impacts of using chemical controls like insecticides have been repeatedly shown to have negative effects on non-target organisms (Demirci & Gungordu, 2020; Pisa et al., 2015). Insecticides are popular because of their ease-of-use and are deployed with multiple methods, like spraying from vehicles (Qin et al., 2016) or direct treatment of seeds (Elbert et al., 2008). Regardless of method of deployment, many commercial insecticides contain active compounds that function similarly, by inhibiting proper central nervous system function, with many classes attacking nicotinic acetylcholine receptors (nAChRs) (Nauen et al., 2015; O'brien, 1963; Simon-Delso et al., 2015; Watson et al., 2011). Amongst these substances, novel groups of insecticides like the neonicotinoids gained prominence as they showed high efficacy at controlling diverse groups of pests (Jeschke & Nauen, 2008), and through widespread use became the most popular class of insecticide on the market, at some point representing more than a quarter of global insecticide sales (Bass et al., 2015). However, after effects they had on non-target organisms, especially bee colonies (Decourtye & Devillers, 2010), became well-known pressure on some governments led to the EU first restricting, and later banning the use of some neonicotinoids (European Commission, 2018).

In the aftermath alternative insecticides from both the neonicotinoids and other classes, like the organophosphates and sulfoximines have become popular (Dáder et al., 2019; Gill & Chong, 2021), but as they are also either receptor agonists or enzyme inhibitors, they thus are still potentially a threat for non-target organisms. Bans on specific compounds are also never global with the neonicotinoids imidacloprid, thiamethoxam and clothianidin banned in the EU and parts of the USA and Canada, but still being sold widely to developing markets like Brazil (Gaboardi et al., 2023). Due to this, there is still a major incentive to study further effects the banned and alternatives pesticides have on the wider environment. One alternative neonicotinoid that is still being used within the EU due to the low risk they have to bees (European Food Safety Authority, 2016) is acetamiprid. Like the other neonicotinoids acetamiprid is applied systemically to crops, and its acute toxicity is lower when compared with other neonicotinoids (Jacob et al., 2019) though in some contexts, like foliar application, it is equally or more toxic (Horowitz et al., 1998). Another recent group of

acetyl choline agonists that have gain notoriety are the butenolides, specifically the first commercially available active ingredient flupyradifurone (Nauen et al., 2015), which is less acutely toxic than most neonicotinoids (Bartlett et al., 2018) but still is an effective pest control. Similarly, the compound sulfoxaflor is the nascent member of the sulfoximine class of insecticides that is being sold to control populations of sap-feeding pest species (Sparks et al., 2013), and like the others is also an acetylcholine receptor agonist. Another mode of action of effective neurotoxic pesticides is inhibiting the function of nervous system enzymes. Of these inhibitors, the organophosphate dimethoate works as an inhibitor for the enzyme acetylcholinesterase (Engenheiro et al., 2005), which is a vital part of proper neuro receptor function in not just pest species but also the non-target organisms.

Of the non-target organisms, the focus historically has mainly been on pollinators, as they fertilize many commercially important crops (Aizen et al., 2009). Even amongst the pollinators, *Apis mellifera* has been a leading model organism being researched and in many countries new approval of active substances requires a scientific risk assessment on the impact the new substance can have on bee species ("Regulation (EC) No. 1107/2009 of the European Parliament and of the council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC," 2009). While this development is positive, using bees as a proxy for beneficial insects in general ignores the role other groups have in the natural cycle of crop plants, especially those that act as predators for pest species. One group of natural enemies that is of great interest is the parasitic wasps, some species of which are actively used in integrated pest management (Niedermayer & Steidle, 2013). Parasitic wasps also, through their function as parasitoids of diverse insect taxa, will have close contact with the environment where insecticides are sprayed and also a potential additional avenue of exposure to contaminants via their hosts (Kazimírová & Ortel, 2000; Kim et al., 2019). As with other insects, parasitic wasps are reliant on their olfactory systems to orient around their environment, using semiochemical signals from conspecifics, hosts, and host-damaged plants for mating and egg laying (Ruther et al., 2000; Schurmann et al., 2009; Whitman & Eller, 1990). These signals are mainly processed via their olfactory systems which is comprised of many receptors and sensory neurons, and in part nAChRs (Bohbot & Pitts, 2015). As such, neurotoxic insecticides have been shown to directly

influence the perception of olfactory signals of parasitic wasps (Tappert et al., 2017). Of the various factors that can be affected by insecticides, recognising conspecifics, and distinguishing potential mates is an important factor in increasing the genetic viability of the offspring of the next generation. As parasitic wasps are also haplodiploid, being able to find a mate over distance will additionally influence the sex ratio of subsequent generations, which can further impact population dynamics. In addition, due to their parasitic nature, finding hosts is a necessary component of their reproduction, mediated by olfactory signals (Quicray et al., 2023) and thus susceptible to changes via insecticides (Delpuech et al., 2005).

Previous research with other parasitic wasps has shown that acetamiprid, dimethoate, flupyradifurone and sulfoxaflor caused decreases in the olfactory orientation of related wasp species *Nasonia vitripennis* (Schöfer et al., 2023). Similar sublethal effects were seen with *Lariophagus distinguendus* (Schöfer et al., 2024) and multiple insecticides have been shown to negatively affect fecundity within related parasitoid wasps (Asadi et al., 2019; Biondi et al., 2013). One must assume that these effects will impact population dynamics of future generations, possibly also leading to local extinctions. Since the olfactory system of insects is highly conserved within insects (Krieger et al., 2003), effects seen within these parasitic wasps species, should also be observed in related species. As both *N. vitripennis* and *L. distinguendus* are part of the *Pteromalidae*, it is also important to see if effects are observed in related wasp families. A good candidate for this is the model system *Leptopilina heterotoma*, a parasitic wasp of the family *Figitidae*.

L. heterotoma is a solitary endoparasitoid wasp that typically parasitises various fly species of the *Drosophila* genera (Papaj & Vet, 1990). They are generally found within decaying substrates, like fermenting vegetables or fruit, where female *L. heterotoma* lay their eggs in *Drosophila* larvae during their 2nd or 3rd instars (Quicray et al., 2023). The *Drosophila* larvae are able to pupate, in which the *L. heterotoma* develop fully and emerge between 21-23 days from the host pupae at 25°C, with the females emerging 1-2 days after the males. *L. heterotoma* is a rigid model system, as one of its common hosts is the widely researched *Drosophila melanogaster*, and the micro trophic system of feeding substrate/*D. melanogaster*/*L. heterotoma* can be effectively manipulated within lab conditions to study the host/parasitoid interactions (Mortimer, 2013; Wertheim et al., 2003). *L. heterotoma* also has shown potential along with other

Leptopilina species in controlling the pest species *Drosophila suzukii* (Mazzetto et al., 2016)*.*

In the present study, we investigate sublethal effects of four topically applied insecticides, acetamiprid, dimethoate, flupyradifurone, sulfoxaflor, on the recognition of female *L. hetertoma* pheromones by males, both in close contact and over distance, and the ability of females to orientate towards host odours. Additionally, we research whether impacts are also observed when the insecticides are taken up via the hosts and their feed.

5.2 Materials and Methods

5.2.1 Insects

Drosophila melanogaster feeding medium (DFM) and a starter culture of adult *D. melanogaster* were provided by the department of developmental biology and neurogenetics at the University of Regensburg, and were reared in a climate-controlled cabinet at 25°C, 50% r.h, and a 16-8h light/ark cycle. On Mondays and Wednesdays 30 *D. melanogaster* adults of both sexes were placed within 5 vials of DFM, a standardised mixture of wheat semolina, sugar beet syrup, yeast and water, and allowed to mate and lay eggs *ad libitum* within the medium. For 2 of the host vials the *D. melanogaster* were left undisturbed to emerge as the next generation, while for the rest, the adults were removed after two days to allow for parasitisation.

Leptopilina heterotoma females lay their eggs in all *D. melanogaster* larval instars, with eggs laid in the L2 Instar being the most successful (Quicray et al., 2023). To allow the female *L. heterotoma* the best chances to parasitise, 20 male and female *L. heterotoma* were stunned with CO_2 gas and placed within the available host vials. The wasps were then allowed to parasitise the larvae in the 3 vials *ad libitum*. At the kept temperature, *L. heterotoma* males emerge after ~21 d, females after ~23 d (Quicray et al., 2023), so parasitised pupae were isolated after 19-20 d and transferred to individual Eppendorf tubes.

5.2.2 Insecticides

All insecticides were dissolved in acetone (ROTISOLV®, ≥ 99.8% purity, Carl Roth GmbH, Karlsruhe, Germany) for application in bioassays. Standards of the four insecticides were purchased at analytical grade. Acetamiprid (≥98.0% purity),

dimethoate (≥98.0% purity) and flupyradifurone (≥98.0% purity) standards were procured from Si Sigma-Aldrich (Taufkirchen, Germany) while Sulfoxaflor (99.23% purity) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

5.2.3 Toxicity tests

Determining toxicity of the four tested compounds on *L. heterotoma* is a necessary step to define which doses are sublethal and thus can be used in subsequent bioassays. Therefore, a dilution series was prepared of each of the substances, all diluted in acetone, starting from a concentration of 1mg/ml. Groups of 8 wasps (n = 3 replicates per dose/sex) were isolated before emergence, cooled in ice and applied topically on the abdominal tips with 210nl dosages of decreasing concentrations, plus control doses of pure acetone, using a microinjector (Nanoliter 2010, World Precision Instruments, USA). As previous experimentation has shown (Jatsch & Ruther, 2021; Schöfer et al., 2023), topical application is effective at determining accurate dosages, and pure acetone did not show adverse effects on longevity within related wasp species. After application, after a 72h period, mortality rates were calculated for each dose. Using the Quest Graph™ Lethal Dose 50 Calculator (AAT Bioquest, 2023), survival curves were plotted for each of the substances, along with functional equations for each curve. These were then further used to determine the Lethal Dose 50 (LD_{50}), as well as 3 sublethal doses (<LD30) to be used in the following bioassays.

Table 5.1 LD_{50}-values and sublethal doses of the four insecticides tested in the bioassays with *Leptopilina heterotoma*. Values in brackets represent the survival rates calculated from the functions of the respective sigmoidal curves using the online tool Quest Graph™ LD50 Calculator (https://www.aatbio.com/tools/ld50-calculator).

	LD_{50} (ng/Wasp)	Tested Sublethal Doses (ng/Wasp)		
Acetamiprid	2.53	0	0.21 (93)	0.42 (87)	0.63 (81)
Dimethoate	1.41	0	0.105 (96)	0.21 (91)	0.42 (81)
Flupyradifurone	31	0	6.3 (84)	10.5 (75)	21 (60)
Sulfoxaflor	1.19	0	0.21 (98)	0.42 (90)	0.63 (80)

5.2.4 Effects on pheromone communication (contact)

As with other parasitic wasps, male *L. heterotoma* display wing-fanning when reacting to the presence of female odours of conspecifics (Weiss et al., 2013). To assess whether the tested doses of the four insecticides affect the males ability to perceive females pheromone extract, 1-2 day old virgin males were treated with either a sublethal dose of one of the insecticides or with the control (pure acetone). The test males were given a small filter paper disk doused in a 50/50 water/honey mixture for nourishment overnight. To produce female extract, females were placed in a sealed glass vial with 10μl dichloromethane (DCM) per wasp, for ten minutes, as was described in (Stökl et al., 2012). This extract was stored at -4°C until needed for experimentation. 1 d after treatment, a 5.5 cm glass arena was prepared by dousing a filter paper disk in 5μl of the female extract and placing that with in the centre of the arena. The DCM was allowed to evaporate for 1 min, before the male was introduced to the arena, which was then sealed with a Petri dish lid. The males were observed for 300s and the time they spent wing fanning in seconds was recorded using The Observer XT 15 Software (Noldus Information Technology, Wageningen, The Netherlands). The males and filter paper disk were removed after each experiment, and the arena was cleaned with ethanol between experiments to remove any residual pheromones.

5.2.5 Effects on pheromone communication (distance)

Male *L. heterotoma* will often disperse before females in their natal patch have started emerging (Quicray et al., 2023), and as such will need to be able to find mates across longer distances. To trial if the active ingredients of the tested insecticides would inhibit such mate finding, 1-2 d old males were applied with either a sublethal or control dose of pure acetone and given the same nourishment over night as the contact experiment. 1 d later the males were tested in a t-olfactometer, a schematic of which is described in another paper (Schöfer et al., 2023). The t-olfactometer was positioned vertically to encourage males in the test chamber to exit their Eppendorf-vial. As with the contact experiment female extract was produced and doused onto a filter paper disk. After the DCM evaporated, the filter paper disk was placed within an Eppendorf-vial, which is then attached to one arm of the t-olfactometer, separated by a fine gauze (mesh width 125 μm). On the opposite arm an empty Eppendorf-vial was attached, again separated

with a gauze. These two zones are considered the pheromone zone and control zone respectively, with the area between the arms defined as the neutral zone. The Eppendorf-vial containing the males was defined as the start zone and attached to the bottom of the olfactometer at the start of each trial. After being attached, the males were observed for 300s, with the time they spent in each zone being recorded using The Observer XT 15 Software. The t-olfactometers were cleaned out with ethanol and pressurized air between uses and the arena setup was rotated 90° after each bioassay to avoid external light influences. The meshes were replaced between every bioassay.

5.2.6 Effects on host finding

Female *L. heterotoma* use *Drosophila* kariomones to identify areas with viable hosts over long distances (Delpuech et al., 2005). To examine how the insecticides influence this host finding behavior, 7-10 d old female *L. heterotoma* were taken directly from the breeding vials, treated with either a control or sublethal dose, and transferred to an Eppendorf vial filled with 1ml DFM and at least 5 *D. melanogaster* larvae, as more experienced females are more successful in host finding (Papaj & Vet, 1990). The females were kept in the vials overnight, and the following day the females were tested in a t-olfactometer. The olfactometer was setup similarly to the t-olfactometer during the distance pheromone experiments, however the pheromone zone vial was replaced with an Eppendorf vial filled with 1ml DFM and at least 5 *D. melanogaster* larvae and called the host zone. Females were transferred from their overnight Eppendorf vial to an empty one and were observed for 300 s, which started when the Eppendorf vial was attached to the bottom of the olfactometer. The time the females spent in each zone was recorded with The Observer XT 15 Software. As with the distance pheromone experiments the olfactometers were cleaned between uses and the arena was rotated between bioassays. The meshes were also replaced.

5.2.7 Rearing on poisoned medium and bioassays

A major part of the study was investigating whether doses of the insecticides can be taken up by *L. heterotoma* via trophic transfer. For this purpose, 20 *D. melanogaster* larvae and 20 1 d old adults (6 replicates per concentration) were separately raised on 1.6g of instant DFM, hydrated with 10 ml of distilled water, were mixed with a 1ml dose of dimethoate in 10% acetone/distilled water. After 8 days the percentage of surviving adults and larvae was recorded for each group. A dilution series of the poisoned

feeding medium was prepared, with concentrations decreasing until next to none of the *D. melanogaster* were affected, with a dose of 10% acetone in distilled water acting as a control group.

After determining 3 doses which did not affect *D. melanogaster* longevity, with the additional control dose, *L. heterotoma* were reared using drosophila fed with the poisoned medium. As the poisoned medium did not affect *L. heterotoma* lethality, adults were extracted from each and tested in the three bioassays explained previously (see 5.2.4-6).

5.2.8 Statistical analyses

All statistical analyses were performed using R 4.0.1 (R Development Core Team, 2023). The contact pheromone data was analysed using a Kruskal-Wallis test with the kruskal.test function (base R package), and following Dunn's tests, using the dunn.test function (dunn.test package) to compare each sublethal dose directly to the control dose. Both the distance pheromone data and the host finding data were analysed using a Wilcoxon signed-ranked test (wilcox.test function, base R package). Boxplots of the individual insecticide doses were plotted with PAST 4.03 software (Hammer et al., 2001) and collated together later.

5.3 Results

5.3.1 Toxicity tests

The dose-mortality curves show large discrepancies in the acute toxicity of the insecticides in *L. heterotoma* (Fig. 5.1, Tab. 5.1). Of the four, flupyradifurone had the lowest toxicity with an LD_{50} of 31 nanograms. The other three insecticides showed stronger toxicities, with acetamiprid being the weakest of the three (LD_{50}= 2.53 ng) and dimethoate (LD_{50}= 1.41 ng) and sulfoxaflor LD_{50}= (1.19 ng) having roughly equivalent toxicities. As the four insecticides have such differing toxicities, different sublethal doses were determined for each insecticide to be used in the bioassays (Tab. 5.1).

$$Y = 0 + \frac{1-0}{1+(\frac{X}{2528.7427})^{1.0641}} \qquad Y = 0 + \frac{1-0}{1+(\frac{X}{1407.5902})^{1.2044}} \qquad Y = 0 + \frac{1-0}{1+(\frac{X}{31009.0337})^{1.0605}} \qquad Y = 0 + \frac{1-0}{1+(\frac{X}{1188.7878})^{2.1591}}$$
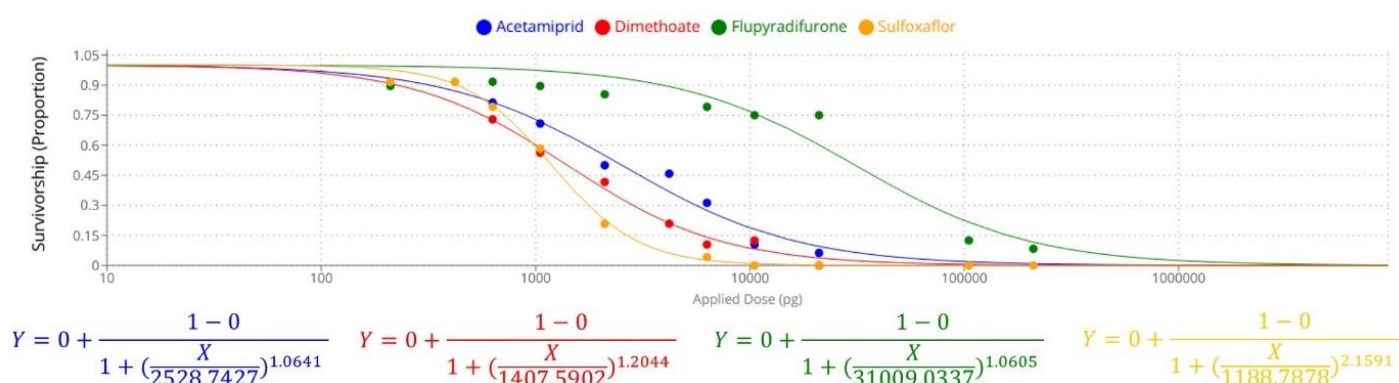
Figure 5.1 Dose-mortality curves after 72h of *Leptopilina heterotoma* treated topically with increasing doses of the insecticides acetamiprid, dimethoate, flupyradifurone and sulfoxaflor dissolved in acetone. Presented are the means of the 6 groups of 8 wasps (3 per sex), all treated with the same dosage. Colour-coded formulas for each curve are given benesth.

5.3.2 Effects on pheromone communication (contact)

In all experiments, the males treated with the control were able to recognise the female *L. heterotoma* extract and exhibited prolonged bouts of the typical wing-fanning behaviour (Fig. 5.2 a-d). In both acetamiprid and dimethoate only the males treated with intermediate doses, 0.42 ng (acetamiprid, Fig. 5.2a) and 0.21 ng (dimethoate, Fig. 2b) respectively, showed significant decreases in the time males spent wing-fanning. For flupyradifurone, all tested doses ($\geq$ 6.3 ng, Fig. 5.2c) reduced the duration males wing fanning significantly while none of the sulfoxaflor treatments were significantly effective (Fig. 5.2d).

70
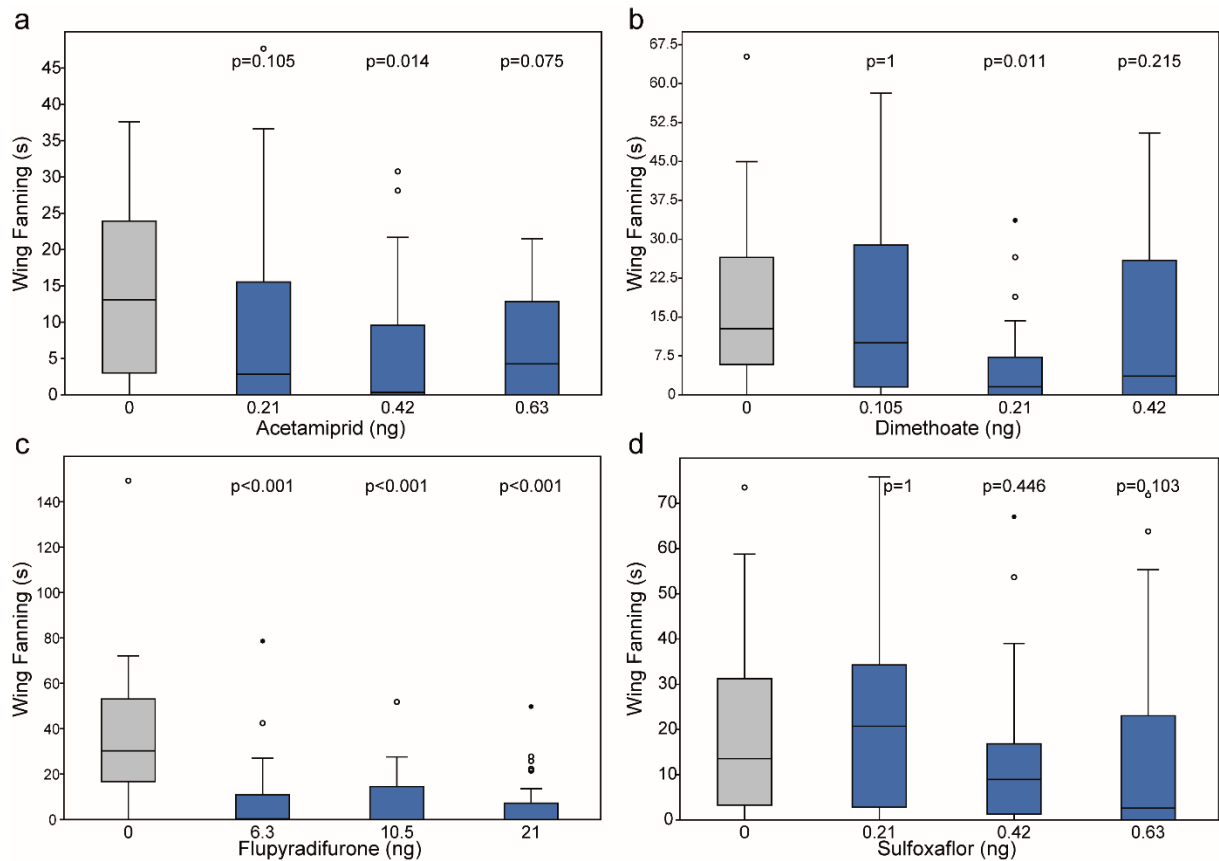
Figure 5.2 Effect of sublethal doses of the four tested insecticides on the wing fanning response of virgin *Leptopilina heterotoma* males to 5µl of female *L. heterotoma* extract mixed in dichloromethane. Represented is the time the males spent wing fanning in seconds during the observed 5 minutes, with the males split into those treated with either a sublethal dose of (a) acetamiprid, (b) dimethoate, (c) flupyradifurone or (d) sulfoxaflor or a control of pure acetone (0 ng). Box-and-whisker plots show median (horizontal line), 25–75% quartiles (box), maximum/minimum range (whiskers) and outliers (° means > 1.5 × and * means > 3 × box height). Statistical comparison between treatments and controls by a Kruskal-Wallis test and subsequent Dunn's tests (n=20).

5.3.3 Effects on pheromone communication (distance)

All males treated with the pure acetone control showed significant preferences for the pheromone zone over the control zone (Fig. 5.3 a-d). When treated with even low doses of acetamiprid ($\geq$ 0.21ng, Fig. 5.3a) or dimethoate ($\geq$ 0.105ng, Fig. 5.3b) the males showed no preference for either zone (p>0.05). Similarly, when treated with any of the tested sulfoxaflor doses ($\geq$ 0.21ng, Fig. 5.3d), males did not distinguish between the pheromone and control zone. An interesting outlier was observed when the males were treated with flupyradifurone (Fig. 5.3c). Males dosed with lower and higher levels of flupyradifurone (6.3ng, 21ng) were affected and spent similar times in each zone,

while those dosed with intermediate levels (10.5ng) showed a significant preference for the control zone (p=0.045), in an apparent avoidance of the female odour.

Figure 5.3 Effect of sublethal doses of the four insecticides on the reaction of virgin *Leptopilina heterotoma* males to 1µl of female *L. heterotoma* extract mixed in dichloromethane, within a t-olfactometer. Given is the time males spent within the pheromone zone and the empty control zone of the olfactometer. Males were treated either with a pure acetone control (0 ng) or a sublethal dose of (a) acetamiprid, (b) dimethoate, (c) flupyradifurone, or (d) sulfoxaflor and tested in a 4-chamber olfactometer 1 d after application. Box-and-whisker plots show median (horizontal line), 25–75% quartiles (box), maximum/minimum range (whiskers) and outliers (° means > 1.5 × and * means > 3 × box height). Statistical analysis for each treatment by Wilcoxon matched pairs test (n = 30).

5.3.4 Effects on host finding

As with previous experiments, the females treated with the acetone control showed a significant preference for the host odour (Fig. 5.4a-d). Acetamiprid showed no effect on the females as groups tested with any of the dosages ($\geq$ 0.21ng) still showed significant preferences for the host zone. In contrast, all sublethal doses of dimethoate ($\geq$ 0.105ng, Fig. 5.4b) and flupyradifurone ($\geq$ 6.3ng, Fig. 5.4c) disrupted the orientation of the females, who spent comparable times in the host and control zones. Only

females treated with the highest tested dose of sulfoxaflor (0.63ng, Fig. 5.4d), were affected and no longer discriminated between the test zones.
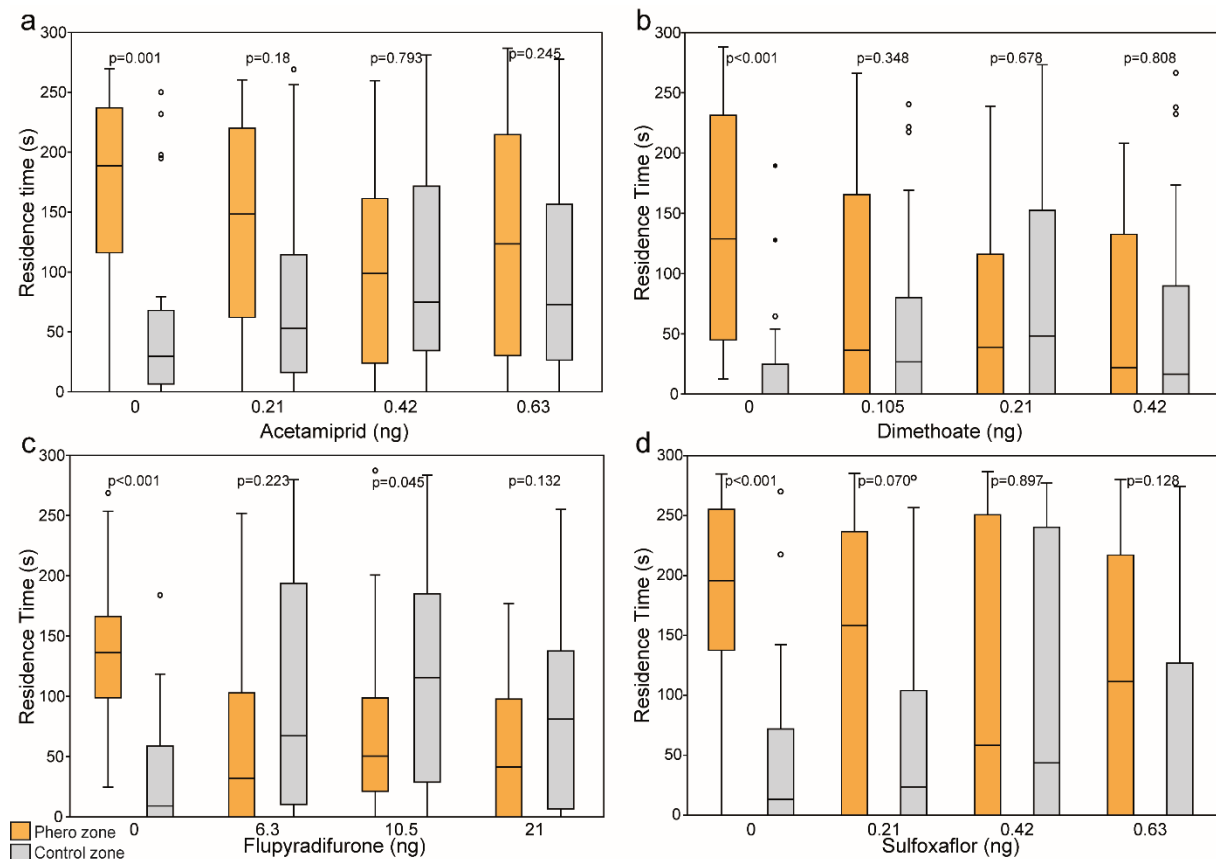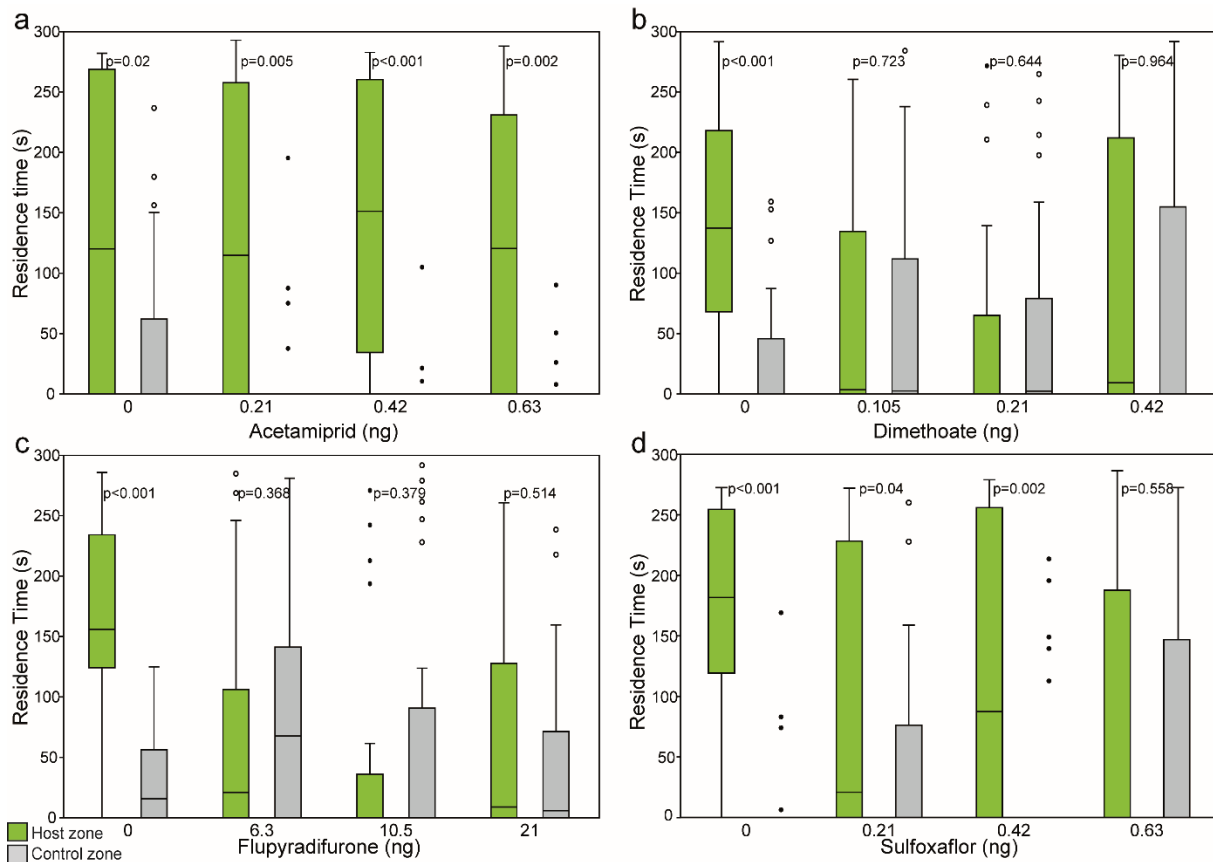

Figure 5.4 Effect of sublethal doses of the four insecticides on the response of mature *Leptopilina heterotoma* females to host odour (1 ml of drosophila feeding medium with 5 *Drosophila melanogaster*). Females were either treated with pure acetone (0 ng, control) or a sublethal dose of (a) acetamiprid, (b) dimethoate, (c) flupyradifurone, or (d) sulfoxaflor and tested in a 4-chamber olfactometer 1 d after application. Shown are the residence times of females in the host zone and the untreated control zone of the olfactometer. Box-and-whisker plots show median (horizontal line), 25–75% quartiles (box), maximum/minimum range (whiskers) and outliers (° means > 1.5 × and * means > 3 × box height). Statistical analysis for each treatment by Wilcoxon matched pairs test (n = 30).

5.3.5 Effects on with poisoned medium

Males and female *L. heterotoma* bred on hosts raised with the control medium (10 ml H_2O + 1ml 10% acetone/H_2O) each showed significant preferences for their pheromone and host zones, respectively (Fig. 5.5b & c). When the males were tested for their wing-fanning reaction to the female extract, males raised via contaminated medium (≥ 0.1 µl/ml) all showed significantly lower duration of wing fanning when directly compared to the control (Fig. 5.5a). Similarly, when tested on their reaction to

the female pheromone at a distance, males from the vials with poisoned medium (≥ 0.1 µl/ml) did not discriminate between the control and pheromone zones (Fig. 5.5b). Tested females on the other hand distinguished significantly between the host and control zone at lower dose levels (≤0.5 µl/ml), and only at the highest tested level (1 µl/ml) was their perception disrupted (Fig. 5.5c).
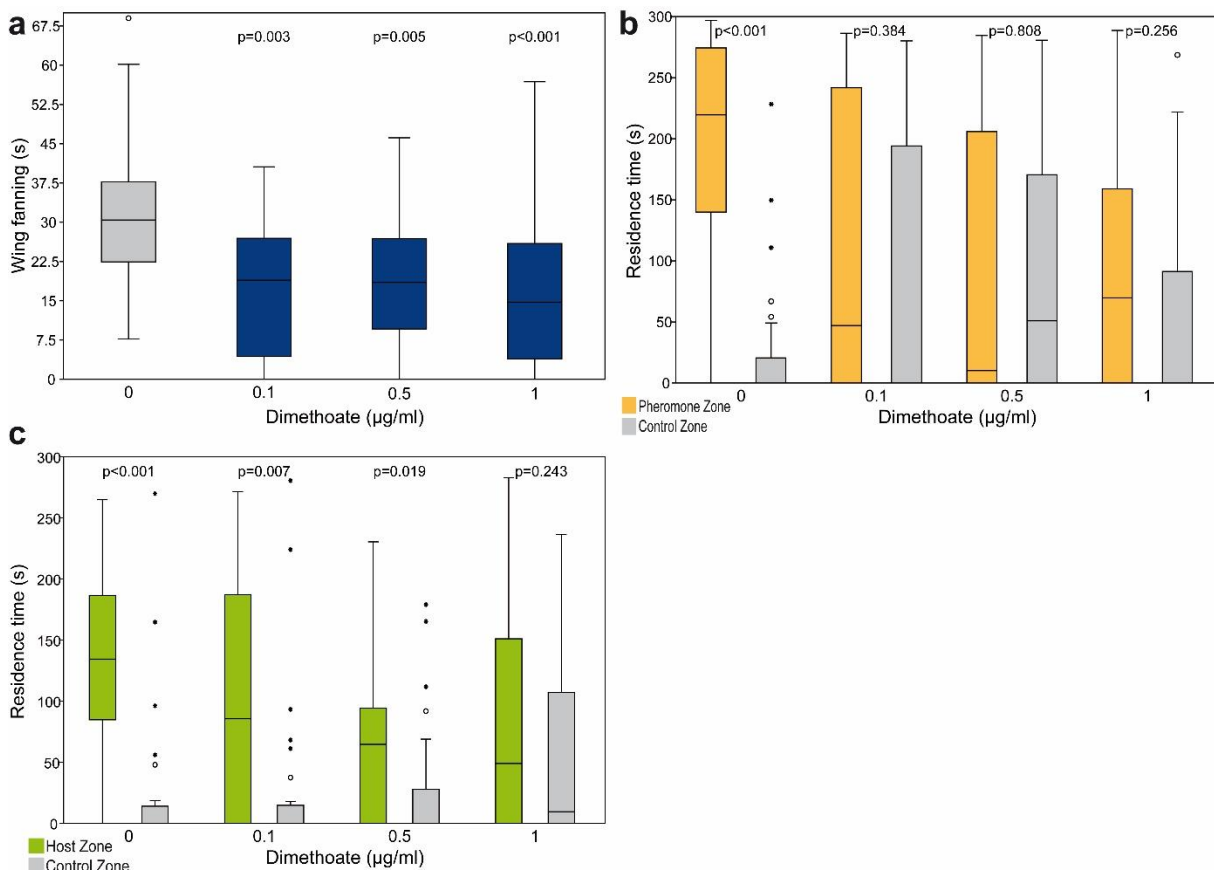
Figure 5.5 Effect of sublethal doses of dimethoate mixed into 1.6g *Drosophila* feeding medium + 10ml distilled water, on either: **a)** The response of virgin *Leptopilina heterotoma* males to 5µl of female *L. heterotoma* extract mixed in dichloromethane, in close contact; **b)** The response of virgin *Leptopilina heterotoma* males to 1µl of female *L. heterotoma* extract mixed in dichloromethane, within a t-olfactometer; **c)** response of mature *Leptopilina heterotoma* females to host odour (1 ml of drosophila feeding medium with 5 *Drosophila melanogaster*) within a t-olfactometer. Males and females were taken from a colony based on either an acetone control (0µg/ml) or colonies based on dimethoate poisoned medium. Represented is either **a)** the time the males spent wing fanning in seconds during the observed 5 minutes; **b)** time males spent in either the pheromone or control zone in a t-olfactormeter or **c)** time females spent in either the host or control zone in a t-olfactormeter. Box-and-whisker plots show median (horizontal line), 25–75% quartiles (box), maximum/minimum range (whiskers) and outliers (° means > 1.5 × and * means > 3 × box height). Statistical analysis for each treatment by Wilcoxon matched pairs test (n = 20).

5.4 Discussion

Proper olfactory system function is a key factor in the ability of predatory wasps to mate properly (Benelli, Giunti, et al., 2013; Quicray et al., 2023) seek out and control their prey populations (Schurmann et al., 2009; Wilson & Woods, 2016; Zhang et al., 1998). Dosages of all four insecticides affected a least one of the three parameters tested, acting as proxies for partner and host finding abilities, with effects being seen at levels of 0.105ng. Furthermore, dimethoate mixed into the feeding medium of the host *D. melanogaster* also affected the pheromone recognition and host finding of *L. heterotoma* raised on said hosts. More broadly, the lessened reaction to female pheromones, which was more prominent when tested at a distance, and female *L. heterotoma* no longer recognising host odours expand on previous understandings on the effects of the four substances on *N. vitripennis* (Schöfer et al., 2023) and *L. distinguendus* (Schöfer et al., 2024). In comparison with those two related species the substances, *L. heterotoma* generally was more susceptible than *N. vitripennis* to acetamiprid, flupyradifurone and sulfoxaflor, but was more resistant to these substances than *L. distinguendus*. Interestingly, all three species have comparable LD50s when dosed with dimethoate, going from 2.2ng/wasp (Nv) over 1.7ng/wasp (Ld) down to 1.41 ng/wasp (Lh). In honey bees dimethoate and other organophosphates are bioactivated by metabolizing enzymes, with their metabolites binding to acetylcholinesterase (Christen et al., 2019). While the targets of these metabolites are generally the same two forms of acetylcholinesterase (Kim & Lee, 2013), insecticides targeting nAChRs have been shown to target discrete receptors and subunits (Moffat et al., 2016), and the form and function of these subunits is different between even closely related species. This disparity may explain the strongly fluctuating sensitivity to the three acetyl choline agonists, while the sensitivity to dimethoate remains similar.

For the contact pheromone experiments, results were mixed, with only intermediate doses of both acetamiprid and dimethoate lessening the wing-fanning bout reactions of male *L. heterotoma*, while all flupyradifurone dosages but none of the sulfoxaflor dosages significantly affected wing-fanning behaviour. While these results may seem inconsistent at first, the olfactory neurons utilised in interpreting olfactory signals are complex and the affinity insecticides for different binding sites within the same organism can also vary widely (Belzunces et al., 2012). In *L. heterotoma* male wing fanning is mediated mainly by the compound (-)-iridomyrmecin, which is the main

volatile exuded by females (Weiss et al., 2015). For male *Leptopilina* distinguishing between conspecifics and other *Leptopilina* species, which also mainly utilise iridoid compounds as volatile mate attractants (Böttinger et al., 2021), is important so as not to waste sperm. Importantly, male *L. heterotoma* must then be sensitive to the volatiles of conspecific females, yet damages to olfactory neurons in the males caused by the insecticide active ingredients seems to inhibit conspecific recognitions. Additionally, as wing-fanning is also a result of motor activity, and as males treated with insecticide doses also displayed some wing-fanning activity, partial inhibition of proper motor function also offer explanation, as insecticides that target neurons have been shown to affect both olfactory and motor function in bees (Hesselbach & Scheiner, 2019), however more research into the motor function of parasitoid wasps is required. Nevertheless, a reduction in wing-fanning activity, which is an important compontent of the intricate courtship ritual of *L. heterotoma,* may reflect negatively on mating success, as has been shown in other species (Benelli et al., 2012; Benelli, Bonsignori, et al., 2013).

Male *L. heterotoma* also differ from even some closely related species, in that they disperse from their natal patch more quickly after emergence and use the more volatile iridoid compounds to orient after females even over larger distances (Böttinger & Stökl, 2020). The results of distance pheromone experiments showed that the orientation towards female pheromone over distances were more susceptible to inhibition from insecticides than the contact experiments. Even the sulfoxaflor doses, which showed no significant effects in the contact experiments reduced the time males spent in the zone adjacent to the female pheromone. In the field, dispersing males have greater success in mating as they are able to mate with females from multiple different natal patches (Böttinger & Stökl, 2020). In healthy populations of *L. heterotoma*, sibling mating will be uncommon, especially if the natal patch is large enough to host multiple foundresses (Fauvergue et al., 1999), but if their sensitivity to the female volatiles is impacted even from the lowest tested doses, their success in finding unrelated females will also decrease. Increased inbreeding in *L. heterotoma* has not been shown to alter the offspring sex-ratio over generations, but inbred females laid female eggs earlier in relation to outbred females (Hey & Gargiulo, 1985). Moreover, decreasing the likelihood of conspecifics interacting and mating, due to males being unable to properly track females, also skews future generations' sex ratios, as the haplodiploid nature of

L. heterotoma sex determination will cause unmated females to produce only male offspring (Gardner, 2014). While it is normal for a proportion of females to remain unmated even in healthy populations (Antolin, 1999), increasing numbers of unmated females due to sublethal insecticide doses damaging mate recognition will result in amplifying the male biased sex-ratio, and potentially leading to local population collapse.

Of the four insecticides, all except acetamiprid affected the orientation of *L. heterotoma* toward volatiles exuded by a feeding medium infested by host larvae. This ability is integral to find suitable hosts and oviposit (Steidle & van Loon, 2002), and as such females less effective at recognising and orienting towards host patch volatiles may have fewer opportunities to oviposit during their lifetime, ultimately resulting in fewer parasitised hosts and lower fecundity. These results are in line with effects shown previously in other species, where the pyrethroid cyfluthrin inhibited the recognition of the host kairomones by female *Telenomus busseolae*, while another pyrethroid, deltamethrin, did not (Bayram et al., 2010). Furthermore, the four insecticides here have previously also been tested with *N. vitripennis* (Schöfer et al., 2023) and *L. distinguendus* (Schöfer et al., 2024), where all four inhibited the recognition of host volatiles. In a slight contrast, a study on *L. heterotoma* treated with chlorpyrifos or deltamethrin found that they increased host kairomone recognition (Delpuech et al., 2005). These results show that an insecticide compound may have a stimulatory, inhibitory or no effect depending on what parasitoid species comes into contact with it, and thus species require individual testing before assumptions about their resistance can be made.

The final part of this study focused on the unique potential of parasitoids being exposed to chemical insecticides via their hosts. Male *L. heterotoma* raised within host on dimethoate contaminated medium displayed less wing-fanning in reaction to female pheromone and oriented themselves less towards female pheromone over distance when compared to males raised in the control environment. With the females, significant effects were observed at the highest tested concentration. While previous research has demonstrated effects of external pollutants on tritrophic interactions (plant - host – natural enemy) (Butler & Trumble, 2008), what has been demonstrated here is an uptake of a contaminant by the host, absorbed via parasitization into the parasitoid, which then exhibited weaker olfactory responses. This is partially in

concordance with research on the transferral of metals between host and parasitoid, where it was found that low proportions of such contaminants were taken up during parasitization (Kazimírová & Ortel, 2000). Unlike metal contaminants, however, insecticides can be metabolized by hosts like *D. melanogaster* (Sparks et al., 2012), and the influence of such metabolites on the olfactory system of *L. heterotoma* has not yet been studied. Additionally, choice experiments to test whether dimethoate contaminated medium acts repellent to egg-laying female *D. melanogaster*, in the same way as heavy metals (Bahadorani & Hilliker, 2009), could also influence the accuracy of this experiment. As such, while the results certainly demonstrate that uptake of insecticides via host species can be sufficient enough to inhibit proper olfactory function in a parasitoid wasp, further research must be conducted before it can be deemed to be a significant avenue of contamination for wild populations.

As a generalist parasitoid of *Drosophila*, *L. heterotoma* can adapt to numerous environments, usually following populations of potential host species (Quicray et al., 2023). Since many drosophilid species breed in decaying fruits, they, and by consequence *L. heterotoma* are often found surrounding fruit orchards. It is common practice in such orchards to utilise various pesticides to control pests (Simon et al., 2011). As all four of the insecticides tested in this study have been or are utilised in fruit orchards (Biddinger et al., 2013; Heller et al., 2020; Schoevaerts et al., 2015), exposure of *L. heterotoma* to these insecticides within such an environment is probable. In such an environment various plant resources can contain various pesticides, with nectar (Azpiazu et al., 2019), pollen (Heller et al., 2020) and guttation water (Schmolke et al., 2018) all having been shown to contain insecticides. In orchards, an additional plant resource that may be contaminated is tree sap, which has been shown to attract both parasitoid wasps and drosophilids (Yoshimoto et al., 2005), but also can contain insecticide compounds when the trees are being treated (Aćimović et al., 2014). While parasitoid wasps do not typically feed on tree sap, many of their hosts do, and as has demonstrated in this study, sublethal doses can be transferred from host to parasitoid.

In the present study all doses were applied topically, to the abdomen and under laboratory conditions. These are not natural environments for parasitoid wasps, but do allow more control to eliminate confounding variables such as irregularities in the amount of contaminated food eaten. This is standard practice for toxicological studies, but it does diminish how realistic the effects are for wild populations. Consequently,

more research is required under both laboratory and field conditions to accurately simulate how much parasitoid wasp utilise different resources. The results of these experiments expand on previous research (Kang et al., 2018; Kremer & King, 2019; Schöfer et al., 2023; Tappert et al., 2017), establishing the importance of parasitoid wasps in their role as biological pest control and indicating the detrimental effects that insecticides can have on their function.

5.5 Supporting Information

Table S1 Results of the toxicity tests with *Leptopilina heterotoma* treated with 210 nl each of acetone solutions of (a) acetamiprid, (b) dimethoate, (c) flupyradifurone, and (d) sulfoxaflor. Given is the number of live/dead wasps (evaluated 72 h after the treatment). Statistical analysis by Fisher's exact test, p-values refer to the comparison between pesticide doses and the respective controls (0 ng, pure acetone).

a) Acetamiprid

Dose (ng)	21	10.5	6.3	4.2	2.1	1.05	0.63	0
Rep. 1	0/8	1/7	3/5	4/4	4/4	7/1	7/1	8/0
Rep. 2	0/8	0/8	5/3	3/5	3/5	7/1	7/1	8/0
Rep. 3	1/7	2/6	2/6	4/4	5/3	3/5	4/4	7/1
Rep. 4	1/7	1/7	2/6	6/2	4/4	6/2	8/0	7/1
Rep. 5	0/8	0/8	1/7	2/6	3/5	5/3	5/3	7/1
Rep. 6	1/7	1/7	2/6	3/5	5/3	6/2	8/0	8/0
total	**3/45**	**5/43**	**15/33**	**22/26**	**24/24**	**34/14**	**39/9**	**45/3**
p-value	<0.001	<0.001	<0.001	<0.001	<0.001	0.006	0.120	

b) Dimethoate

Dose (ng)	21	10.5	6.3	4.2	2.1	1.05	0.63	0
Rep. 1	0/8	1/7	0/8	0/8	3/5	4/4	5/3	7/1
Rep. 2	0/8	3/5	4/4	7/1	2/6	4/4	6/2	8/0
Rep. 3	0/8	0/8	0/8	0/8	0/8	4/4	4/4	8/0
Rep. 4	0/8	0/8	0/8	1/7	1/7	4/4	8/0	8/0
Rep. 5	0/8	0/8	0/8	0/8	1/7	3/5	4/4	7/1
Rep. 6	0/8	2/6	1/7	2/6	8/0	8/0	8/0	8/0
total	**0/48**	**6/42**	**5/43**	**10/38**	**15/33**	**27/21**	**35/13**	**46/2**
p-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.0037	

c) Flupyradifurone

Dose (ng)	210	105	21	10.5	6.3	2.1	1.05	0.63	0.21	0
Rep. 1	0/8	1/7	6/2	7/1	6/2	6/2	7/1	8/0	7/1	8/0
Rep. 2	3/5	3/5	8/0	8/0	8/0	7/1	7/1	7/1	8/0	8/0
Rep. 3	0/8	0/8	7/1	7/1	7/1	7/1	8/0	8/0	6/2	8/0
Rep. 4	0/8	1/7	7/1	7/1	7/1	7/1	8/0	8/0	7/1	8/0
Rep. 5	1/7	1/7	3/5	4/4	5/3	7/1	6/2	7/1	7/1	8/0
Rep. 6	0/8	0/8	5/3	3/5	5/3	7/1	7/1	6/2	8/0	6/2
total	**4/44**	**6/42**	**36/12**	**36/12**	**38/10**	**41/7**	**43/5**	**44/4**	**43/5**	**46/2**
p-value	<0.001	<0.001	0.007	0.007	0.027	0.159	0.435	0.677	0.435	

d) Sulfoxaflor

Dose (ng)	210	105	21	10.5	6.3	2.1	1.05	0.63	0.42	0.21	0
Rep. 1	0/8	0/8	0/8	0/8	0/8	0/8	4/4	3/5	6/2	7/1	7/1
Rep. 2	0/8	0/8	0/8	0/8	0/8	3/5	6/2	8/0	8/0	6/2	8/0

Rep. 3	0/8	0/8	0/8	0/8	0/8	1/7	2/6	6/2	8/0	8/0	8/0
Rep. 4	0/8	0/8	0/8	0/8	1/7	1/7	7/1	8/0	8/0	8/0	8/0
Rep. 5	0/8	0/8	0/8	0/8	0/8	2/6	5/3	6/2	7/1	7/1	8/0
Rep. 6	0/8	0/8	0/8	0/8	1/7	3/5	4/4	7/1	7/1	8/0	8/0
total	**0/48**	**0/48**	**0/48**	**0/48**	**2/46**	**10/38**	**28/20**	**38/10**	**44/4**	**44/4**	**47/1**
p-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.008	0.362	0.362	

5.6 Author Contributions

Nils Schöfer: Writing – original draft, review and editing; Methodology; Investigation; Formal analysis; Visualization

Natalie Saxinger: Writing – review and editing; Investigation; Formal analysis

Katrin Braumandl: Writing – review and editing; Investigation; Formal analysis

Joachim Ruther: Conceptualization; Funding acquisition; Methodology; Project administration; Supervision; Writing-review & editing.

6. Conclusion

Insecticides, many of which are wide spectrum and are used to kill/control a variety of pests, are a necessary part of modern agriculture. However, if these chemicals are used indiscriminately, beneficial insects, especially those in similar classes to the target species, can be exposed to and affected negatively by pesticides. Previous research has been primarily concentrated on the lethal and partial sublethal effects of individual substances on individual species, and within the insect clade there is a bias towards pollinators, specifically bees. This thesis aims to study four still-in-use insecticidal compounds and their lethal and sublethal effects on 3 species of parasitic wasp, *Nasonia vitirpennis*, *Lariophagus distinguendus* and *Leptopilina heterotoma*, comparing both the insecticides and species against each other, and ultimately demonstrating how parasitic wasps may be uniquely exposed to insecticides.

6.1 Toxicity

The methodology used to expose the wasps to the toxins during this thesis was topical application of the substances diluted in acetone to the abdomen tip of the wasps, a methodology (Tappert et al., 2017) shown to be effective. The methodology sacrifices its slightly unnatural exposure pathway for more accurately being able to dose individuals. Alternatively, it would be possible to expose the wasps orally via contaminated sugar water as a feeding medium, but this way would not guarantee that each wasp would consume the same amount of medium, which could invalidate any results. By exposing groups of wasps to equal amounts of acetone/insecticide solution and repeating this with solutions of diminishing concentration, the toxicity of each species and insecticide constellation was able to be determined and easily compared.

Within each species the toxicity of the active ingredients was quite variable. Fluypradifurone was the weakest of the four substances when *N. vitripennis* or *L. heterotoma* was exposed to it, with LD_{50} of 102 and 31 ng/wasp, respectively, but was much more toxic to *L. distinguendus* (0.80 ng/wasp). *L. distinguendus* was much more sensitive to the other two nAChR agonists, acetamiprid and sulfoxaflor, as well, with decreases in LD_{50} by a factor of 16 and 50 respectively (Tab. 4.2) when compared to *N. vitripennis*. On the other hand, the toxicity of dimethoate between the three species stays relatively similar. As discussed in chapter 5, this relatively stable toxicity between

species may in part be explained by the relative simplicity of the acetylcholineesterases (Kim & Lee, 2013) when compared to the complexity of nAChR subunits. *L. distinguendus*' weakness to the nAChR subunits was also discussed in chapter 4, where the possible lack of metabolizing enzymes were hypothesized to be a significant factor, though more research on this topic is needed for definitive answers.

6.2 Partner-finding

The proper functioning of the insect olfactory system is a determining part of their fitness. Depending on the mating specificities of the species being tested, either the female or male was tested. In *N. vitripennis,* females were tested on their reaction to synthetic male pheromone, which was described in (Ruther et al., 2008) and (Ruther et al., 2016) and was shown to cause arrestment in females in (Tappert et al., 2017). In *L. distinguendus*, the male reaction was assessed through counting the number of wing-fanning bouts to conspecific female "dummies". These "dummies" were shown to elicit the wing-fanning behaviour in males in (Kühbandner et al., 2013) as a response to cuticular hydrocarbons the "dummies", recently freeze-killed female *L. distinguendus*, still exude. Male *L. heterotoma* also display wing-fanning behaviour in response to female conspecifics, but due to them not reacting to *L. heterotoma* "dummies", female extract was used. Additionally, as the wing-fanning reactions of male *L. heterotoma* observed were fewer in number, but longer in average duration, the wing-fanning bout time in seconds was analysed. While there are differences in the parameters assessed between species, as each of the parameters involve an actively searching individual and an unresponsive proxy for a potential partner, the results between the species are still comparable.

Acetamiprid had significant effects on all three tested species. In *N. vitripennis* females, doses from as low as 0.63ng, the lowest tested dose in this constellation, showed an effect, with females spending similar time in the zone close to the synthetic pheromone and apart from it (Fig. 3.3a), when compared to the control group. The females were clearly impaired in their ability to sense the synthetic male pheromones, which is an essential part of the *N. vitripennis* courtship ritual (A. Moynihan & D. Shuker, 2011). Similarly, when *L. distinguendus* males were tested, a dose of 0.21ng was enough to significantly lower the number of wing-fanning bouts, though this was not the lowest dose tested (Fig 4.2a). For *L. heterotoma* two partner finding parameters were tested,

as unlike the other two species, mating does not only take place at the natal host patch (Fauvergue et al., 1999). Interestingly, while all tested acetamiprid sublethal doses affected the ability of males to orient to the female extract over distance (Fig. 5.3a), only the intermediate dose affected the wing-fanning duration when the male *L. heterotoma* came in close contact with the extract (Fig. 5.2a). Such a biphasic response is not uncommon when working with toxins (Calabrese, 2013), and may be a sign of possible stimulatory effects of acetamiprid at higher doses.

Dimethoate had a drastic effect when female *N. vitripennis* were exposed to it. Dosages as low as 0.105ng were able to disrupt the recognition of the male pheromone (Fig 3.3b). Male *L. distinguendus* exposed to dimethoate also displayed gradually decreasing rates of wing-fanning bouts, though here only those treated with the two higher doses were significantly lower (Fig. 4.2b). Dimethoate-treated *L. heterotoma* males again showed a biphasic response in the contact experiment (Fig. 5.2b), while the distance experiments again had significant results at all tested levels (Fig.5.3b).

Flupyradifurone was less effective in disrupting the *N. vitripennis* females, as only the highest tested dose of 21ng was able to weaken the preference for the pheromone (Fig. 3.3c). In contrast, all the much lower tested doses were able to decrease wing fanning in *L. distinguendus* (Fig. 4.2c), and while the doses *L. heterotoma* males were exposed to were equal to the doses treated to *N. vitripennis* females, *L. heterotoma* males showed both lower wing-fanning (Fig 5.2c) and weakened recognition of the female extracts (Fig. 5.3c) even at low and intermediate levels.

For sulfoxaflor the results were even more interesting, as it showed no effects on the female *N. vitripennis'* ability to distinguish the male pheromone even at doses of 5.25ng (Fig. 3.3d). Then, when *L. distinguendus* males were treated, their wing-fanning bouts significantly went down from doses as low as 21 picograms (Fig. 4.2d). However, when *L. heterotoma* males were treated in the contact experiments, again sulfoxaflor showed no effect at any tested doses (Fig. 5.2d), but all doses affected the long-range orientation of the males (Fig. 5.3d).

All in all, the effects observed across the three species were diverse, with the results from one experiment not being indicative of the substances effect with another species. That said, effects were seen with all species, with multiple active ingredients showing effects across two or even all three species. As was discussed partially in chapter 3,

the effect on *N. vitripennis* females can be detrimental to the fitness of *N. vitripennis* populations. Females utilize the male pheromone to assess mate quality (Ruther et al., 2009); if they are not able to distinguish the male pheromone properly, this will affect mate choice and their willingness to mate. Furthermore, as mating in *N. vitripennis* happens exclusively on the natal host patch, and males do not leave their natal patch (Grillenberger et al., 2009), if females do not feel attracted to stay on the host patch and disperse before mating, the fitness of the population will be significantly affected.

In both *L. distinguendus* and *L. heterotoma* wing fanning is an important factor determining the quality of males as mates (Benelli, Bonsignori, et al., 2013; Van Den Assem, 1968). Furthermore, in both species wing-fanning is a reaction to the perception of a conspecific female (Ruther et al., 2000; Weiss et al., 2015). If the recognition of the female sex pheromones, on either a *L. distinguendus* "dummy" or in female *L. heterotoma* extract, is impaired to the point that males no longer exhibit as much wing-fanning, this would both impact the likelihood of males interacting with females and affect their quality as mates, further decreasing their reproductive success. Uniquely for *L. heterotoma,* mating also routinely occurs apart from the natal patch (Fauvergue et al., 1999), but here the problems caused by the insecticides are even greater, as males are even less able to distinguish the female pheromones at distance, a skill vital to find potential mates in the wild (Quicray et al., 2023). These results are reflected in other species of parasitoid wasp and other insects (Shi et al., 2024; Tappert et al., 2017), and expand on potential differences in the way that parasitoid wasp species are able to compensate different insecticides.

6.3 Mating

As all parasitoid wasps are haplodiploid, mating success is a strong determinant of the demographics of succeeding generations (Antolin, 1999). Insecticides, as observed in bees, can hamper the olfactory reception of partner pheromones that individuals use to recognise conspecifics and potential partners (Boff & Ayasse, 2023; Strobl et al., 2021),. Of the three species, mating experiments were undertaken only with *N. vitirpennis* and *L. distinguendus*. While mating experiments were trialed with *L. heterotoma*, low acceptance of males by females made these experiments infeasible. Female *L. heterotoma* rarely mated with the first male they encountered, and prefer off-patch mating (Quicray et al., 2023), which is possibly to reduce sibling-mating and

local mate competition. The mating experiments showed some interesting differences between *N. vitripennis* and *L. distinguendus*, even amongst those in the control groups. Acetone, the control medium, has been demonstrated to have no effect on mating success (Jatsch & Ruther, 2021). However, the mating rate of *L. distinguendus* pairs was not always 100% (Fig. 4.3). In contrast, the *N. vitripennis* control pairs were always successful (Fig 3.4). This result proposes that *L. distinguendus* are more discriminatory than *N. vitripennis*, or that the mating rituals between the two species are moderated differently.

Sublethal doses of acetamiprid affected males more than females in both species. In *N. vitripennis* only the highest tested dose (6.3ng) significantly reduced the mating rate, and only when the male or both partners were applied with a dose (Fig 3.4a). Females were not significantly affected and continued to mate at similar rates to the control. This may be a factor of size differences between male and female *N. vitripennis*, as females are typically larger than males (Mair & Ruther, 2019), and as such are able to compensate higher doses of acetamiprid than males can. Likewise, in *L. distinguendus* effects were seen only when the male or both were treated (Fig 4.3a), though again the doses were relatively smaller. In *L. distinguendus* as well, males are smaller than females (Van den Assem et al., 1989), and such sexual dimorphism will affect susceptibility to insecticides. A curious point here is that when only male *L. distinguendus* were treated, there was a biphasic response, while when both were treated there was a gradual decrease, with the mating rate dropping to 35% at the highest tested dose.

Dimethoate had singular effects with *N. vitripennis*, when only males were treated and only at the highest tested dose of 1.05ng (Fig 3.4b). Females were unaffected, while even when both were treated no effects were seen. Conversely, *L. distinguendus* was more affected by sublethal doses, with significant effects observed when treatment was applied to females or both partners (Fig 4.3b). Strikingly, when males were treated or both were treated, there were drops in mating rates, from 85% (control) down to 60%, but this was not a statistically significant effect. As was discussed in chapter 4, this result may be due to the limitations of the small sample used, and though this is what the Fisher's exact test was designed for (Jung, 2014), additional replicates could aid in clearing up such irregularities.

Of all four insecticides, Flupyradifurone had the strongest impact on mating rate, both on *N. vitripennis* and *L. distinguendus*. When either the *N. vitripennis* males or females were treated, only the highest dose had an effect (21ng), but when both of the pair was treated, all three tested doses reduced the mating rate, which went down to 30% (Fig 3.4c). Similar results were seen with *L. distinguendus*, though when females or both sexes were treated, all three doses caused significant declines in mating success (Fig 4.3c). This strong reaction to flupyradifurone is corroborated in mating trials with other hymenoptera (Boff & Ayasse, 2023; Tabebordbar et al., 2020), where lack of recognition to a partner's pheromones impeded successful courtship.

Only the highest dose of sulfoxaflor (2.63ng) had a significant effect on *N. vitirpennis* pairs, though this effect was observed in every constellation (Fig. 3.4d). In contrast, the much lower doses applied to *L. distinguendus* all had lower mating rates in comparison to the control pairs. Again, sometimes higher doses had slightly higher mating rates than lower ones, likely an artefact of sample size.

Occasionally, when both partners were treated, effects were stronger (i.e effects seen at lower doses or lower mating rate at equivalent doses) compared to when only one partner was treated. Clearly an interaction of damages to both males and females can amplify negative effects, as has been documented in other research (Haynes, 1988), albeit this was not always the case. Regardless, the results of both mating trials established that all four insecticides affected the courtship rituals negatively, with differences between the species assumably attributed to the different mating structures of the species (Van den Assem, 1969; Van den Assem et al., 1980).

Mating in both *N. vitripennis* and *L. distinguendus* is regulated by pheromones (Abdel-Latief et al., 2008; Benelli, Bonsignori, et al., 2013; Ruther et al., 2000; Steiner et al., 2006), and as observed in the previous chapter, recognition of partner pheromones was disrupted by sublethal doses. While both the arrestment of females in *N. vitripennis* and wing-fanning in *L. distinguendus* are parts of courtship in the respective species (Abdel-Latief et al., 2008; Benelli, Bonsignori, et al., 2013), the mating rituals are more complex and require input from both partners. This explains why the results of the partner-finding experiments were not prognostic of the insecticides effect on mating rate. In *Heriades truncorum*, treatment with flupyradifurone made potential partners unattractive relative to untreated controls and altered the pheromone profile

of treated individuals (Boff & Ayasse, 2023), a development that could also happen in the parasitoid wasps.

The impact of lower mating rates is evident. Mating success in parasitoid wasps determines sex ratio, clutch size and can even affect egg-laying behaviour Morag et al., 2011; Darrouzet et al., 2007). Depending on the level of exposure and the active ingredient, the majority of females in a local population may stay virgins, resulting in a heavily male-skewed next generation. If exposure continues throughout subsequent generations, interactions between sexes will decrease, further dropping fertility and ultimately leading to local extinctions.

6.4 Host Finding

In all parastoid wasps, generalist or specialist, the fitness of populations is dependent upon the ability of females to find hosts (Bailey et al., 2009), as parasitoid eggs are not viable unless they are able to feed on a host shortly after hatching. Host-finding assay were undertaken with all three species as they parasitise different taxa; *N. vitripennis* is a parasitoid of flies belonging to the *Sarcophagidae* and *Calliphoridae* (Godfray, 2010), *L. distinguendus* parasitises some species of *Curculionidae* beetles (Niedermayer et al., 2016) and *L. heterotoma* utilises *Drosophila* species as hosts (Quicray et al., 2023). Species also uses different odour cues from the host surroundings as partial cues (Mair & Ruther, 2019). There were also some interesting differences between the species. While *N. vitripennis* females were innately able to orient to the *Lucilia caesar* in the preliminary experiments, both *L. distinguendus* and *L. heterotoma* females required some prior association with their respective hosts (*Sitophilus granarius* larvae and *Drosophila melanogaster* larvae) to increase their responsiveness, as was discussed in chapters 4 and 5.

Acetamiprid showed interesting effects across the three species, as in *N. vitripennis* (Fig. 3.5a) the preference for the host zone present in all control groups only disappears at higher doses (≤ 2.1ng), though females treated with an intermediate dose actually displayed a preference for the host zone. Such an effect was not found in *L. distinguendus* females, as here all tested insecticide doses did not hold a preference for the host zone (Fig. 4.4a), whereas in *L. heterotoma* none of the tested doses showed any effect (Fig. 5.4a). The varying responses between the species may be explained by differences in associative learning between species, which can vary

widely between species (Lewis & Takasu, 1990; Schurmann et al., 2009), and even between host habitat variations (Meiners et al., 2003).

N. vitripennis females applied with dimethoate doses showed an unusual dose-response relationship, with higher doses consistently showing an avoidance of the host odour (Fig 3.5b). Host odours are generally innately attractive, though associated odours are also learned (Tumlinson et al., 1993), but this intrinsic attractiveness can be overwritten (Benelli et al., 2014), positing that dimethoate can nullify the positive association with host odours. Dimethoate did not have as strong an effect on *L. distinguendus*, as only the highest tested dose negated the preference for the host odour (Fig. 4.4b), while *L. heterotoma* was affected from even the lowest test dose (Fig. 5.4b).

In contrast to its strong effects on mating rate in *N. vitripennis*, flupyradifurone only had an effect on the host-finding behaviour at the higher tested level (Fig. 3.5c). Curiously, in *L. distinguendus* only those treated with the lowest dose (Fig. 4.4c, 0.105ng) did not have a significant preference for the host odour, while higher doses again found the host odour attractive. Such an inverse hormesis is not an uncommon statistical model in toxicological studies (Nweke & Ogbonna, 2017), though its occurrence here may be resolved with increased replicates. On the other hand, the host finding capabilities of *L. heterotoma* females was damaged at all tested levels (Fig. 5.4c).

Sulfoxaflor disrupted the recognition of host odours in *N. vitripennis* females at even the lowest sublethal doses (≤0.525ng, Fig. 3.5d). This is a clear contrast to sulfoxaflor's effect on females' ability to distinguish partner pheromones, as was discussed previously, suggesting that the partner and host cues are interpreted differently in the parasitoid wasp olfactory system. In both *L. distinguendus* (Fig. 4.4d) and *L. heterotoma* (Fig. 5.4d) the highest tested doses were the only ones to display significant disruption.

Altogether, while the results were quite variable in their severity, each insecticide did inhibit proper olfactory system function in at least one species. Other insecticides, when tested on their effects on parasitoid wasps host finding, have shown both negative effects (Desneux, Pham-Delegue, et al., 2004), and positive stimulation (Rafalimanana et al., 2002). This further demonstrates that all of the tested insecticides attack neurotransmitters, both the particular kinetics of the specific insecticide

(Taillebois et al., 2018) and the ability of different species to metabolise the active ingredients (Hayward et al., 2019) affect responses at different dose levels.

Many factors of a parasitoid wasps development are determined by the quality of the host they emerge from (Mackauer et al., 1997; Werren & Simbolotti, 1989). The host's resources are important for size, sex allocation and in gregarious wasps, like *N. vitripennis*, the amount of progeny allocated per host (Rivers & Denlinger, 1995). Moreover, oviposition experience in female parasitoid wasps can affect how capable females are at host finding (Steidle, 1998; Ueno & Ueno, 2005), meaning that impaired ability in finding hosts, like the insecticides cause, can have knock-on effects. Ultimately, impaired host finding ability will limit the amount of hosts a female parasitoid wasp will interact with, hence limiting the host quality an individual female will have access to. Consequently, both solitary and gregarious wasps would be limited in the number of offspring that individual females would be able to produce, leading to increased pressure on the next generation and population decline.

6.5 Trophic transfer

A common criticism of lab analyses of sublethal dose effects of insecticides is that they are unrealistic of dosages that insects would encounter in the wild. However, chapters 3 and 4 both discussed various mediums that, through feeding or contact, could expose individuals of the three species to sublethal equivalent dosages over their lifetimes (Tab. S3.2, Tab. 4.2, Tab. S4.2). Furthermore, parasitoid wasps may have an additional avenue of uptake, via their hosts. Ecotoxic substances like metals have been known to accumulate in hosts, such as fruit flies, and transfer to parasitoids (Kazimírová & Ortel, 2000; Kim et al., 2019). In chapter 5, the feeding medium - *D. melanogaster – L. heterotoma* system was deemed the simplest system to artificially contaminate, as unlike *N. vitripennis* the hosts were alive and could be fed, and it was simpler to uniformly contaminate the *Drosophila* feeding medium than the wheat grains.

As this was an exploratory part of the study, only one contaminating insecticide was tested. Dimethoate was chosen as it had significant effects in all three trials. In all three trials, the control groups showed normal wing fanning and orientation behaviour (Fig. 5.5), demonstrating that similar to how acetone does not affect *N. vitripennis* behaviour (Jatsch & Ruther, 2021), the acetone did not affect the hosts ability to develop and did not noticeably affect *L. heterotoma* behaviour. Male reaction to the female extract, both

in contact and over distance, was significantly affected by contaminated hosts (Fig. 5.5a-b) at all tested concentrations of dimethoate in the medium, whereas female reaction to host odours was only affected at the highest tested concentration (Fig. 5.5c). The results clearly demonstrate that contaminated mediums, even at concentrations that are sublethal for the host species, can affect the behaviour of parasitoids. As it is still unclear whether the host quality is lowered by the contimanants and therefore affects the capabilities of the wasp offspring (Mackauer et al., 1997), or if the host metabolizes the contaminants (Sparks et al., 2012) and those metabolites are responsible, further research on this topic is needed to clarify the mechanisms of such a transfer.

6.6 Outlook

Though the research covered in this thesis has answered a number of questions on the sublethal effects of multiple insecticides on the olfactory system of parasitoid wasps, as is the case with all research, more questions have come up and remain unanswered. One open question pertains to possible synergistic effects of multiple insecticides, or combinations of insecticides and other control agents. Both questions have been studied with *Apis mellifera* (Tosi & Nieh, 2019; Y. H. Wang et al., 2020), but as we have seen with various results in previous chapters, these results are not indicative of potential effects on parasiotid wasps. It is unknown whether commercial formulations of control agents, in which the active insecticide is supplemented with either inert ingredients and/or additional control agents, have the same effects at sublethal levels.

Behavioural assays with exposure in semi-field and field conditions could also provide further information on the influences of various abiotic and biotic factors on parasitoid wasps' life history, and how these may intersect with the influences of insecticides (Giovanni Tamburini et al., 2021). Studies based wholly in a lab setting are useful for isolating parameters so that external influences are kept to a minimum, yet in nature these external factors may amplify or reduce effects seen in laboratory settings and as such are more valuable for informing contemporary agricultural practices like Integrated Pest Management. Said studies could also be used to further study the accumulation of contaminants like pesticides via hosts and their food medium in a more natural context.

Finally, part of the rationale for this thesis research is the establishing of the three species, *N. vitripennis, L. distinguendus* and *L. heterotoma*, each model systems in their own rights (Mair & Ruther, 2019; Niedermayer et al., 2016; Quicray et al., 2023), so they can be used in the process of future regulation for the allowance of novel insecticide compounds. Both the topical application and behavioural assays are easily reproducibly with novel chemicals. Furthermore -as was shown in chapters 3, 4 and 5- the mandatory research with *A. mellifera* and *Osmia bicornis* (Authority, 2016) are insufficient at describing if a substance truly is safe for beneficial insects. Future risks assessments could, with little issue integrate the aforementioned experiments and thereby decrease the impact of potential original compounds on non-target beneficial organisms. While these tests would ultimately demand more resources of those developing novel pesticides, ultimately considering the potential impacts those compounds can have on beneficial insects, like the parasitoid wasps, it seems an appropriate area to heed caution, especially in the current era of anthropogenic mass extinction.

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