Acute and chronic neonicotinoid effects on navigation and hive development of the honey bee *Apis mellifera*

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1. Zusammenfassung

Diese Dissertation befasst sich mit den Auswirkungen von nicht letalen Dosen von Neonikotinoiden auf Bienen. Neonikotinoide stellen eine Klasse von Insektiziden dar, die auf den nikotinischen Acetylcholin Rezeptor wirken. Sie werden im großen Maße in der Landwirtschaft als Spritzmittel und zur Saatgutbeize eingesetzt. Dabei können sie in Rückständen von Bienen beim Sammeln von Nektar und Pollen aufgenommen und zum Stock gebracht werden. Damit wirken diese Stoffe sowohl auf die Sammlerinnen die primär mit ihnen konfrontiert werden, als auch auf die restlichen Bienen im Stock. Um einen weiten Blick auf die Auswirkungen der Neonikotinoide zu werfen wurden deshalb Experimente an einzelnen Sammlerinnen durchgeführt, die mit den Substanzen gefüttert wurden, ebenso wie Experimente an Bienenvölkern, bei denen die Substanzen in die einzige vorhandene Futterquelle gegeben wurde. Damit wurde sichergestellt, dass das gesamte Volk diese mit der Nahrung zu sich nahm.

In dieser Dissertation wurden die Neonikotinoide Imidacloprid, Clothianidin und Thiacloprid benutzt. Die beiden erst genannten unterliegen zum Zeitpunkt des Verfassens dieser Arbeit einem temporären Verkaufs- und Ausbringungs-Stopp, bei Agrarpflanzen die von Bienen angeflogen werden. Auf Grund dessen sind die Ergebnisse dieser Arbeit, in der Hinsicht auf die Bewertung der Gefahren von Neonikotinoiden, wichtig.

Als neuronal aktive Substanzen können Neonikotinoide die normale Funktion des Nervensystems von Bienen beeinflussen. Die Aufnahme durch die Nahrung sorgt für eine breite Verteilung im Körper der Biene und damit einer Beeinträchtigung des gesamten Nervensystems. Dies kann zu Veränderungen im Verhalten bewirken, die in Veränderungen in der Bewegung, Orientierung oder auch Interaktion mit anderen Bienen beobachtet werden können. Die Wirkung am Rezeptor variiert, trotz gleichen molekularen Ziels an der Zelle, stark zwischen den verwendeten Neonikotinoiden. Clothianidin wurde als voll Agonist beschrieben, der sogar stärkere Ströme als Acetylcholin bei gleicher Konzentration hervorrufen kann. Imidacloprid dagegen wurde bereits als partieller Agonist beschrieben, der geringere Ströme über den Rezeptor

auslöst. In dieser Arbeit wurde ein erster Versuch durchgeführt um Thiacloprid ebenfalls als Agonist am nikotinischen Acetylcholin Rezeptor der Biene zu beschreiben. Hierbei wurde, wie beim Imidacloprid, durch die Applikation an einer Zelle in Kultur ein geringerer Strom als durch Acetylcholin ausgelöst. Allen Neonikotinoiden ist gleich, dass sie eine hohe Affinität für den nikotinischen Acetylcholin Rezeptor aufweisen und den natürlichen Liganden dort verdrängen können. Eine Wirkung an anderen Zellen oder anderen Molekülen kann nicht ausgeschlossen werden, jedoch wurde bisher keine offensichtliche Wirkung von Neonikotinoiden an einem anderen Zielort beschrieben. Die Wirkung dieser Substanzen ist hochspezifisch für Insekten. Zwar wurden in Zellmodellen eine leichte Aktivität am nikotinischen Acetylcholin Rezeptor von Säugetieren gefunden, jedoch sind hierfür sehr hohe Konzentrationen nötig. Bei Insekten wirken die Substanzen schon in kleinsten Mengen. Die wirksamen Konzentrationen können jedoch auch innerhalb dieser Stoffgruppe variieren. Das, auch in dieser Arbeit genutzte, Thiacloprid hat einen LD_{50} Wert der etwa um den Faktor 1000 über den von Imidacloprid und Clothianidin liegt.

Zur Untersuchung zum Einfluss von Neonikotinoiden wurden Bienenvölker unter kontrollierten Bedingungen gehalten, bei denen je eins der Neonikotinoide Clothianidin, Imidacloprid oder Thiacloprid in das Futter gemischt wurden. Die Experimente fanden im Flugraum des Instituts für Bienenkunde statt, in dem die Temperatur, Luftfeuchtigkeit und die Beleuchtung reguliert werden konnte. Es wurden Neonikotinoid Dosen gewählt, von denen durch vorhergehende Tests oder durch frühere veröffentlichte Arbeiten davon ausgegangen werden konnte, dass keine akute Beeinflussung der Sammlerinnen, welche mit dem behandelten Futter primär in Kontakt kamen, bestand. Allen Völkern stand nur das gegebene Futter zur Verfügung, welches auch bei allen Behandlungen abgenommen wurde. Hierbei handelte es sich um eine Zuckerlösung der jeweils das gewünschte Neonikotinoid beigemischt wurde. Es konnte festgestellt werden, dass chronisches Füttern mit einer Zuckerlösung mit 8,876 mg/kg Thiacloprid zu einer verringerten Sammelleistung führte.

Der drastischste Effekt konnte jedoch im Brutnest beobachtet werden. Die Königin legt konstant Eier in unbesetzte Zellen ab. Normalerweise entwickeln sich diese zu Larven,

die von den Ammen Bienen gefüttert und gepflegt werden. Die Zellen in denen diese Larven liegen werden dann nach einigen Tagen verdeckelt um ihre Entwicklung abzuschließen. Die verdeckelten Zellen sind optisch sehr gut zu erkennen, weshalb sie als Maß für die Entwicklung des Brutnest gezählt wurden. Bei der Behandlung mit Zuckerlösung mit 8,876 mg/kg Thiacloprid konnten kaum verdeckelte Brutzellen gefunden werden. Die Königin legte jedoch weiterhin Eier ab. Das Fehlen der folgenden Entwicklungsstadien ist am besten durch eine gestörte Kommunikation zwischen Eiern oder Larven und den Ammenbienen zu erklären. Diese duftgesteuerte Kommunikation könnte durch die Zugabe von Thiacloprid gestört werden. Denkbar ist dies sowohl in der Larve, indem die Abgabe der Duftstoffe beeinflusst wird, vor allem aber bei den Ammenbienen. Hier kann davon ausgegangen werden, dass die, durch Thiacloprid gestörte, neuronale Verarbeitung die Signale nicht korrekt verarbeitet. Eine Beeinträchtigung der Königin selbst ist unwahrscheinlicher, da die Eiablage weiterhin stattfand. Damit konnte gezeigt werden, dass geringe Dosen die Larval-Entwicklung von Bienen beeinflussen, eventuell durch Einflüsse auf die Larven selbst, oder auf die Kommunikation zwischen Ammenbienen und der Brut.

Um Auswirkungen auf einzelne Tiere zu zeigen, wurden unterschiedliche Parameter im Heimflug von Bienen nach Fütterung mit je einem der Neonikotinoide Clothianidin, Imidacloprid oder Thiacloprid analysiert. Bienen wurden beim Nahrungssammeln gefangen und an einer anderen Stelle freigelassen. Dem zu Folge mussten sie sich orientieren um ihre neue Position zu bestimmen und den Heimweg zum Stock zu finden. Der Heimflug wurde mit Hilfe von harmonischem Radar verfolgt. Die Biene trug hierzu einen Transponder, der das Radarsignal verändert zurückstrahlte und so die Ortung von einzelnen Bienen möglich machte. Dadurch konnte ein Flugprofil erstellt werden, das aus zwei Flugphasen bestand. Zuerst absolvierten die Bienen den Flug der sie von der ursprünglichen Futterquelle zum Stock zurückbringen würde, dann mussten sie vom Ende dieser Position den wahren Standort des Stocks finden. Die beiden Flugphasen setzen zwei verschiedene Navigationskonzepte voraus, deren sich die Bienen bedienen mussten um die Aufgabe zu lösen. In der ersten Phase absolvierten die Bienen den so genannten Vektorflug. Hier kehren sie die Flugrichtung und Länge des Hinflugs zu einer

Sammelstelle um, um zu ihrem Bienenstock zurückzukehren. In der zweiten Phase befanden sich die Bienen nun, da sie an einer anderen Ausgangsposition freigelassen wurden, an einer Position, die nicht mit dem erwarteten Ergebnis des Vektorflugs entsprach (Ankunft am Bienenstock). Hier mussten die Bienen nun lokale Landmarken aus früheren Orientierungs- und Sammelflügen wiedererkennen und daraus einen Weg zurück zum Stock zu finden.

Aus dem Flugprofil konnte abgelesen werden, wie lange die Bienen für die Phasen des Flugs benötigten, in welchem Hauptflugwinkel sie die erste Flugphase absolvierten, in welche Richtung sie am Ende der ersten Flugphase flogen und wie gerichtet der Flug war. Auch wurde erfasst, ob die Bienen überhaupt in der Lage waren zum Stock zurückzukehren. Verfüttert wurden jeweils 50 µl Zuckerlösung mit 2,5 ng Clothianidin, 7,5 ng Imidacloprid, 11,25 ng Imidacloprid oder 1,25 µg Thiacloprid.
Hier zeigte sich, dass die Fütterung mit 50 µl Zuckerwasser mit 0,6 µM und 0,9 µM Imidacloprid, ebenso wie mit 0,1 mM Thiacloprid zu einer verringerten Heimkehrwahrscheinlichkeit führte. In der ersten Flugphase konnte auch gezeigt werden, dass 0,2 µM Clothianidin im Zuckerwasser zu einem schnelleren Flug führte und dass der Flugwinkel im Vergleich zur Kontrolle in Richtung der wahren Position des Stocks verschoben war. Beide Imidacloprid-Gruppen zeigten eine ähnliche, signifikante Verschiebung des Flugwinkels, ebenso konnte im Flug selbst eine häufige Änderung der Richtung festgestellt werden.

In der zweiten Flugphase konnten ebenfalls Einflüsse gefunden werden. Am Ende des ersten Flugabschnitts flogen die Bienen über einen Bewässerungskanal, der nicht auf der ursprünglichen Flugroute zwischen Stock und Sammelstelle lag. Bienen welche mit Thiacloprid behandelt wurden wählten hier häufiger eine inkorrekte initiale Heimflugrichtung, was in länger dauernden Heimflügen resultierte. Im Heimflug dieser Bienen konnten auch häufiger Richtungswechsel beobachtet werden. Die mit Clothianidin behandelten Bienen legten eine längere Flugstrecke zurück, zeigten jedoch keine häufigen Richtungswechsel. Bienen, welche Imidacloprid beider Konzentrationen konsumierten, zeigten einen häufigen Wechsel ihrer Flugrichtung. Interessanter Weise konnten damit bei den mit Clothianidin gefütterten Bienen im Schnitt die längsten

Flugstrecken beobachtete werden, während die mit Thiacloprid gefütterten Bienen die meiste Zeit für den Flug benötigten. Diese Diskrepanz ist teilweise dadurch zu erklären, dass die Thiacloprid behandelten Bienen zwischendurch im Grass landeten und ihren Flug später fortsetzten

Damit konnten bei allen drei gewählten Neonikotinoiden Einflüsse auf spezifische Komponenten der Navigation von Bienen gefunden werden. Die deutlichsten Effekte konnten in der zweiten Flugphase beobachtet werden. Hier muss die Biene zum einen optische Signale verarbeiten um Landmarken zu erkennen, zum anderen muss sie auf ein, bei zuvor absolvierten Flügen gebildetes, Gedächtnis zurückgreifen, die interne Repräsentation der Umgebung des Bienenstocks und der dort vorhandenen Landmarken. Diese Prozesse könnten durch die Fütterung mit Neonikotinoiden beeinträchtigt werden.

Die vorliegende Dissertation untersuchte sowohl einzelne Bienen als auch den Superorganismus Bienenvolk auf Beeinträchtigungen durch Neonikotinoide. Diese konnten durch Verabreichung von nicht letalen Mengen herbeigeführt werden. Da das molekulare Ziel der Neonikotinoide im Nervensystem sitzt konnten in verschiedensten Essays Einflüsse gezeigt werden.

Die Erklärung der beobachteten Beeinflussungen liegt, nach Schlussfolgerung der vorliegenden Ergebnisse in der Beeinträchtigung der korrekten neuronalen Verarbeitung von Informationen. Damit konnten die eingehenden Fragen zumindest teilweise beantwortet werden und die Datenlage zur Frage der Schädlichkeit der, auch politisch umstrittenen, Substanzen erweitert werden.

Imidacloprid und Clothianidin unterliegen momentan einem begrenzten Verkaufsverbot in der EU bis zum Dezember 2015 . Als Teil des EU-Projektes „Auswirkungen von chronischem Insektizid Eintrag auf die Vitalität von Bienenvölkern“ werden diese Daten helfen die Gefährlichkeit der getesteten Substanzen zu bewerten.

2. Introduction

Neonicotinoids can influence the behavior of honey bees in non-lethal doses. They are a class of chemicals designed to affect insects and, if possible, not mammals. They have widespread use in agriculture and are important for the agrochemical industry. Individual worker bees can encounter several neonicotinoids during their foraging trips. An important focus of current research is therefore, to investigate the influence of these substances on bees which gather from plants treated with neonicotinoids. Bees provide a large quantity of pollinators and are as such very important for a stable environment. This thesis aims to investigate effects on single bees tries and to relate this to the development of the entire hive.

Two of the three substances which were tested in this thesis underlie an ongoing moratorium in the EU, which prevents their sale. Banned are the neonicotinoids clothianidin, imidacloprid and thiamethoxam (EFSA press release 2013) until December 2015. To allow for a balanced decision it is important to further the knowledge of these substances.

2.1. Bees in the environment

Bees play an important role in the environment. They are proposed to exist as a phylogenic group for over 100 million years (Cardinal and Danforth 2013). It is likely that they evolved after the occurrence of the first flowering plants approximately 130 million years ago (Crane et al. 2004). While there are bee species that live solitary, many species evolved to eusocial species. The honey bee is such a eusocial animal with a division of labor between the individuals of a hive.

Bees are primary pollinators for many plants. 35% of crop-based food production benefits from pollination (Klein et al. 2007). A review by van Engelsdorp and Meixner collected the data for the global numbers of hives (van Engelsdorp and Meixner 2010). They concluded that although the numbers of hives have increased over the last five decades, the main growth was in Asia. European and North American regions suffered

especially in the last years more and more colony losses. The usage of insecticides is a popular explanation for these colony losses.

Due to the fact that many insects use plants as food sources, the plants developed ways to defend themselves against insects. There are different ways in which plants are getting attacked by insects, but every part of a plant can be eaten by some insects. Their leaves or roots can be eaten or their sap can be siphoned. These are attacks that weaken the plant but do not necessarily kill it. Also their seeds or the seedlings can be eaten, which results in a complete loss of a plant. As such, there are different levels of threat and different ways to defend against these predators. The predator's nervous system is a good target to defend themselves from insects. It is a vulnerable part in every animal, as a normal functioning nervous system requires the correct function of several different ion channels and receptors in multiple cells to work together in a controlled matter. When an insect eats a part of a plant which contains a substance that is able to disturb these correct functions, it can be killed or at least incapacitated. If the part of the plant that is eaten is not vital to the survival it can also be enough to irritate the insect to avoid further aggression.

A well-known example of such an aversive substance is nicotine. The most prominent producers of nicotine are plants of the genus *Nicotiana*. Nicotine is produced in the leaves and affects predators which eat the leaves. When it is eaten it acts on the nervous system of the animal and can disturb the normal nervous function, leading to different effects, depending on the different potential predators. This aversive effect led to the usage of nicotine and nicotine related substances, so called nicotines in agricultural pest management. Later this would lead to the creation of neonicotinoids, which were designed after nicotine.

2.2. The honey bee brain and cholinergic transmission in the bee

Invertebrates use acetylcholine (ACh) as their main excitatory neurotransmitter in their central nervous system (Pitman and Kerkut 1970, Breer and Sattelle 1987, Kreissl and Bicker 1989, Albert and Lingle 1993, Buckingham et al. 1997, Eastham et al. 1998, Goldberg et al. 1999, Déglise et al. 2002). Bees have been shown to have a concentration of up to 2.56 pmol/µg protein in their brain (Fuchs et al. 1989). The targets of acetylcholine are primarily two types of receptors. One is the nicotinic acetylcholine receptor (nAChR). This receptor consists of five subunits and constitutes an ion channel. Upon activation by acetylcholine, by binding of two molecules, the receptor changes its conformation and opens a cation channel over the membrane, permitting the flow of mainly potassium and sodium ions, but also calcium ions from the extracellular in the intracellular space.

Immuno-reactive staining against the nicotinic acetylcholine receptor and histochemical staining of the acetylcholine esterase activity were used to locate the places where this acetylcholine is used for neuronal information transfer (Kreissl and Bicker 1989). A strong accumulation of receptor and enzyme can be found in the olfactory system. The antennal nerves and the glomeruli of the antennal lobes (AL) show a strong staining for nAChR and the acetylcholine esterase (AChE). The AL is the primary processing center for olfactory information. Further downstream in the processing pathway the mushroom bodies can be found. Here the lip region, where the input from antennal lobe converges with other information, shows also a strong nAChR staining and a lighter staining for the AChE. Almost no AChE can be found in the pendunculus of the mushroom body. The mushroom body is a center for multimodal information processing and is involved in memory formation and decision making (review Menzel 1999). The visual neuropils show a similar importance for acetylcholine as a transmitter. The lamina shows nAChR reactivity, but almost no AChE staining. This can then be found similarly in the medulla and lobula. The lobula shows even a very strong staining for the AChE. At last, the central body, which is involved in orientation, also shows nAChR and AChE staining. Memory formation is highly dependent on such a functional cholinergic system (Gauthier et al. 2006). ACh was also shown to be prevalent in the brain of other insect

species (e.g., *Manduca sexta*: (Homberg et al. 1995), *Schistocerca gregaria*: (Leitinger and Simmons 2000), *Periplaneta Americana* (Fusca et al. 2013), *Drosophila melanogaster*: (Buchner et al. 1986, Schuster et al. 1993, Yasuyama et al. 2002, Yasuyama et al. 2003).

A variety of different subunits of the nAChR have been described in the honey bee. In the honey bee genome eleven genes coding for nAChR subunits have been found. Nine different variations of the α-subunit (Amelα1-9) and two variations of the β- subunit (Amelβ1-2) were described (Thany et al. 2003, Thany et al. 2005, Jones et al. 2006). For another important invertebrate model organism, the fruit fly *Drosophila melanogaster,* ten nAChR subunits have been described, with seven α and three β-subunits (Baumann et al. 1990, Gundelfinger and Hess 1992, Grauso et al. 2002, review: Sattelle et al. 2005). The formation of a functional receptor and ion channel depends on the correct paring of subunits. A functional channel consisting of only bee α subunits was found when a homomere of five bee α7 subunits was expressed. The different composition of the nAChRs is important, because they respond differently to acetylcholine and also neonicotinoid application (Dupuis et al. 2012). Four α- subunits have been found in the honey bee brain through in situ hybridization (Thany et al. 2003, Thany et al. 2005). The different subunits are differently expressed in the brain, in regard to region and also to developmental stage. Amelα8 for example was found in pupal Kenyon and antennal lobe neurons, whereas Amelα5 and Amelα7 were found in cells in the mushroom body in type II Kenyon cells and in the antennal lobe of adult bees. Amelα2 was found in type I and type II Kenyon cells but not in the antennal lobes. These different receptors react differently to application of acetylcholine. Substances that mimic the actions of acetylcholine, such as neonicotinoids, could thereby have different effects on the nerve cells, regarding which type is activated.

The other receptor type is the muscarinic acetylcholine receptor. It is named after its sensitivity to muscarine, a neurotoxin found in members of the *Agaricomycetes*, with mushrooms like the fly amanita. The receptor is metabotropic. This means, that the receptor itself is not an ion channel, but that the receptor is coupled to an intracellular mechanism that triggers either an ion channel located near the receptor or another

intracellular reaction. Although acetylcholine can activate both receptors, they are different in the binding to the receptor. Acetylcholine interacts with a carbonyl group of the nicotinic receptor in a distance of 5.9 Å, while it interacts with an ethereal oxygen atom in a distance of 4.4 Å to the muscarinic receptor (Beers and Reich 1970). No muscarinic-receptor specific binding site for neonicotinoids has been described. This shows a difference in the binding features of neonicotinoids to the natural agonist, acetylcholine.

2.3. Neonicotinoids

Nicotine is a naturally occurring neural active substance. It is an alkaloid found in plants of the *Solanaceae* family and is produced to protect itself against herbivores. It is active against neurons of vertebrates as well as invertebrates by acting on the nicotinic acetylcholine receptor. It was used in the early 20th century as an insecticide and is still used in some parts of the world. The neurons with nAChR of vertebrates as well as of invertebrates respond to administration of nicotine with an excitation. Whereas nicotine binds to the nAChR and causes a reaction, it is not able to block all binding of other ligands to the nAChR of *Musca domestica* and *Torpedo marmorta* (Eldefrawi et al. 1971). Since nicotine is toxic to humans many modifications to the molecule where tried over the time to improve its activity as an insecticide. This led to the synthesis of nithiazine, the first substance later known as “neonicotinoid”. The term “neonicotinoid” was suggested by Tomizawa and Yamamoto (1993). It was introduced to differentiate between substances that act in a similar way on the nAChR but differ in their activity on insects. One of the main motifs of acetylcholine is a positively charged nitrogen atom as part of its molecule, as pictured in figure 2.1. a. This is crucial for the interaction with the nAChR. Neonicotinoids on the other hand have, similar to nicotine, a partially positive charge in their molecular structure, due to various electron- withdrawing substituents. Structural examples are shown in figure 2.1. b-e. This allows them to interact with the nAChR, without the need for an ionized atom. The missing ion allows easier passage through organic compounds such as the cuticle of insects or protective membranes

(Tomizawa and Yamamoto, 1993). Nithiazine was synthesized 1970 by Soloway and colleagues (Soloway et al. 1978). But this chemical was not photo stable enough to be used under field conditions. Several modifications of nithiazine resulted years later in the creation of imidacloprid in 1985 by Shiokawa and colleagues (Shiokawa et al. 1986). And they showed only a low affinity for nAChRs of several non-target organisms like mammals (Tomizawa et al. 2000a). This high selectivity is one of the main reasons why neonicotinoids achieved such a quick popularity. The handling of these substances constitutes less direct risks from the substance itself for the person handling it. This is an important difference to other substances used for plant protection. Examples for these are pyrethoids, which act on voltage gated sodium channels (review: Bhupinder 2002), organophosphates, which inhibit the acetylcholine esterase (Namba 1971) or cyclodienes, which act in GABA-gated ion channels (Buckingham et al. 2005). Many of those other substances have molecular targets that are quite similar in insects and in mammals and can thereby affect and harm also humans. Examples are the organophosphates dichlordiphenyltrichlorethan (DDT) and sarin with well-known effects on non-target organisms. Another important quality is the high photo stability. This feature was first added to neonicotinoids with the creation of imidacloprid and was a necessary step to be effective under field conditions. This allows spreading a solution with neonicotinoids onto crops and having it active for at least several days. The water solubility is another important feature. There are two main ways to bring the neonicotinoid to the desired targets. For one, it allows for spraying a water based neonicotinoid solution on plants. The second way is seed coating. Here the seed gets coated with a layer of formulated neonicotinoid. After such a seed is planted the plant absorbs the neonicotinoid throughout the growth phase. The water solubility allows the insecticide to enter the plant and be distributed systemically, throughout the entire plant. The important characteristics of neonicotinoids and their specificity for the target insects were recently reviewed by Jeschke et al. 2013.

The use of neonicotinoid is already restricted to concentrations and forms of spreading to minimize the effect on honey bees in many countries. But these restrictions cover only high concentrations to prevent immediate deaths of foraging bees.

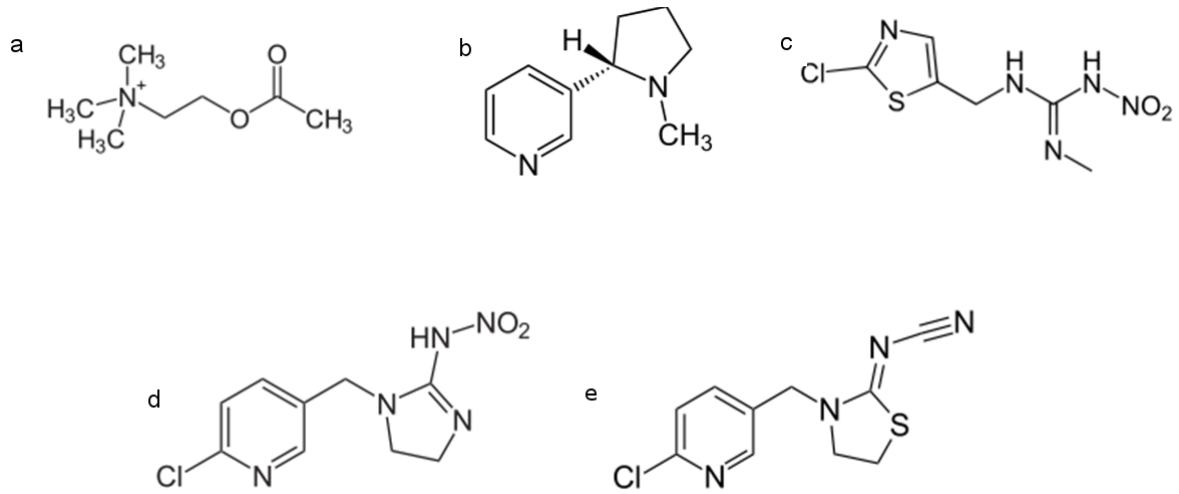

Figure 2.1. Ligands of the insect nicotinic acetylcholine receptor. Shown are the natural ligand acetylcholine (a), nicotine (b) and three neonicotinoids, clothianidin (c), imidacloprid (d) and thiacloprid (e).

Neonicotinoids can be differentiated in subclasses, according to their structural differences. Clothianidin has an acyclic group; thiacloprid and imidacloprid have a chloro-substituted heterocyclic ring (CN-groups and NO-groups). The nitrogen atom is important, as it provides one of the important interaction points of the molecule with the receptor. This nitrogen atom becomes partially positively charged due to the rest of the molecule. It becomes however not ionized in the bee cellular substrate, unlike nicotinoids (Tomizawa and Yamamoto 1993).

As described earlier, the opening of the nAChR leads to increased cation permeability. Binding of the neonicotinoid at the nAChRs will activate the receptor and via depolarization cause excitation of the postsynaptic membrane (Bai et al. 1991, Brown et al. 2006, Jepson et al. 2006, Palmer et al. 2013). This current can be measured and the current elicited by neonicotinoids can be compared to the natural ligand acetylcholine. Imidacloprid was found to act as a partial agonist, eliciting a smaller current than acetylcholine, on cultured Kenyon cells (Déglise et al. 2002) and antennal lobe neurons (Nauen et al. 2001, Barbara et al. 2005, Barbara et al. 2008). In *Drosophila* neurons, clothianidin was found to be an effective agonist and elicits a greater current than acetylcholine (Brown et al. 2006). It was shown that applications of imidacloprid or clothianidin depolarize Kenyon cells in isolated honey bee brains via nAChR activation

with different efficiencies (Palmer et al. 2013). However, both substances block the transmitter binding and thus act as blockers of cholinergic receptors upon prolonged applications in honey bees (Déglise et al. 2002, Palmer et al. 2013). This is a result of the fact that neonicotinoids cannot be degraded by the acetyl cholinesterase.

Given the wide-spread central nervous distribution of the nAChR, it is not surprising that sub lethal neonicotinoid doses compromise behavior and cognitive abilities also in honey bees including memory formation and retrieval, social interactions, navigation and communication. An overview of studies of these effects can be found in table 2.1.

Table 2.1. List of studies regarding neonicotinoid effects. All studies were performed on bees unless stated otherwise. Abbreviations: PER = proboscis extension response, ltm = long term memory, RFID = radio frequency identification, HPLC = high pressure liquid chromatography.

Neonicotinoids	Method	Key findings	Author/s
Imidacloprid	Radio ligand binding	Partial positive nitrogen atom interacts with invertebrate nAChR	Yamamoto et al. 1995
Imidacloprid, Thiacloprid	Radio ligand binding	Upregulate human α4β2 nAChR expression in cell models	Tomizawa and Casida 2000
Imidacloprid	Whole cell patch clamp recordings	Imidacloprid evokes weaker currents than acetylcholine	Déglise et al. 2002
Imidacloprid	Bee marking and observation	High concentrations repel bees (>500 ppb)	Bortolotti et al. 2003
Clothianidin, Thiamethoxam	HPLC, Mass spectrometry	Plants metabolize thiamethoxam to clothianidin	Nauen et al. 2003
Clothianidin	Whole cell patch clamp recordings	clothianidin evokes stronger currents than acetylcholine	Brown et al. 2006
Acetamiprid, Thiamethoxam	PER – conditioning, monitored walking distance	Increased water uptake, lowered learning performance	Aliouane et al. 2009
Thiacloprid	Infection with *Nosema ceranae*	Increased mortality when coupled with a *Nosema* infection	Vidau et al. 2011

Imidacloprid, Clothianidin	RFID tracking	Reduced foraging activity, no flight activity after consumption	Schneider et al. 2012
Imidacloprid	camera tracking	Reduced mobility	Medrzycki et al. 2013
Imidacloprid	RFID tracking	Reduced foraging performance in bumble bees	Gill and Raine 2012
imidacloprid, clothianidin, thiacloprid	Radar – tracking	Reduced likelihood to return home, impaired orientation	Fischer et al. 2014

Neonicotinoids are designed in a way that especially the mammalian nAChR has almost no response to common neonicotinoids (Tomizawa et al. 2000a). However, it was shown that clothianidin and imidacloprid can influence the normal activation of the mammalian α4β2 receptor in cell culture (Li 2011). While both reagents are only able to elicit a very low current at the receptor, co-application with acetylcholine showed more visible effects. Imidacloprid reduced the current elicited by acetylcholine greatly, while clothianidin co-application can amplify the effect of acetylcholine in a low concentration. Also a weak activation of rat muscle nAChR in a heterologous expression system in *Xenopus* oocytes was reported (Methfessel 1992)

Exposure to imidacloprid and thiacloprid was shown to upregulate the amount nAChR in heterologous expression systems, with mouse fibroblasts expressing the chick nAChR variant α4(2)β2(3) (Tomizawa et al. 2000b). The mechanism of this upregulation is not entirely clear. It is proposed that a normal or higher ionic current is not required for this, as shown in studies with nicotine (Peng et al. 1994). The authors suggest that binding by the agonist changes the conformation of the channel what impedes the degradation of the channel proteins.

A mechanism with which neonicotinoids bind to the receptor is known. Neonicotinoids contain a halogen atom, which exerts the needed electronegativity to create a partial positive charge with which the molecule can interact with the nAChR (Tomizawa 2011). It is assumed that all neonicotinoids have only one binding site on the α-subunit of the receptor (Zhang et al. 2000). A method to specify the binding region is to use neurotoxins which are known to block the binding site of the natural ligand.

Acetylcholine can be prohibited to interact with the nAChR by blocking the channel with α-bungarotoxin. Imidacloprid was shown to displace α-bungarotoxin from the nAChR in cockroach nerve preparations. This indicates that this is the binding site for neonicotinoids at the receptor (Bai et al.1991). Additionally, a specific point mutation, Y151S, in the α-subunit was found which could greatly decrease the responsiveness of a receptor to imidacloprid, but not to acetylcholine (Liu et al. 2006). The substitution of the tyrosine at position 151 by methionine in the α1 subunit, expressed in a heterologous system with rat β2 subunits in *Xenopus* oocytes led to an agonist-like action of imidacloprid. Responses to acetylcholine were blocked and the effect was slowly reversible (Zhang et al. 2008).

It was also shown that, when using a cell model *with Xenopus laevis* oocytes expressing a hybrid nAChR with *Drosophila melanogaster* α4 and chicken β2 subunits, the initial current through this receptor elicited by imidacloprid can be greatly increased by introducing a two point mutation in the D-loop of the β2 subunits. In the T77R mutation, the polar threonine is substituted by the basic arginine. The positive charge of arginine is supposed to help to attract imidacloprid. A positive charge in the D-loop, presented by a glutamate at the 79th position is then still an electrostatic hindrance. By substituting this with a neutral valine in the E79V mutation, the channel shows a greatly increased current after application of imidacloprid (Shimomura et al. 2006).

After exposition to neonicotinoids insects, but also the plants which are treated, are able to break those substances down through detoxification. In this process the neonicotinoid becomes partially digested and changed. One of the mayor enzymes in the detoxification is the cytochrome oxidase P450 (Schulz-Jander and Casida 2002). It was shown that when neonicotinoids lose the electronegative part of their molecule, they also become more active and bind better to nAChRs in cell models with vertebrate receptor subunits (Tomizawa et al. 2000b). This may lead to concerns, for example when bees are poisoned and get eaten by animals which are normally not affected by neonicotinoids. It is possible that a bee which is undergoing detoxification has at least some catabolic products which can then affect non insect species. For example, a neonicotinoid that was not used in this study, but which is also used in agriculture,

thiamethoxam, was shown to be metabolized to clothianidin in *Spodoptera frugiperda* larvae, as well in cotton (Nauen et al. 2003). This led to a much higher toxicity than initially assumed, as thiamethoxam has a low affinity to the nicotinic acetylcholine receptor, while clothianidin shows a very high affinity.

The brain of a bee is not the only target of neonicotinoids. Other tissues also show reactions after exposure to neonicotinoids. A continuous exposure to imidacloprid changes the expression levels of several genes in the gut of larval bees, especially immune-related genes (Derecka et al. 2013). It is however not clear, if this is a direct reaction of the cells of the intestines or if the nervous system is again affected and sends signals to other cells which then react to this stimulus.

Insects had time to develop targeted resistance mechanisms against natural occurring neurotoxins. The caterpillar of *Manduca sexta* for example is able to eat nicotine-rich leaves (Snyder et al. 1993, Wink and Theile 2002). Neonicotinoids exists now for 40 years. First reports indicate that some pest insects might be subject for selective pressure for resistance development. An example for this is the brown plant hopper *Nilaparvata lugens* (Liu et al. 2005) and the Colorado potato beetle *Leptinotarsa decemlineata (*Szendrei et al. 2011). But it can be assumed that honey bees which are cared for by a bee keeper do not undergo the same selective pressure, assuming that free mating between the hives can occur. Bees, like other livestock, can also be subject to targeted breeding. And selection for neonicotinoid resistance might be difficult to achieve.

2.4. Disturbances of global bee population

Plants are generally stationary life forms. As such, they have to rely on various transmission mechanisms to achieve sexual reproduction with another individual of their species. This can be done by releasing their pollen in the air and let it be transmitted by the airflow. A similar mechanism is true for plants living under water, where the sperms

are just released in the water and transmitted by the water flow. Both transmission mechanisms have the advantage that they are independent of other carrier systems.

An alternative way to ensure pollination is to rely on animals to carry the pollen to a blossom of the same species. This is a mechanism that evolved later than the air dependent pollination, as it requires an animal to move between plants of the same species. To ensure that this mechanism works the plant has to give an incentive to a mobile transmitter, like an insect. In this case, the pollinator visits a plant to gather resources, like nectar or the pollen themselves. During this, some pollen sticks to the surface of the pollinator. When it visits another individual of this species, this pollen can enter the blossom and thereby ensure pollination.

Often there is a specific coevolution or adaptation between plants and some specific pollinators. This ensures that the plant has an animal, often an insect, to carry pollen to another plant of the same species, allowing pollination. On the other hand, this can ensure that the animal has a carbohydrate source that can be difficult to access for animals which did not adapt to the specific flower. Honey bees on the other hand pollinate very indiscriminately many flowering plants. Also, where other insects can also act as carriers for pollen, they normally do not occur in such a high density as honey bees. Thereby bees are very important for the reproduction of a large number of plants. Beekeeping as a profession also takes advantage of this, as colonies are kept in transportable boxes which can be transported to agricultural land for targeted pollination.

During this bees can encounter neonicotinoids, as they are used for plant protection by spraying or seed coating. But other sources, such as flea-collars for pets, can also contain neonicotinoids and disperse them in the environment. These substances are then often combined with chemicals against other targets like fungi. If several of such products are combined they can have synergistic effects which then affect the bee even stronger.

There are also first reports that bees are not repelled by neonicotinoids in their food sources and that they may even be attracted by low doses (Kessler et al. 2015). During my own experiments I could also observe that bees showed no aversion against

solutions with non-lethal doses of neonicotinoids, together with sugar as an appetitive stimulus and without.

Sub lethal behavioral effects on pollinating bees may thus be the most likely exposure scenario in agriculture from neonicotinoid plant treatment. Although the concentrations detected in pollen and nectar from seed-treated crops with neonicotinoids are generally too low to cause immediate death from acute poisoning (Blacquiere et al. 2012, Belzunces et al. 2012), neonicotinoid residues in pollen and nectar often lead to long-term pesticide exposure when honey bees are foraging on treated crops.

A disturbance in this pollination task, committed by bees, could possibly be compensated by other pollinators. But the targeted pollination with an organism where thousands of pollinators can be kept in high density and can be moved to desired areas would certainly make a negative impact on modern agriculture.

2.5. Main goals of my experiments

The main hypothesis of this thesis is that neonicotinoids influence the behavior of bees when administered in non-lethal doses. Individual worker bees can encounter several neonicotinoids during their foraging trips. While a lethal dose would incapacitate them, prevent their return to the hive and killing them, a non-lethal dose can result in the return of a bee to the hive with contaminated food.

In the first experimental chapter influences of such low concentrations over long periods are investigated. Bees have to gather sugar syrup, laced with different neonicotinoids. Acute effects on individual bees are not expected, although during the course of the experiment accumulation might affect the bees and show altered behavior. The development of the brood nest is monitored to see how the offspring which then had to develop under neonicotinoid influence is developing. The larvae might show deficits in their development. Another factor might already be the eggs, or the egg laying. The queen will be under constant exposure to neonicotinoids, which might compromise the ability to lay eggs or compromise the early development.

The other main part is a detailed investigation of individual effects. The navigational skills of bees are the focus of interest for this. Orientation is an essential skill for every foraging bee and neonicotinoids can interfere with this (Schneider et al. 2012). The flight path of bees which return to their hive is monitored via radar to get a detailed view how neonicotinoids interfere with their orientation. This is highly dependent on several brain functions, such as perception, memory retrieval and motor coordination. Interferences are expected in all components. While the effects on the motor coordination should be minimal to ensure the ability to fly, it can be expected that neonicotinoids will disturb the correct information flow in the brain during the return home which then will lead to an altered flight course.

The combination of these experiments can then be used to assess some of the influences of non-lethal neonicotinoid doses on individual bees. It also sheds light on if a bee returns to the hive after exposure to a neonicotinoid and if there is a delay that might indicate a detoxification before the return. If the bees return in time, it can then illustrate what can be expected effects on the developing brood inside the hive.

Even with the presented problems for bees, neonicotinoids present an interesting option in agriculture, as they are very specific in their target sites and show almost no activity on human nervous cells (Tomizawa et al. 2000a). This makes them a desirable solution against common pest insects in agriculture. Many alternative substance classes like pyrethoids or organophosphates can still be dangerous for humans and other mammals in high enough concentrations. The current risk assessment for bees contains only a toxicity essay to estimate the LD_{50} value. A desirable goal might then be to find a dose range in which the effects of non-lethal doses can be kept to tolerable parameters under inclusion of the here new found data.

3. Thiacloprid is an agonist of the nAChR from larval bees

3.1. Introduction

Neonicotinoids have been described as agonists of the nAChR. It was shown that imidacloprid acts as a α-bungarotoxin sensitive, as well as insensitive nAChRs in *Periplanata americana* dorsal unpaired median neurons (Buckingham et al. 1997). Imidacloprid was also described in honey bees as a partial agonist of the nAChR in antennal lobe cells (Nauen et al. 2001) and in Kenyon cells (Déglise et al. 2002). Clothianidin was described as a full agonist of the nAChR in *Drosophila* and the authors even described a current greater than a current elicited by similar acetylcholine stimulation (Brown et al. 2006). Both neonicotinoids have also been shown to inhibit cholinergic responses in the mushroom body of bees by triggering tonic inward currents which lead to a depolarization block (Palmer et al. 20013).

Thiacloprid is expected to also act as agonist of the nAChR, but was not already described as such in bees. Behavioral observations the lab at the Institut für Bienenkunde showed similarities between imidacloprid and thiacloprid. This led to the assumption that thiacloprid may also act as partial agonist. To investigate this, currents were measured from pupal antennal lobe neurons after application of thiacloprid. For comparison the natural agonist acetylcholine was also applied in the same concentration. The currents and the length of the current were then compared.

3.2. Materials and Methods

This study was performed on cultured cells from pupal antennal lobe neurons from honey bees. The used methods were based on the protocol published by Kreissl and Bicker (1992). Pupae of stages P 6-7 were collected from the hives and the antennal lobes dissected. The tissues were first dissociated standard saline (containing NaCl 130 mM, KCl 5 mM, $MgCl_2$ 10 mM, glucose 25 mM, sucrose 180 mM and Hepes 10 mM with a pH of 7.2). This was followed by a transfer in a preparation medium (Leibovitz L15 medium, Gibco BRL, supplemented with sucrose 22.2 mM, fructose 22.2 mM,

glucose 0.09 mM, proline 0.029 mM; with penicillin/streptomycin 0.5 ml/l and gentamycin 50 µl/l at 500±10 mOsmol/l). After obtaining the cells in the medium, a dish coated with Concavalin was filled with 20 µl of the suspension. The loaded dishes were then filled with 2 ml of culture medium (containing the previous described Leibovitz L15 medium with Pipes 2 mM, 14.9% FCS and 1.2% yeastolate) and kept at 26°C in an incubator. The experiments were performed 2-4 days later.

The measurements were done with patch clamp in whole cell configuration following the methods described in the dissertation by Himmelreich (2013). The recordings were done with an ECP9 amplifier using the software patch-master v 2.4 (both from HEKA Elektronik, Lamprecht, Germany). The data were low-pass filtered at 2.9 kHz with a Bessel filter. Capacitance caused by the pipette and the cell itself was compensated by the “c-fast” and “c-slow” mode of the amplifier.

A borosilicate glass pipette (GB150-8P, Science Products GmbH, Hofheim, Germany) was used for the recording. It was filled with an internal standard solution (containing potassium gluconate 115 mM, KF 40 mM, KCL 20 mM, $MgCl_2$ 4 mM, Bapta 5 mM, Na_2ATP 3 mM, Na_2GTP 0.1 mM, glutathione 6 mM, sucrose 150 mM and HEPES-Bis-tris 10 mM with pH 6.7 and 490±10 mOsmol/1; the KF was added shortly before the recording).

The cells were kept under a continuous flow of standard saline.

Thiacloprid was prepared by dissolving 18.78 mg in 1 ml of acetone, leading to a 50 mM solution and kept overnight to ensure full dissolution. This was then further diluted with external standard saline to a concentration of 1 mM which was then stored frozen. The final concentration was achieved by further dilution with external saline shortly before the experiments. The same was true for Acetylcholine, which was already prepared in 10 mM concentration.

Transmitters were applied by pressure in the flow of the external solution. Acetylcholine and thiacloprid were kept at a concentration of 100 µM. The application was also controlled by the patch-master software and was set to last for 400 ms after triggered.

Cells were clamped to a holding potential of -70 mV after the patch was achieved. This ensured that no voltage sensitive channels were opened during the recording.

3.3. Results

The measured currents on antennal lobe neurons could always be classified as slowly desensitizing currents. These were found to constitute the majority of cholinergic currents in antennal lobe neurons (Barbara et al. 2008). Acetylcholine or respective thiacloprid were pressure applied in a concentration of 100 µM each and the elicited current measured. Example traces can be seen in figure 3.1. The application of acetylcholine elicited a mean peak current of 629±220 pA (n = 6). Thiacloprid elicited a peak current measured of 344±306 pA (n = 8). With this, thiacloprid was only able to trigger 43.3% of the mean current by the same concentration of acetylcholine.

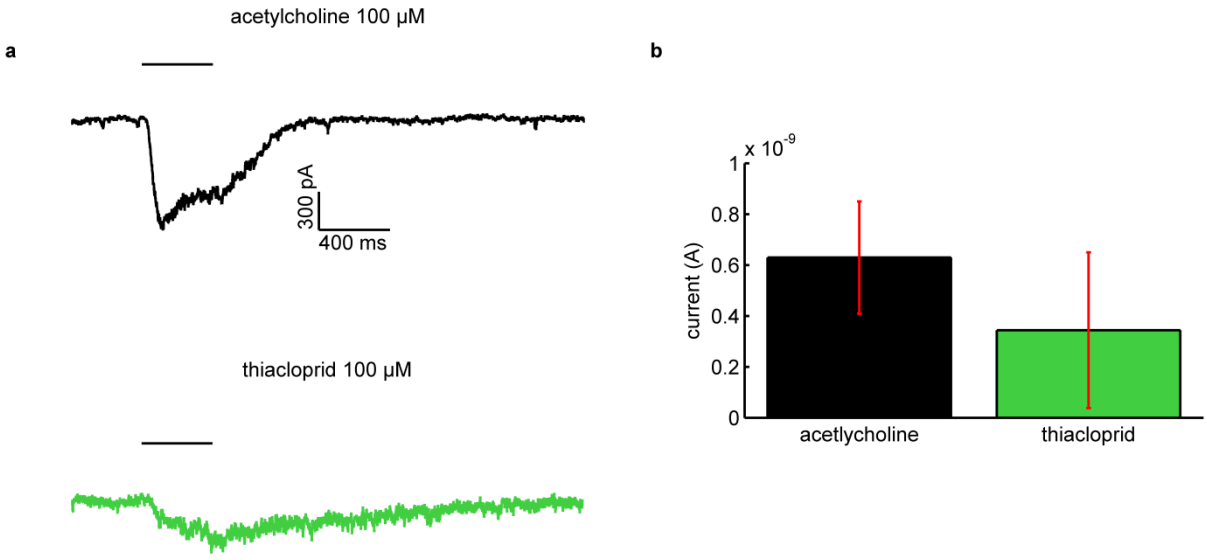

Figure 3.1. Acetylcholine and thiacloprid act as agonists of pupal antennal lobe cells. a shows example traces for acetylcholine (upper) elicited currents and thiacloprid (lower) elicited currents. The black bar indicates the duration of the transmitter application. Both substances were applied with a concentration of 100 µM for 400 ms. **b** shows the mean currents measured. Acetylcholine led to a mean peak current of 794 pA, thiacloprid to 344 pA. The standard deviation is shown as a red bar.

Another observation was the length of the elicited current. The pulse of 400 ms acetylcholine elicited on average a current with a length of 0.95 s. The initial desensitization can be observed during the pulse and the currents cease shortly after. Application of thiacloprid led to a different kinetic, with a slower onset and a longer time until the initial level was reached an average length of 1.93 s, with a much slower closing kinetic.

3.4. Conclusion

The found data are consistent with literature. Currents of about 700 pA have been described in cultured pupal antennal lobe neurons (Barbara et al. 2005). The data suggest that thiacloprid acts as agonist on antennal lobe neurons of honey bees. Although the current triggered by the agonist is weaker than by acetylcholine. This may help to explain some of the findings of influence of thiacloprid on different parameters of honey bee behavior or other measurements. A general trend in experiments with thiacloprid is a reduced activity of the animal. This is comparable to the effects of imidacloprid, which also leads to weaker currents in Kenyon cells of bees (Déglise et al. 2002). The longer duration of the weaker current is also an interesting effect. As described earlier, a common feature of neonicotinoids is a high affinity for the receptor (Nauen et al. 2001). They are also not affected by the acetylcholinesterase. Although this enzyme can be excluded in this experiment, the high affinity could be an explanation for the prolonged opening of the receptors. The actions of thiacloprid on the neurons still need to be fully characterized but the properties as a partial agonist, like imidacloprid becomes more evident.

4. Hive development after chronic exposure to neonicotinoids

4.1. Introduction

Experiments were planned to prove the concept of a long term exposure experiment. The goal was to establish a closed environment in which we could keep a bee colony and control the environmental parameters such as temperature, length of day, humidity and available food sources. This reduced the parameters to explain the observable effects. Otherwise, fluctuations in weather like long periods of rain or aridity and extreme temperatures affect bee colonies. Also, the used concentrations for the chronic feeding are thought to have no effect on a bee after consumption of a single dose. By constructing an environment in which the bees are bound to consume the tested substances over a long time a comparison can be achieved to test the effects of long time exposure to neonicotinoids. Whether this is due to accumulation of these substances in a bee by multiple feeding sessions or accumulation in the hive remains unclear. Due to the length of the experiment the used substances should affect already existing bees in the hive, as well as newly emerged bees, which were raised under the influence of neonicotinoids. An influence can be assumed, as the primary energy and carbohydrate source, the offered sugar syrup was spiked with the neonicotinoids. Although the pollen, which was also present in the flight cages, can supply the bees with carbohydrates, one can assume that this is not enough to sustain the energy needs of a hive and that the bees have to rely on the sugar water for their energy needs.

A perquisite for this experiment was that bees forage from food sources containing low amounts of neonicotinoids. Early studies showed that individual bees can be fed with sugar water containing neonicotinoids (Schneider et al 2012). It was recently published that bees might even have a preference to forage from food sources containing imidacloprid or thiamethoxam in low concentrations (Kessler et al. 2015). The authors could also not find a reaction from the gustatory sensory neurons to application of imidacloprid, clothianidin or thiamethoxam. This implies that the neonicotinoids do not

act as a deterrent during foraging, but could encourage bees to forage more of the syrup spiked with neonicotinoids.

Emerging bees can have different modes of exposure to the neonicotinoids during their different developmental stages. In the first days, after the queen has laid an egg in a cell, the organism has no contact to food. An effect of neonicotinoids in this phase should only occur if the eggs in the queen were affected earlier. To reach the next developmental stage, a nurse bee has to attend the egg, rearrange it and start to feed it. In this stage, the developing bee can come in contact to contaminated food, as well as it is dependant on a nurse bee which can also be affected by neonicotinoids. The next step in development is then the larval state, which requires constant feeding while the larva grows. After several days the cell becomes capped and the developing bee should be independent of the nurse bees and additional food. The complete time frame for the development from an egg to the adult bee varies between the different forms of adult bees. A worker bee emerges after 21 days, a queen already after 16 to 17 days and a drone after 25-32 days (Rembold 1964). Larvae are known to emit chemical cues which can be received by nurse bees (Carroll and Duehl 2012). The interactions between the nurse bees and the eggs, larvae and pupae can then be regulated by signals between them, mainly transmitted via odor (Traynor et al. 2014, Slessor et al. 2005).

From this three main points emerge at which neonicotinoids can interfere with the production of offspring in the hive.

First, the queen can be affected, by consuming the food which was collected by the workers. The neonicotinoids could be able to interfere with her behavior and thus the egg laying process. Second, the behavior of worker bees could be affected. Brood rearing requires regularly attention and care by the workers. During this the worker bee has to feed the developing bee and later seal the cell with wax. As these processes are partly regulated by signals from the developing bee, the nurse bee has to receive the, primarily olfactory, signals and process them in the right way. As we know that the olfactory pathways are heavily reliant on cholinergic transmission one could assume that neonicotinoids would be able to interfere with this mechanism. Third, the

developing bee itself could be affected. Normal cholinergic transmission is supposed to be required for the development of the organism, as it is one of the main excitatory transmitters in the bee nervous system. A disruption in this system could lead to either developmental defects, or could affect the emission of the signals which are used to communicate with the nurse bees.

Some research exists on colony development of bumblebees and how it is altered by neonicotinoids. It was shown that 10 ppb imidacloprid in the food solution leads to an increase in the number of bees which decide to forage instead of remaining in the nest. Also the production of new brood is reduced and foragers are more inclined to become lost during the foraging (Gill et al. 2012). These concentrations are three times as high as used in this experiment. Wu et al. showed in 2011 that combs with pesticide residues, among them neonicotinoids, can lead to a reduced longevity in worker bees.

4.2. Materials and methods

The animals used in this experiment were honey bees, *Apis mellifera*, subspecies *carnica* from the stock of the Institut für Bienenkunde. The bees were cared for by professional beekeepers of the Institute.

The used hives were transferred in the end of fall from the outside into a controlled environment.

4.2.1. Experimental setup

The experiments were conducted in a closed environment, called the flight room. The closed room allowed a complete control of all environmental parameters. This allowed us to conduct the experiments during the winter months in three consecutive years by simulating a warm and sunny environment. Bee colonies outside of the flight room hibernate during this time, with almost no flight activity and without foraging for food.

The room was equipped with electric high frequency lights. The flicker-fuse frequency for bees is supposed to be around 220 Hz (Autrum and Stöcker 1950). The 60 Hz frequency in the electric current would lead to a 120 Hz flicker frequency in normal lights. The used lights produced a flicker frequency of 300 Hz which was sufficient to produce an acceptable environment for the bees. A reflective foil was also mounted on the inner side of the roof over the lights to increase the acceptance of this light source by the bees.

The lights were separated in four groups, each coupled with a shared fuse and a timer. This allowed separate timing of the four light groups.

A 12 hour day/ 12 hour night cycle was chosen, with a transition period of one hour between each phase. For the "dawn" period, first only one light group was activated; the second group followed 15 minutes later and so on until all lights were lit. The "dusk" period was achieved by shutting the lights down in reverse order.

The temperature was also controlled to achieve the desired environment. A normal radiator, connected to the central heating system of the building in which the flight room was located, provided the heating of the room. During the day period we set the temperature to 26°C. During the night time the temperature would decrease by heat dissipation trough the walls.

At last we could also control the air humidity in the flight room, which was set to 60% humidity.

The flight room has an area of 300 m² (15 m x 20 m). We deployed two flight nets, each containing one bee colony for each treatment. This setup ensured two copies of each treatment group for each year. The flight nets had a base area of 16 m² (4 m x 4 m) with a height of 2.3 m.

Bee colonies were kept in the hives that were normally used by the Institut für Bienenkunde for regular bee keeping activities. These hives were only filled with five combs, half the amount that is used for regular beekeeping. This was done to reduce the amount of bees in the flight nets while it still ensured a normal hive activity. Each hive had a plastic feeder on top (manufactured by Bergwinkel-Werkstätten Schlüchtern) in which a sugar solution, spiked with the specific neonicotinoid for the colony, was offered. The feeder was connected to the inside of

the hive. With this, the bees could walk to the feeder without having to leave their hive, ensuring that each colony would forage from their own food source.

In addition to the sugar solution, each flight net contained a bowl with pollen and a bowl with water. Those food sources required the bees to leave their hives and fly to the bowls to collect these necessary resources. The water and pollen bowls were refilled every three to four days and cleaned when necessary.

A clean, uncontaminated start was necessary for the colonies. To ensure that only the offered neonicotinoids affected the development of the colonies, we started with fresh frames, containing new wax. Then we added two kg bees to each hive. Two days later a queen was assigned to each colony.

In the first year three hives were kept in each flight net, one control hive, one thiacloprid group (8.876 ppm) and one clothianidin group (1.87 ppb). In the next year a fourth hive was added per net, resulting in one control group, two thiacloprid groups (2 ppm and 8.876 ppm) and one imidacloprid group (3.74 ppb). In the third year we also used four groups per net, one control group, two thiacloprid groups (2 ppm and 8.876 ppm) and one clothianidin group (3.74 ppb). An overview for the used concentrations and the related assumed doses for a single bee are shown in table 4.1.

The used concentrations were calculated using the formula from Schmuck et al. (2003). $a = \frac{b}{(20\mu l * 1.335\ mg/ml)} * 1000$. *a* is the desired concentration of neonicotinoid in the food syrup, *b* is the corresponding LD_{50} for a single animal. The 20 µl refers to the anticipated uptake of food from a bee and the 1.335 mg/ml is the density of the food syrup. With this we could estimate the concentrations of neonicotinoids in the sugar syrup relative to the LD_{50} for a single bee. Basis for the used LD_{50} values was the publication by Schneider et al. (2012). This dose is later also represented in the report of the European food safety authority 2013, which is the source for the other oral LD_{50} values. The lowest used acute dose without an observable effect was 0.05 ng/bee. At a LD_{50} of 3.7 ng/bee (EFSA report 2012) this equates to 1/73 of the LD_{50}. This equivalent was used to estimate the first dose for the usage of thiacloprid, as there were no available data to this point in time for a dose that had no acute effect. The finally used dose then equates to 0.237 µg/bee, estimated from

a LD_{50} of 17.32 µg/bee (EFSA report 2012). After the first year we concluded to also use a higher dose of clothianidin and doubled it from 1.87 ppb to 3.74 ppb. We also choose to use a second lower concentration of thiacloprid. All neonicotinoids were dissolved in acetone. These solutions were further diluted with water up to a final step in which the solutions were mixed with Apiinvert©. This dilution led to a final acetone concentration of approximately 0.05% in the highest concentration. The same amount of acetone was added to the control solution. Clothianidin and imidacloprid were bought from Sigma-Aldrich. Thiacloprid was provided by the Bayer Ag in the first year and bought from Sigma-Aldrich in the consecutive years.

Table 4.1. Overview for the used concentrations of neonicotinoids in the different treatment groups. The relative dose for a single bee assumed to result from uptake of this syrup is shown for comparison. The last row shows the oral LD_{50} values which were assumed for this study. The LD_{50} values are taken from the official EFSA report (2012).

Substance	Clothianidin		Thiacloprid		Imidacloprid
Concentration (sugar syrup)	1.87 ppb	3.74 ppb	8.876 ppm	2 ppm	3.74 ppb
Relative Dose for a single bee per day	0.05 ng	0.1 ng	0.237 µg	0.057 µg	0.1 ng
Oral LD_{50} per bee	3.7 ng		17.32 µg		3.7 ng

As shown in table 4.1., all used treatments are below the LD_{50}.Therefore, an acute lethal effect can be excluded as an explanation for the observed effects. The monitored timeframe in which the hives were observed varied between the years, and was adapted to the different conditions each year concerning availability of hives, starting weather conditions and availability of beekeepers for help with the inspection of the brood. The concept of the flight room is, of course, to be independent from the weather conditions in the winter. Nevertheless, the hives were outside prior to the transfer in the flight room. As such the bees experienced the changing climate and could be influenced by it. We tried to start the experiment with hives in which the queen was still laying eggs.

The timeframe varied from 61 days in the first year to 132 days in the second year to 53 days in the third year. In the first year a decline in hive health was observed and assessed by the beekeepers in the later days of the experiment. The longer period in the second year was then used to investigate how an addition of 500 g bees to each hive affected the monitored parameters and if this could be used as a rescue mechanism to prolong the availability of a hive after we observed a decline in hive health (qualitative assessment by the beekeepers). This process was designated as revitalization. Bees can be added to a hive and can be accepted by the old bees. During this period, it was important to protect the queen from the new bees until those were accommodated in the hive. The queen was placed in a cage to achieve this and thereby no egg laying could take place for three to four days. The experimental timeframe was then again set to approximately two months in the third year.

Over the course of the experiment two hives lost a queen. In one control hive and one hive from the imidacloprid group no queen could be found during the first inspection. Both hives received new queens, although the queen in the control hive did not start to lay eggs. This hive had to be excluded for the brood analysis. The bees were still foraging an amount of syrup comparable to other control hives, therefore it was included in the foraging analysis.

The presence of a queen was controlled and confirmed by a beekeeper at each brood inspection interval. The mentioned missing queens were replaced. As some treatments resulted in a lower number of brood cells it was important to guarantee the presence of a queen.

4.2.2. Statistical analysis

To measure the influence of neonicotinoids on hive development two factors were investigated. The first parameter was the foraging activity of each hive. To quantify this, the amount of sugar syrup taken from the feeder of each hive was measured. Every three to four days the bowl containing the sugar solution was taken from the

feeder, weighted, refilled and weighted again. This allowed for calculating the weight of foraged food in grams. These data could then be compared between the groups. Were possible the mean data between hives collecting from syrup with the same treatment (control or neonicotinoids) were compared. For groups with only two hives the individual data are shown. Additionally, the analysis was carried for all 132 days in the second year. To visualize the amount of foraged food, the data were added over the time course of the experiment. After 53 days the maximal amount of food was measured as the sum of all collected food solution over the course of the experiment. To compare different slopes of the food collection curve the half maximal value was calculated. This was done by calculating the half maximal amount and finding the next corresponding real measured value and the day at which this was reached. These data are visualized with a linear interpolation between the measurements.

The second parameters for the analysis were the size of the brood area and the size of the capped brood area. These data were obtained multiple times during the experiment, but not often to avoid a disturbance in the brood care and the hive environment. To achieve this, the brood combs were taken out of the hive and the bees were removed with a brush. Then a transparent plastic sheet was placed over the comb and the outline of the brood area was copied in this sheet. All capped cells were also marked in the sheet to look for effects on the development of the brood. These sheets were then scanned and digitalized. GIMP v.2.8 (GNU Image Manipulation Program, published under the GNU-general public license) was used for further image processing. The images were then transformed to grayscale images. The size of the brood nest was read out by a custom script, written in Matlab v.R2012a (The MathWorks, Inc., USA) and based on the build in help function for the chapter “basic image enhancements and analysis techniques”. The script can be seen in figure 4.1.

```
% creation of a empty matrix to keep the data
gesamtbrut=[]
% loop to process all images intended for thje current group
for k=1:17
    xbild= input('welches Bild        ' ,'s')
I = imread(xbild);
% Images have to be greyscaled, occupied cells have to be white
% visual check the images before processing!
level = graythresh(I); % convert grayscale to B/W
bw = im2bw(I,level);
bw = bwareaopen(bw, 50); % count area in pixel, set threshold to
% reduce noise Count of areas, if the brood nest is fragmented, set
% threshold for individual areas if they appear connected
cc = bwconncomp(bw, 18)
cc.NumObjects
brooddata = regionprops(cc, 'basic')
brood_areas = [brooddata.Area];
[area, idx] = max(brood_areas)
brood = false(size(bw));
brood(cc.PixelIdxList{idx}) = true;
imshow(brood);
% total area count is shown here, together with the number of areas
% use number of areas as correction, must fit to the image!
Brutsize=sum (brood_areas)
gesamtbrut=[gesamtbrut;Brutsize]
% variable gesamtbrut contains area and area count for all images
% used in the loop
end
```

Figure 4.1. This script was used to determine the size of the brood nest. Black/ white images were used as input, with white being the brood cells. The script counts the pixels of coherent areas, adds those and displays the number of areas counted for each image. All data from one read out cycle are then combined in one matrix, containing the size of the brood nest and the number of individual areas per image.

The amount of foraged food was weighted. Due to the low amount of comparable replications only the two thiacloprid concentrations and the control were analysed with a rank-sum test for non-normal distributed data by comparison of the amount of foraged food after 53 days.

The size of the brood nest and the amount of capped cells were taken. Differentiation between capped brood cells and capped honey cells was possible due to the fact that bees tend to arrange their brood nests in a way that the brood cells are kept close to each other, while food is stored in the outer rim of the comb. This mechanism also helps to keep the temperature controlled and warm.

The number of brood cells was taken by measuring the brood nest size and dividing it by the size of a single cell. Capped brood cells were counted individually.

The total numbers of observed cells were compared, as well as the number of capped brood cells. These data could be compared between the treatment groups. Also a Pearson-correlation could be drawn between the total size of the brood nest and the capped brood cells. The graphs were produced using custom scripts in Matlab v.R2012a (The MathWorks, Inc., USA). The statistical analysis was done with the same program.

4.3. Results

4.3.1. Food uptake

The first observed parameter was the amount of collected sugar syrup in each hive. The results are shown in grams. Data from the first 53 days were compared for all groups, as this was the common observation period in all years. In the second year a substantially longer timeframe was also tested, with revitalization after 60 days in which 500 g bees were added to the hives. The observation then continued for additional 72 days to a total of 132 days. These results are shown separately, but the first 53 days of data from these hives are included in the graphs for all groups.

Effects of different neonicotinoids mixed in the food on the amount of foraged food solution are shown in the following figures 4.2. – 4.5. The figures are separated into treatment groups. All figures show in the upper portion a graph with a curve for the accumulated amount of food that was collected over 53 days for each used concentration. The days at which the half maximal food amount was collected are shown by a vertical line. The lower portion depicts a graph with the individual measurements at each time point. From these one can infer the progression of the slope of the foraging.

Table 4.2. Overview over the maximal amount of foraged food. Data are shown for each hive over the course of 53 days in gram. Displayed is first the maximal amount of food, the cumulated value over the duration of the experiment at the final day. The half maximal value corresponds to the closest value of the means to the mathematical half maximal value. The last column shows the day when this value was reached. (* = Hives without a queen at the first inspection)

Treatment group, hive	Maximal foraged food (g)	Half maximal value (g)	Day max_{half} was foraged (from 53 days)
Control, 1	**2702**	**1470**	**29**
2	**1580**	**888**	**29**
3 *	**2792**	**1427**	**18**
4	**2621**	**1435**	**17**
5	**1610**	**914**	**16**
6	**1815**	**1033**	**13**
clothianidin 1.87 µg/kg,1	**1986**	**1114**	**31**
2	**1716**	**948**	**28**
clothianidin 3.74 µg/kg,1	**1839**	**994**	**16**
2	**1685**	**949**	**13**
imidacloprid 3.74 µg/kg, 1	**2302**	**1355**	**17**
2 *	**1290**	**756**	**17**
thiacloprid 2 mg/kg, 1	**1710**	**952**	**13**
2	**1686**	**963**	**17**
3	**858**	**463**	**25**
4	**1627**	**931**	**13**
thiacloprid 8.876 mg/kg, 1	**1310**	**668**	**13**
2	**526**	**276**	**28**
3	**1584**	**802**	**17**
4	**978**	**504**	**13**
5	**1180**	**644**	**13**
6	**496**	**306**	**11**

Each dataset was analyzed for the maximal amount of foraged food in gram, the half maximal value and the day when this was reached. The half maximal value corresponded to the next real value to the mathematical max_{half}. This allowed assigning a discrete day when this value was achieved. The results can be seen in table 4.2. The control group showed on average the highest amount of collected food solution with 2.1 kg collected syrup. All other treatment groups had lower average maximal values. The largest difference could be found in the 8.876 mg/kg group. Here we could determine a significant difference to the control group ($p = 0.0043$; rank sum-test). Such a significant analysis could not be conducted for the other groups, as not enough replications were available for a reliable analysis. Two hives, one in the control group

and one in the imidacloprid group had no queen present at the first brood inspection (marked with a * in table 4.2.).

The day when half of the final food amount was collected serves as a relative assessment tool to compare the slope for the collected food between the hives from the different groups. This day was reached earlier when compared to the control group for all treatment groups, except the hive which foraged from the food solution containing the high clothianidin concentration.

Figure 4.2. illustrates the course of missing sugar solution in the feeder of each corresponding hive over 53 days. These data were interpreted as foraged food. Shown in the upper half is the mean of the data from 6 hives from the control group with the standard deviation. The hives had collected on average over 2.1 kg feeding solution over the course of the 53 days. The lower half shows an interpolation of the foraging progress for each hive at each measurement day. Only a very small amount of solution was foraged in the first 3 days. After this period in which the bees familiarized themselves with the feeding apparatus they started to forage. This can be seen by a sharp incline which is followed by a steady foraging activity up until days 20 – 30. After 19 days half of the maximal foraged amount was collected by the bees. This value almost coincides with the onset of a decline in foraging activity.

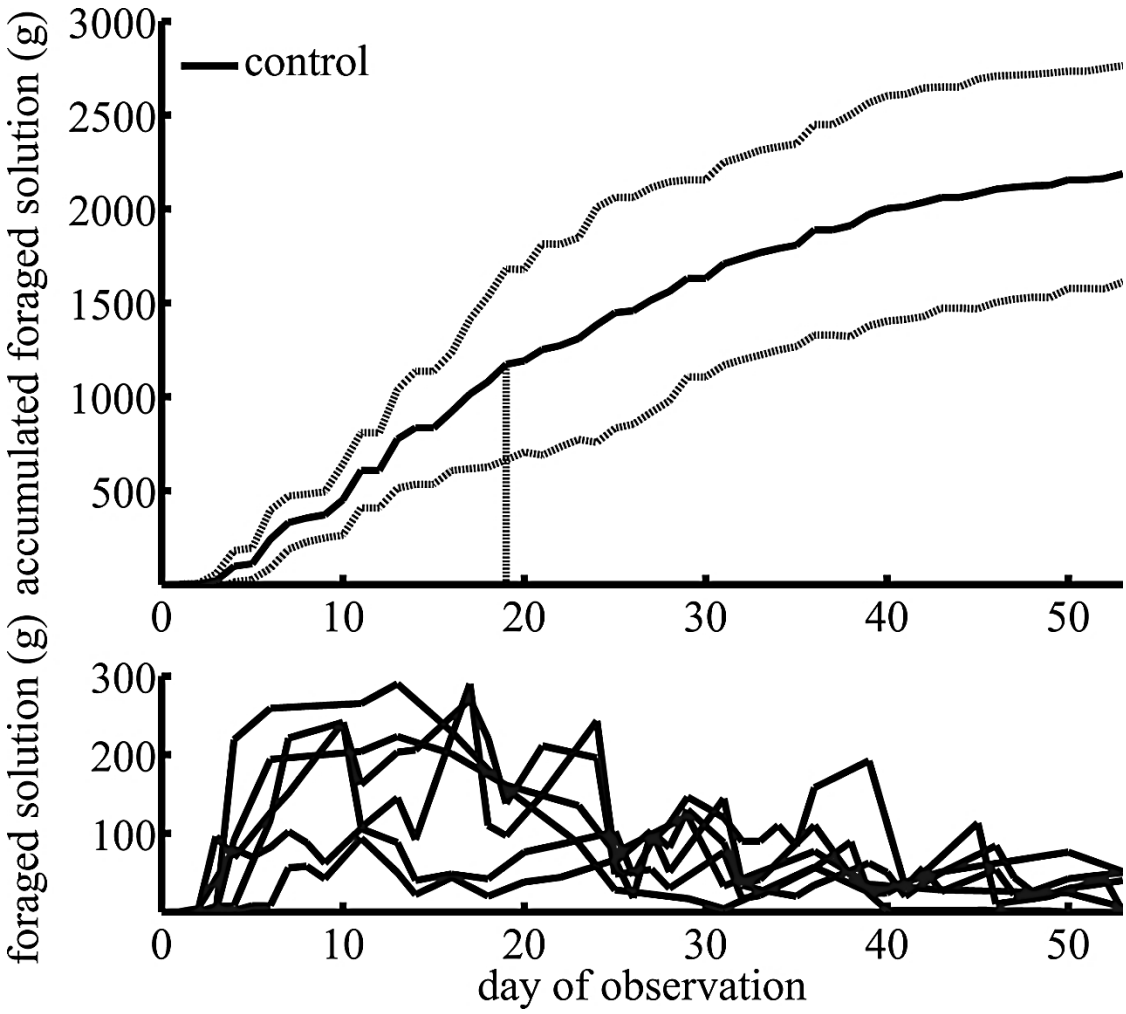

Figure 4.2. Food solution foraged by bees from the control group. The upper graph shows the amount of food accumulated over 53 days. The data for each day are displayed in grams by the mean of all groups as black line, with the standard deviation as dotted lines. The data from the control group were obtained from six hives over the course of three years. The dashed vertical bar indicates the day 19, at which the half maximal amount was foraged. The lower graph shows the uptake at each measurement as a continuous line for each group.

Figure 4.3. shows the data for the bees which had to collect a sugar solution containing either 1.87 µg/kg or 3.74 µg/kg clothianidin, divided in an upper and a lower graph like figure 3.2. The curves for all hives are shown, but the courses of the data for hives from the same treatment were quite similar. The hives which foraged from the lower concentration collected 1986 g and 1716 g syrup in total. The initial slope was also more flat with an increase after ~25 days. Half of the total collected food was foraged after 28 days from one hive and after 31 days from the other hive. No other treatment group required so long to collect the half maximal amount. The hives which foraged

syrup containing 3.74 µg/kg clothianidin had collected a comparable total amount of food after 53 days, with 1839 g and 1685 g. But the initial slope was much steeper. This was reflected by the fact that the half maximal amount of foraged solution was already achieved after 13 and respective 16 days. After 25 days the hives that collected the 3.74 µg/kg clothianidin solution showed a decline in the foraging activity, this is especially true for one hive, as can be seen by the dark blue line in the lower graph.

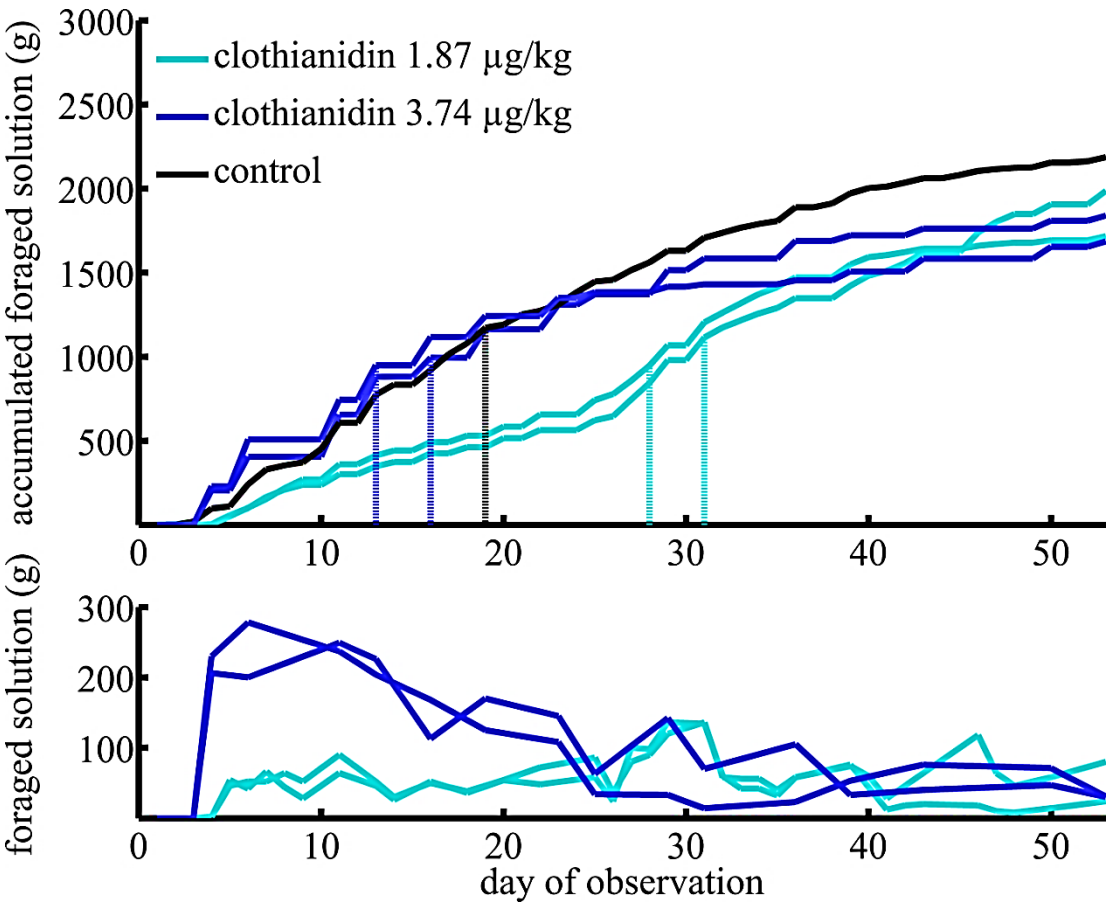

Figure 4.3. Food solution foraged by bees from both clothianidin groups. The upper graph shows the amount of food accumulated over 53 days. The course of the light blue lines represents the accumulated foraged amount in gram of food from syrup containing 1.87 µg/kg clothianidin. The course for the foraging data for bees collecting from syrup with 3.74 µg/kg clothianidin group is shown in grams by dark blue lines. Each line represents the data form one hive. In black the mean values of the control group are shown. The data from both clothianidin groups were obtained from two hives each in one year. Dashed vertical bars indicate the day at which the half maximal amount was foraged, days 28 and 31 for the lower concentration and days 13 and 16 for the higher concentration. The grey vertical bar displays the value for the control group. The lower graph shows the uptake at each measurement as a continuous line for each hive in the same colors.

The hives which collected sugar solution with 3.74 µg/kg imidacloprid had initially a progress of foraged food similar to the control group as seen in figure 4.4. After 17 days half the maximal amount of collected syrup was reached in both hives. But the final amount of collected syrup differed greatly between the groups. One hive collected 2302 g, whereas the other hive collected only 1290 g. Shortly after the half maximal

value was reached the curve flattened and separated from the control values. The lower graph of the figure 4.4. shows the strong decline in foraging activity for both hives.

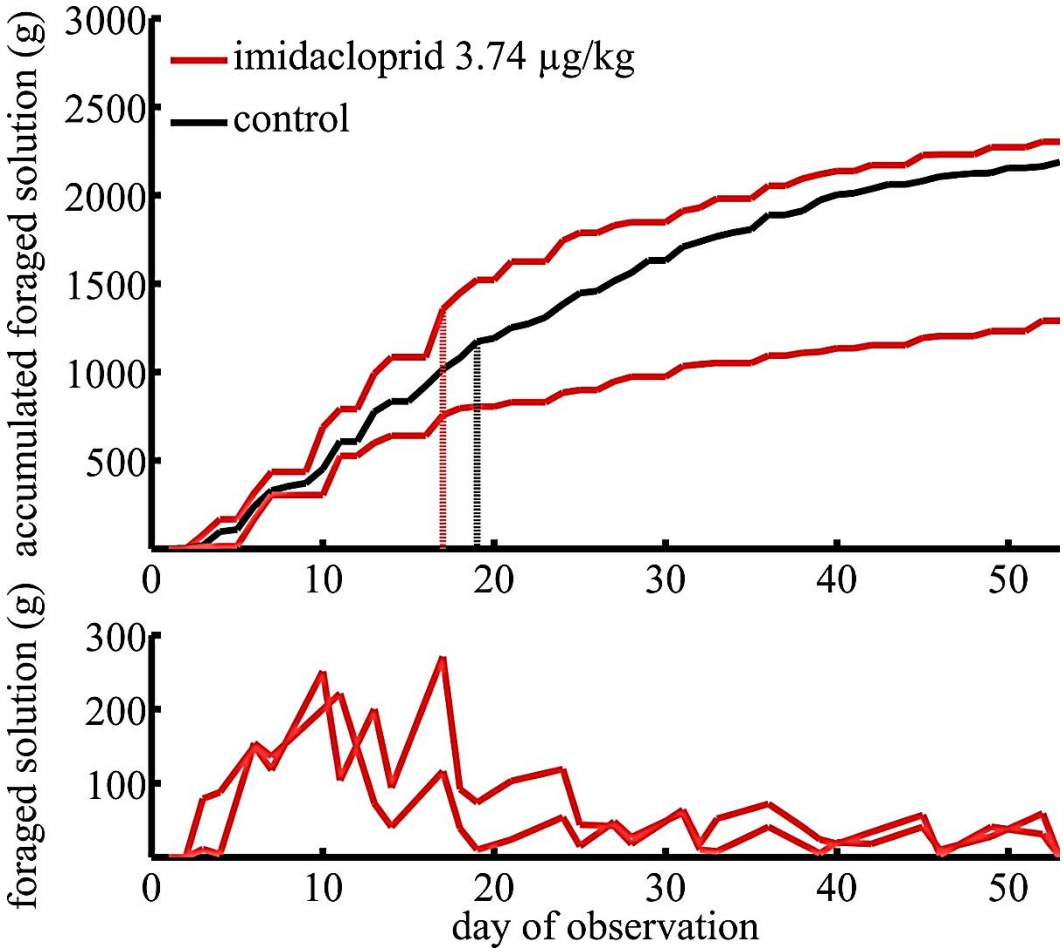

Figure 4.4. Food solution foraged by bees from the imidacloprid group. The upper graph shows the amount of food accumulated over 53 days. The values for both hives which collected from syrup with 3.74 µg/kg imidacloprid are shown in grams by a red line. In black the mean values of the control group are shown. The data from the imidacloprid group were obtained from two hives in one year. The dashed vertical bar indicates the day 17, at which the half maximal amount was foraged in both hives. The grey vertical bar displays the value for the control group. The lower graph shows the uptake at each measurement as a continuous line for each group in the same colors.

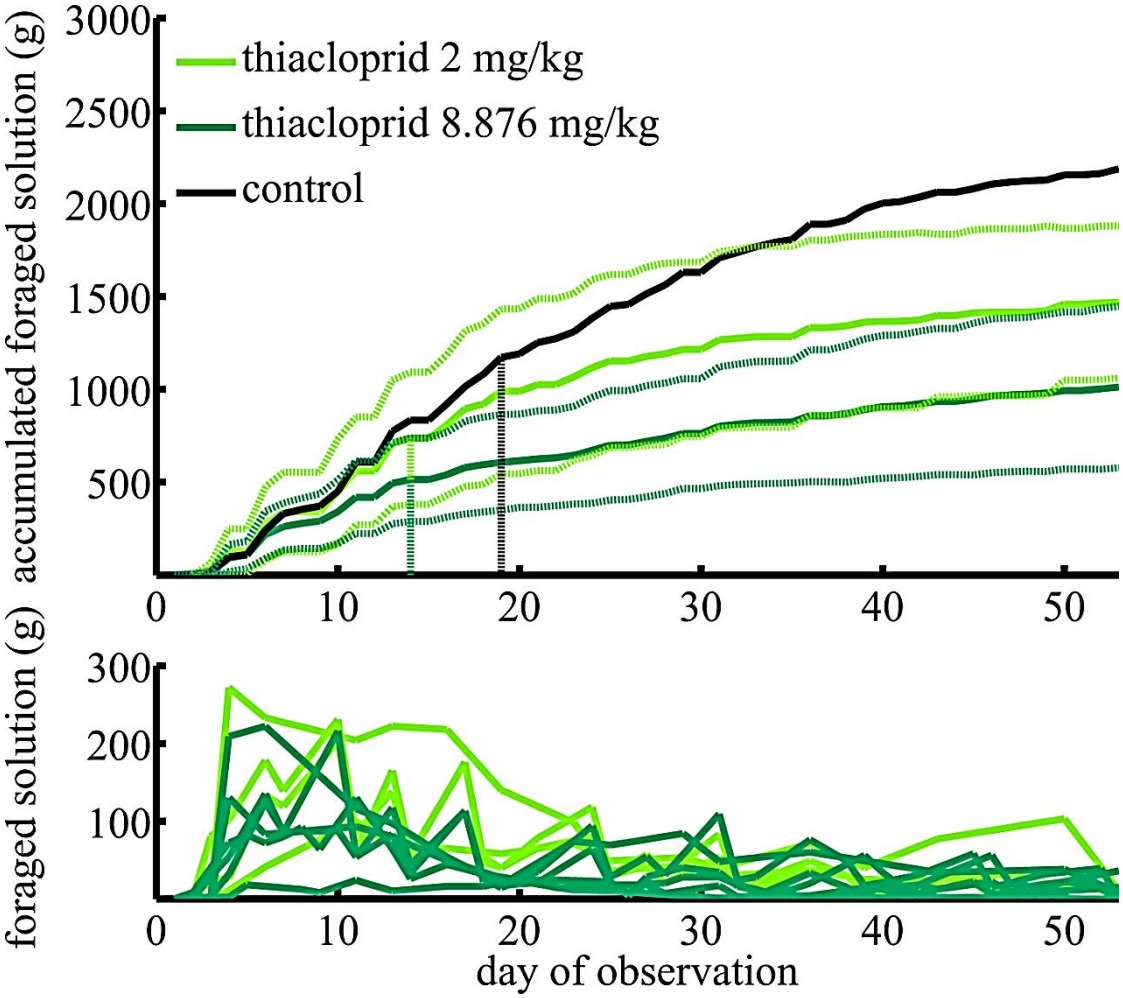

Figure 4.5. Food solution foraged by bees from both thiacloprid groups. The upper graph shows the amount of food accumulated over 53 days. The mean of the 2 mg/kg thiacloprid group is shown in grams by a light green line with the dotted light green lines as standard deviation. The mean of the 8.876 mg/kg thiacloprid group is shown in grams by a dark green line with the dotted dark green lines as standard deviation. The data from the thiacloprid 2 mg/kg group were obtained from four hives over two years. The data from the thiacloprid 8.876 mg/kg group were obtained from six hives over the course of three years. Dashed vertical bars indicate the day at which the half maximal amount was foraged. This was reached at 14 days by both groups. The grey vertical bar displays the value for the control group. The lower graph shows the uptake at each measurement as a continuous line for each group in the same colors.

Figure 4.5. shows the results for both thiacloprid groups. Both concentrations showed the strongest difference to the control groups, compared to the other neonicotinoids. Hives that foraged from food sources with 2 mg/kg thiacloprid collected on average 1.47 kg food solution. The hives that foraged the food solution with 8.876 mg/kg thiacloprid collected on average only 1.012 kg. But in hives from both groups the half

maximal value was collected after 14 days. The foraging activity was very low, when compared to the other groups.

In the second year a revitalization of the hives was tested. 500 g bees were added to each hive to compensate for the loss of bees during the time. This loss occurred in all groups and can therefore not be explained only by the effects of neonicotinoids. Figure 4.6. shows the amount of foraged food for this year with all groups in one graph. After an initial increase in foraging activity the steepness of the slopes for all groups declined. The collection of sugar solution came to an almost complete stop after the mentioned two months. At this point, 500 g bees were added to each hive.

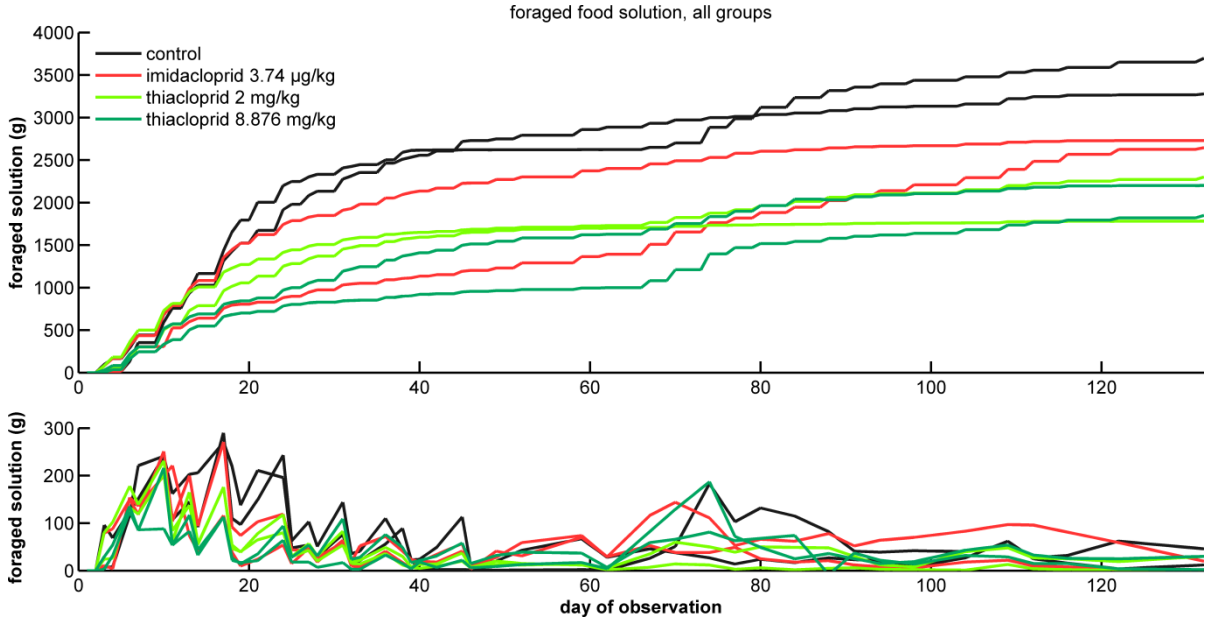

Figure 4.6. Amount of foraged food for all groups in the second year over 132 days. Each line presents the data from a single hive. Tested groups in this year were two control hives, two hives fed with 3.74 µg/kg imidacloprid, two hives fed with 2 mg/kg thiacloprid and two hives fed with 8.876 mg/kg thiacloprid. The upper graph shows the accumulated food amount over 132 days. The lower graph shows the uptake at each measurement as a continuous line for each group in the same colors. After 60 days 500 g bees were added to each hive. Hives in all groups increased their foraging activity after this event. The final accumulated values of collected food were 3697 g and 3278 g for the control group, 2730 g and 2645 g for the imidacloprid group, 2300 g and 1782 g for the lower and 2201 g and 1849 g for the higher thiacloprid concentration.

After the revitalization an increase in foraging activity could be observed. This increase in foraging activity was very notable in the thiacloprid groups and one hive in the control group and on in the imidacloprid group.

4.3.2. Brood development

In addition to the amount of foraged food, the number of brood cells was measured. The cells in the brood nest were divided in two groups, all cells and capped brood cells. While the open cells contain the eggs and early larval stages, the capped cells contain the late larval stages and then the pupal stages of development.

Figure 4.7. shows an overview for the data from all hives at all time points. The hives treated with thiacloprid show overall a lower number of brood cells, and capped cells. There is also a large amount of brood nests without capped cells present in half of the data points for the high thiacloprid concentration group.

Regardless of treatment, an increase of the cell count could be observed initially, with a decrease over the course of the experiment could be observed. This overall trend is to be expected, as the colonies started without brood, which naturally leads to an increasing number of cells when the queen starts to lay eggs. The decline at the end was a sign that the conditions in the flight room and the treatments led to not sustainable brood conditions.

When observing the brood development from hives whose workers foraged from 8.876 mg/kg thiacloprid spiked food it became clear that only very few eggs developed further to larvae and pupae. It has to be noted that the observed eggs might stay longer in the cells than the anticipated 3 days. Normally the worker bees would eat or remove an egg with an impaired development. The observed brood nest contained eggs which seemed dried out and should have been removed.

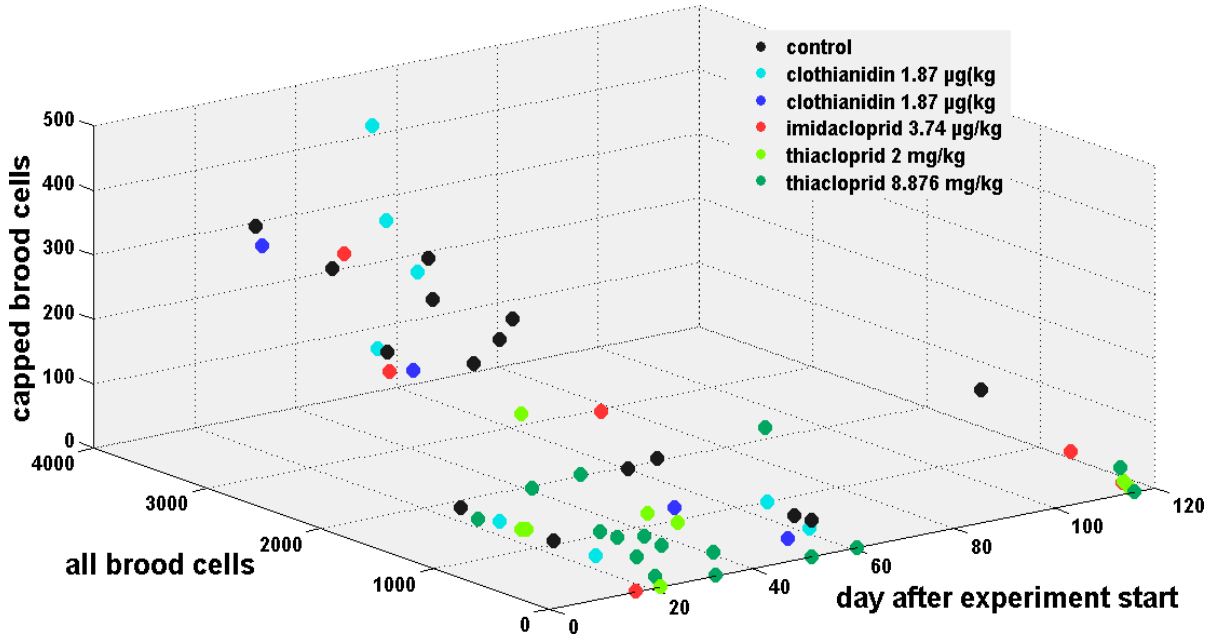

Figure 4.7. Comparison between the number of brood cells plotted against the number of capped cells for all time points. Each point represents a hive with the values for the total number of brood cells in the x-position and the corresponding number of capped brood cells on the y-position. On the z- axis the day of counting is plotted. The groups are plotted in the colors used in the previous figures.

A more detailed view on the data can be seen in figure 4.8. Here a direct comparison is drawn exemplarily between the two hives from the control group in the first year. Shown are the course of the brood nest size, measured in the number of cells and the numbers of the capped brood cells. The left y-axis shows the values for the entire brood nest, whereas the right y-axis shows the values for the capped brood cells. At the first meassument day (25 days after the beginn of the experiment) one hive had 1889 cells, the other one 1070 cells. But none of those cells contained brood developed enough to be capped. Nine days later those capped cells could be observed with 269 capped cells from a brood nest with 2529 cells in one hive and 162 capped cells from a total of 2931 cells. Then, while uncapped cells could be found, there were also always capped cells. At the last observation day, 61 days after the start of the experiments the brood nest was shrunken substantially and the number of capped cells was also reduced again.

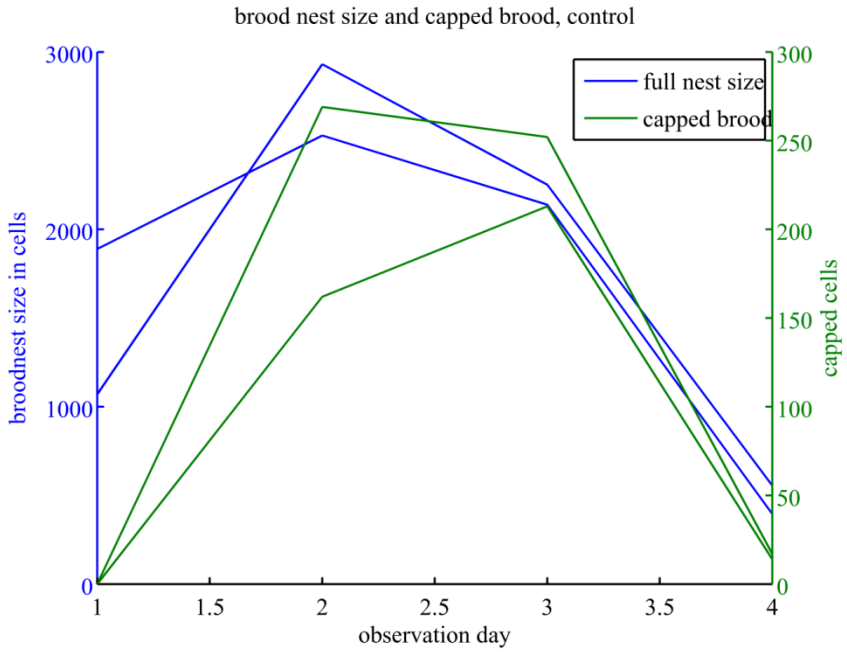

Figure 4.8. Brood nest size of two hives from the control group. Shown are the numbers of brood cells in blue over the course of four measurements on the left y-axis. The related numbers of capped cells are shown in green with the right y-axis as reference.

A correlation was done after approximately one month between the total cell count and the number of capped brood cells for all treatment groups. The results are shown in figure 4.9. The control group showed the expected positive correlation between the total amount of brood cells and the number of capped cells. This was to be expected, as healthy eggs continue to develop into larvae which sooner or later get capped. These observations were also true for both clothianidin groups and the imidacloprid group.

Both thiacloprid groups, however, differed from this trend. Here no correlation could be found between both parameters, meaning that either no capped cells were present, or that there was no clear trend and occasionally occurring capped cells could not be linked to a higher total cell count.

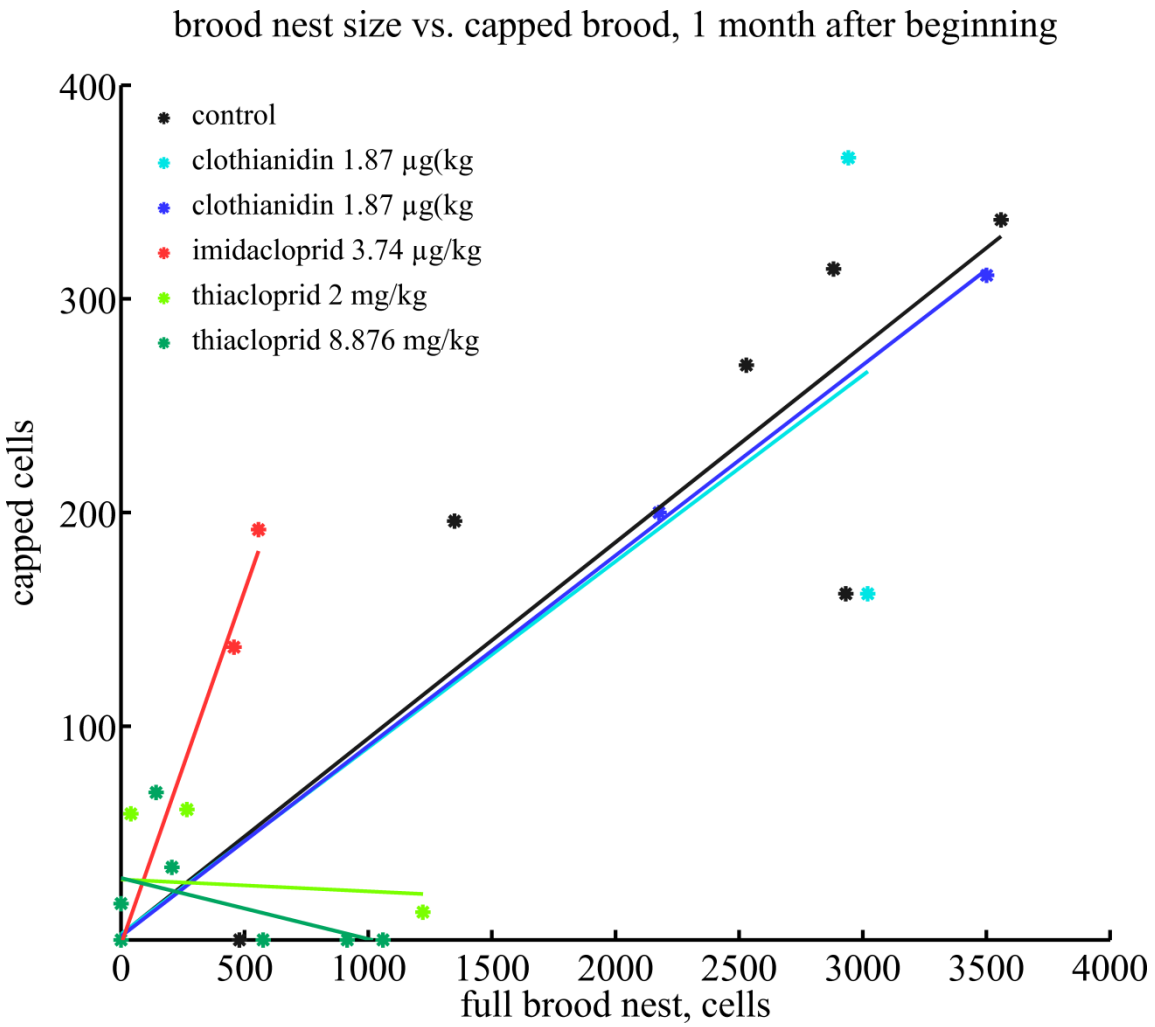

Figure 4.9. Relation between all brood cells and the capped cells after one month. Data are taken from hives from all observation periods, but approximately one month after the experiment started. This is the period in which the hives show a good health. Each dot represents the data from one hive. Different colors show hives from the different treatment groups according to the legend. Plotted are the data for the number of brood cells against the number of capped brood cells. For each treatment a linear fit was calculated, with 0/0 as fixed point. The control group, both clothianidin and the imidacloprid group showed a positive trend between the total cell count and the capped brood cells. No positive trend could be found for both thiacloprid treatment groups.

The most staggering observation in this experiment was the observable lack of developed brood in hives treated with thiacloprid, while still finding a considerable amount of eggs in the cells. The larval development of a bee starts with the queen laying an egg in a cell. This egg then has three days to develop, until it reaches the larval stage, at which point it requires food for its further development. The larva then starts to grow and undergoes 4 larval molts until the cell gets capped by worker bees

with a thin wax sheet. While it can be difficult to distinguish the developmental stages of the larva, a capped brood cell is easily spotted. Therefore, the data were divided in brood cells (containing eggs and larvae) and capped brood cells.

To investigate the observed effect further, a correlation was done by plotting the total amount of brood cells to the amount of capped brood cells. Due to the different time points at which the numbers were taken each year the best correlation could be drawn approximately one month after begin of the experiment. Additionally, this was a time point at which a full larval developmental cycle could take place, providing all larval developmental stages for the observation. One control hive had to be excluded from this analysis. Whereas the bees showed a food gathering behavior that was comparable to the other hive, the queen was not accepted by the worker bees and no eggs were laid. A second change of the queen also did not result in egg laying. The data are shown in figure 4.6. Control hives had a positive correlation between both parameters, as had both clothianidin treatments. With the imidacloprid treatment, a slightly higher percentage of capped brood cells could be observed. The thiacloprid treatments had the most striking results. While there were some hives with a comparably normal brood cell numbers, the majority of hives had a reduced amount of total brood cells. On top of this a massive reduction in capped brood cells could be observed. This area also encountered change during the experiment, showing that these cells were not filled with old eggs which were not excluded from the comb, but that the queen was still laying new eggs.

Figure 4.10. Bees accumulating in the feeder. The bees start to cluster in the feeder itself and around it. The bees in the sugar solution are not dead. They stay in the feeding solution and show only slow if not none movement.

A last important discovery was the observation that bees fed with both thiacloprid solutions tend to stay in the feeder area. Figure 4.10. shows an example of the observed scene. The sugar solution was sticky enough to immobilize bees, presumably after the consumed the solution. Bees that went into the feeder and managed to leave it tend to stay in the surrounding area. This led to an accumulation of foragers in the feeding area. The bees in the sugar solution were still alive and slowly moving. While the sugar solution itself was enough to keep the bees stuck to the feeder by immobilizing them, the same effect could not be observed for bees in the other treatment groups. This led to the conclusion that the thiacloprid itself impairs the motor activity of the bees up to a level where they couldn't free themselves from the sticky sugar solution. I could observe bees which tried to free other bees by licking the sugar solution from their bodies. No successful attempt could be observed for this behavior and it can be assumed that the helping bees became affected by the thiacloprid.

Bees that collected from either clothianidin solution seemed contrary to this more aroused and more active, even when compared to the control group.

4.4. Conclusion

These experiments showed that bees can be kept in the described flight room conditions under a steady diet with neonicotinoids for a longer period (>two months). The bees accepted the food containing neonicotinoids and started to forage it. Pollen was also foraged by bees from all treatment groups. This was confirmed, as pollen could be found in cells from all hives.

Chronic feeding of the colonies (flight room) with sub lethal doses of thiacloprid (8.8 ppm or 2 ppm) resulted in a decreased amount of foraged contaminated sugar water as compared to control colonies (sugar syrup only). Additionally, we observed a descent amount of brood cells occupied with eggs, while the other development stages were largely absent. While this can happen occasionally, due to defect larvae and or other difficulties in the complex brood care environment in the hive, a loss of brood on this scale is a clear indication of a disturbance in the brood development. Chronic colony feeding with sugar syrup containing 3.74 µg/kg clothianidin did not impair the foraging activity or the brood development. Although it has to be noted that the bees in the feeder compartment appeared anxious in comparison to bees from other hives. The feeding with 1.87 µg/kg clothianidin however resulted in a much lower foraging activity in the first 20 days when compared to the higher clothianidin treatment group or the control group. But the hives still produced developing brood. It seemed that the bees were not collecting a surplus of syrup to store it for later use. The foraged amount in the first days was similar to the amount collected by the bees foraging from a food source containing 8.876 mg/kg thiacloprid. But whereas the bees from the thiacloprid group showed a visible impairment, reflected in slow movement, this could not be noted for the bees from the clothianidin group. This leads to the assumption that the lower quantity of foraged syrup might be a consequence of a change in behavior regarding foraging.

The feeding of imidacloprid (3.74 ppb) did not result in a change of foraging activity or brood development. One important observation could be made. The queen in one hive started later to lay eggs. This coincided with a lower foraging activity. A control hive that was also lacking brood however was not showing this reduced foraging activity.

The experiments showed in hives from all groups a decline in hive health after approximately two months. This was even true for the control hives. This overlapped with a decrease in the foraging activity. As the combs in all hives had still enough empty space to store the solution, one can assume that this maximum might be a result of the declining hive health and a reduced number of bees capable to forage food.

An influence from the neonicotinoids could be observed on the amount of foraged food. Hives which had to forage a food solution containing 8.876 mg/kg thiacloprid had collected a significant lower amount of food. The 2 mg/kg thiacloprid group also showed a reduced amount of collected food, although not as significant. During the experiment the bees from these hives could be observed to be very lethargic and only very few returned from the feeding apparatus to the hive to deliver the food solution. In a similar way, stored sugar solution could be found in the cells in the hive, but only a small amount. Thiacloprid in the tested concentrations seems to affect the bees in a way that they are not able to fulfill their task as foragers.

The bees that collected solution containing either clothianidin concentration showed an opposite behavior. They were more aroused. This led however not to more foraged food solution than in the control hives. The higher activity might have been of more chaotic nature, so that it could not be translated in a better foraging strategy by the bees. It can be excluded however, that the increased activity used the collected food up, as there were plenty of cells filled with sugar solution in the hive.

In the second year we conducted an experiment in which we added additional bees to the hives to attempt a rescue of these hives. A rescue was necessary, because the health of the hives declined after two months. This decline can be explained by the not optimal conditions in the flight room, which might have led to a loss of worker bees and an accumulation of sickness, mainly the gut parasite *Nosema spec*. This disease is the

most probable culprit, as I could observe lots of bee feces-drops on the ground of the flight room, which can be a sign of a *Nosema* infection. In the flight room the bees have limited space to move, which might improve the transmission rate of parasites.

The attempt to rescue the hive consisted of adding 500 g bees to each hive. These bees were supposed to replace the missing bees and add assumedly healthy bees to the population.

The acquired data showed that such a rescue is possible, which allows prolonging the timeframe of such experiments. In this experiment an increase in foraging activity could be observed for the 8.876 mg/kg thiacloprid collecting bees. This is probably explainable by the fact that when these bees start to forage they first have to consume a critical amount of food to accumulate a concentration high enough to lead to the observed sluggishness.

During this “rescue” phase an additional effect could be monitored. One control hive had no queen over a longer period in the first 60 days. Nevertheless, the bees in those hives collected a quantity of food comparable to the other control hive. After the bees were added, the brood-less hive had a sharp spike in the amount of collected food. While this could be attributed to the new bees, this spike could not be observed in the hive containing brood. Both hives had already collected enough sugar solution to store it in capped food cells and the stored solution might have been sufficient for the new bees to be fed. A possible explanation for this would be that the absence of brood in one hive motivated the new bees to collect food, as there was not as much work to be done inside the hive. At the same time, the bees in the hive with brood had enough food present and could be tasked with brood-care.

The hives which had to collect sugar syrup with 3.74 µg/kg imidacloprid showed another interesting phenomenon. Here the queen in one hive started to lay eggs earlier than the queen in the other hive. This resulted directly in a drastic reduction of foraged food. After the egg-laying had begun, the bees started to forage more sugar syrup, but they never collected the same amount as the other hive from the same treatment group. The addition of new bees was then visible as a peak in foraging activity. The assumption

here is, that the bees from the imidacloprid treatment were not inclined to collect a surplus of syrup, in the same way that the brood-less control hive did. The addition of new bees, then improved the foraging rate, leading finally to a cumulated foraged food amount, comparable to the other imidacloprid hive which had brood for the entire period. It might be possible that the communication between the bees in this group was disturbed, which led to a lower foraging activity. This cannot be attributed to the absence of brood, as the bees from the control hive collected syrup without any brood.

My experiments provide data for a chronic feeding essay which can be used for further experiments to determine if the observed effect is a result of damaged brood or an impaired brood - nurse bee interaction or a combination of these.

One proposed test for this would be a transfer of brood from a colony treated with 8.876 ppm thiacloprid to a control colony and vice versa. If the eggs are already damaged, one should not see a development of these eggs in the control colony.

The same would be true for larvae. Here one should be able to see a difference between newly emerged larvae and old larvae which already received food in the thiacloprid colony (although the data in this experiment showed that a larva with food is only rarely found in a colony treated with 8.876 ppm thiacloprid). Here are also two kinds of damage conceivable. The different development stages could be impaired in their biological development or the interaction between egg and nurse bees could be disturbed.

An experiment conducted by the PhD-student Hedwig-Annabell Gärtner from the Institut für Bienenkunde was dedicated to this question and will be discussed in her doctoral thesis. Artificial rearing showed a normal development of bees, even when fed with a solution containing the equivalent of 8.876 ppm thiacloprid (oral communication, Hedwig-Annabell Gärtner). This implicates the conclusion that the interaction between the egg and the worker is impaired.

A third explanation comes from an observation acquired during the feeding. The bees from hives fed with thiacloprid in both concentrations seemed paralyzed by the food and

started to accumulate in the feeding area. Figure 4.10 gives an example of the early stage of this observation. Later in the experiments the number of bees in this area only increases, leading to a visible lack of worker bees in the hive. This lack of foragers could encourage the remaining bees in the hive to abandon the brood and start to forage themselves, which eventually traps them also in the feeder. This assumption is based on the observation that bees can change their dedicated task in the colony under stress (Rösch 1930).

5. Neonicotinoids affect navigation of honey bees

Results from this chapter were already published for the most part (Fischer et al. 2014, PlosOne: “Neonicotinoids Interfere with Specific Components of Navigation in Honey bees”). The experiment was performed in collaboration with Prof. Menzel from the Freie Universität Berlin. It was conducted in two consecutive years. I conducted the experiment personally in 2012; therefore, I will describe the procedure like it was done in this year. Some changes were made between the years. Thiacloprid was introduced as a new test substance and one test group from the first year was no longer used. There was also a slight change in the location of fix points in the field, due to the fact that the equipment had to be set up each year again. The data from 2011 were obtained under similar conditions, what allowed the pooling of the data from the same treatment groups. Although parts of the data were already analyzed in the bachelor thesis of Teresa Müller (2011), all data were reevaluated and treated as not-analyzed for the publication and for this thesis. I was involved in planning the experiment (in both years), in the conduction of the experiment, the data evaluation and processing and in the writing of the paper.

Individual exemplary flight paths of the tested bees can be found as animations on the attached CD.

The original paper can be found as an open access paper under the URL:

http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0091364

Or under the DOI:

10.1371/journal.pone.0091364

5.1. Introduction

One of the great cognitive traits of honey bees is their ability to fly and successfully navigate in their foraging territory. They are known to fly distances of up to 5 km when gathering food. This equates to an area of up to 78 km² of foraging ground for the hive. Bees can't rely on their hive as the sole navigational fix point when flying, as they often leave the near area and travel distances long enough that the hive is no longer visible to them.

In addition, they are able to relay information about the location of food sources to other bees using the waggle dance, for long distances or the round dance, for short distances (von Frisch 1967, Seeley 2011). Acquiring this information and communicating it to other individuals requires multiple cognitive processes. Bees are able to use input from several sensory organs to assess a food source, navigate to its location and back to the hive and pass this information on to other individuals. Bees are able to use the position of the sun as a factor for navigation, by relating the angle of the flight vector to the sun. They can even correct this relative navigational cue over the course of the day, when the sun changes its relative position in the sky. This vector can be communicated to other bees via the waggle dance (Grüter and Farina 2009, Menzel et al. 2006, Menzel et al. 2012, Galizia et al. 2012). For this, the bee waggles her abdomen on the cells in the hive. The frequency of the waggling is related to the distance of the food source. The direction is given by the angle between the direction in which the bee dances and the "down" direction in the hive, given by the gravitational pull. Bees can continue this dance over long periods and will correct the angle according to the positional changes of the sun during the day, as shown by experiments in which the bees were anaesthetized and thereby their internal clock manipulated (Cheeseman et al. 2012).

Another mechanism is the navigation using known landmarks. For this the bee is required to know prominent structures in its flight area, information that is learned during the orientation flight, when the bees leave their hive for the first time to explore the surrounding. Of course, during foraging flights they can also learn new landmarks. For this it has been concluded that bees use a frame of spatial reference.

Both mechanisms can also be combined when a bee follows the instructions from a waggle dance and then decides to visit another known location. In this case the new location has to be related to the known reference system to allow a successful navigation.

This experiment uses both mechanisms to investigate influences of non-lethal neonicotinoid doses on the navigation of honey bees.

We trained honey bees that were old enough to have already performed their orientation flights to an artificial feeding site and caught them there, to release them at a different location. In such a catch-and-release experiment the bees follow at first the vector that would have led them from the feeder back to the hive (Menzel et al. 2005). This component of the flight requires the bee to store the route between the two fix locations, the hive and the feeder, and remember the vector that can be computed from this. Therefore, this component was referred to as "vector flight".

When the bee arrives at the end of the vector flight and the hive is not at the expected location, the second flight phase begins. Here the bees had to perform search flights to recognize landmarks that were required on earlier flights and which were not related to the initial task, the simple return from the feeder. For this the bee needs to activate its memory from its former orientation flights and use the information to compute its current location in the field (Menzel et al. 2012). Then the bee has to relate this location to a new path back to the hive. This second phase was then referred to as "homing flight", as this was the phase were the bee could return home.

These two flight components refer thereby to different navigational memories. The goal of this experiment was to investigate how neonicotinoids interfere with the navigation of honey bees in both flight phases. It is known that insecticides interfere with neural functions in the brain of insects and can possibly compromise the acquiring of sensory information, the processing of those information and the correct output of this processing, by altering the motor functions (Bortolotti et al. 2003, Decourtye et al. 2011, Gill et al. 2012, Henry et al. 2012, Schneider et al. 2012, Medrzycki et al. 2013). It was already shown that neonicotinoids can affect the ability of bees to return successfully to

the hive (Schneider et al 2012). But this could not explain if the underlying reason is an inhibition of normal motor function or if the neural processing necessary for the flight is affected. Non-lethal doses were used as we only searched for effects on the neural processing and tried to avoid general poison effects.

5.2. Materials and methods

The experiments were carried out during August 2011 and 2012. At this time there was only a minimal amount of local flowers as alternative sugar sources for the bees. The surrounding area was used for agriculture while the experimental area itself was not used during August. The grass was cut in parts of the area at the beginning of the experiment and a second time halfway through the experiment. The area itself was located near Wittenberge in Brandenburg, Germany. The geo coordinates of the field are: N 52.97555, E 11.83677 (location of the radar station used for tracking the bees). The field was located in a remote area, without major streets or traffic. The landscape consists of no visible mountains and the only visible landmarks on the horizon were trees. The area is used as a mating area for bees. Because of this there were no other hives located near the field and we could assume that all bees involved in the experiment were part of the colony. The Hive was set up two weeks prior to the first experiment and consisted of a full colony. The bees were supplied by a local bee keeper.

5.2.1. Training procedure

Bees from a hive located 634 m away from the radar were trained to an artificial feeder, filled with 2 M sugar water. This training procedure was executed over a time span of two days. The feeder, a transparent plastic cylinder with a bottom part that allowed minimal flow of sugar water, was placed in front of the hive until 3-5 bees started to collect sugar water from it (see figure 5.1). After we could observe the bees returning to this feeder we increased the distance of the feeder by some centimeters and waited for

the bees to return. When the bees returned, the feeder was again removed to a further away location. The increase in distance was at first only approximately 5 cm. Those steps were increased to approximately 10-15 cm after a distance of 1 m to the hive was achieved. The steps where further increased up to 2 m at a distance of 20 m from the hive, while reassuring that the bees were still able to find the feeder and return. This allowed us to train the bees to the final feeder location, 250 m eastwards of the hive.

Figure 5.1. Bees at the feeder. The transparent feeder is filled with a sugar water solution. The bees were trained to fly to the feeder to forage the sugar water. After the initial training the bees continued to return to the area at which the feeder was placed for the whole duration of the experiment.

After the initial training the bees kept returning to the trained final feeder location and also started to recruit new foragers. The feeder was set up each morning at 9 am. Each day 20 - 30 bees were marked with a color pen. This color was changed each day with a reuse of the same color after five days. This allowed us to find bees that knew the feeder location. When bees marked with the color of an earlier day returned, they were captured using a holding device and a plastic number tag was glued on their back with

shellac (figure 5.2.). The number tags were numbered with two digits and had different colors. The used colors were white, yellow, pink, red, blue and green. This allowed a specific color and number combination for every bee. The marked bees were allowed to fly again after approximately five minutes, when the shellac was dried. We obtained trained and marked bees which had visited the feeder for at least two days.

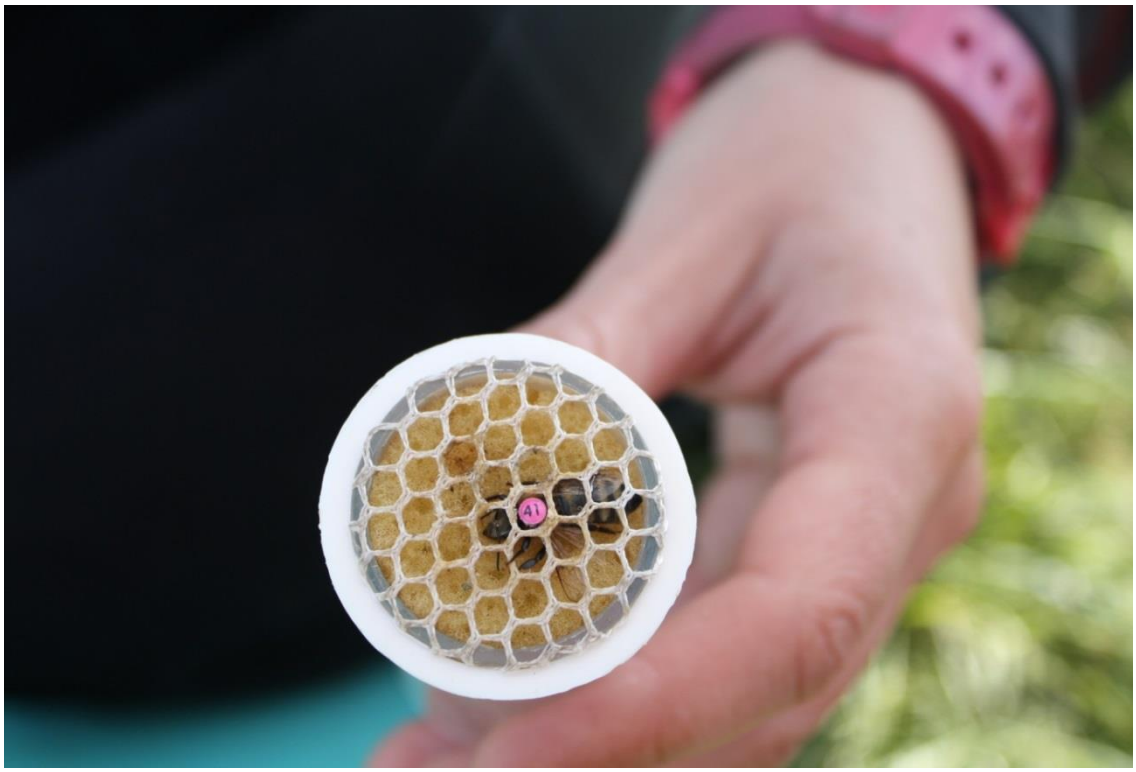

Figure 5.2. Bee with an individual number tag in a holding devise, held by a student. All tested bees received a tag with a unique combination of a two-digit number and a specific color.

The test bees where caught at the feeder before they were able to drink and quickly transferred individually in the holding device that was also used to mark the bees. They were equipped with a miniature feeder providing 49 µl of a sucrose solution plus 1 µl of the neonicotinoids clothianidin, imidacloprid or thiacloprid, leading to 50 µl of total consumed volume. The bees in the holding devices were kept in a dark Styrofoam box for 90 minutes (incubation) during which they imbibed all of the sucrose solution. Each day six bees were caught at 15-minute intervals in the morning. 90 min after the first bee was caught the Styrofoam box, containing all six bees in individual holding devices, equipped with the miniature feeder was moved to the release site, 450 m south of the

feeding site. The four different treatment groups were randomly assigned to the bees, while trying to balance the numbers of the groups.

90 min after the first bee was caught and fed with the respective substance, a radar transponder was attached to the plastic tag on the back of the thorax. The transponder was prepared earlier with a double sided sticky tape that allowed a quick attachment of the transponder to the bee. The bee was then released and the flight path recorded.

The bees were released every consecutive 15 minutes after each other to ensure an equal incubation time. It occurred occasionally that the bees did not start to fly immediately, but normally they started to fly during this 15-minute time window. If the time up to the next release was very short, 1-2 minutes, the release of the next bee was delayed for up to 2 minutes. This was done to ensure a clean tracking by the radar, as the signals from multiple bees could not be differentiated by each other and so a spatial distinction was necessary.

At least three people had to work together to acquire the displayed data. One person released the bee, one person ran the radar device and one person waited at the hive for the returning bee. The returning bee was captured before entering the hive, the transponder removed and the bee was then killed. In some rare cases the successfully returning bee could not be caught immediately. All of those bees could be found at the feeder the next day, either still carrying the transponder or they could be identified by their number tag. This ensured that each bee was only tested once in the experiment.

Data were collected during two experimental seasons (2011, 2012). Since we did not observe any differences in the flight behaviors between the years, we pooled the data. The location of the hive and the feeder were slightly different, but with the same positions relative to each other. The total number of bees tested was 98, in 2011 and 110 in 2012.

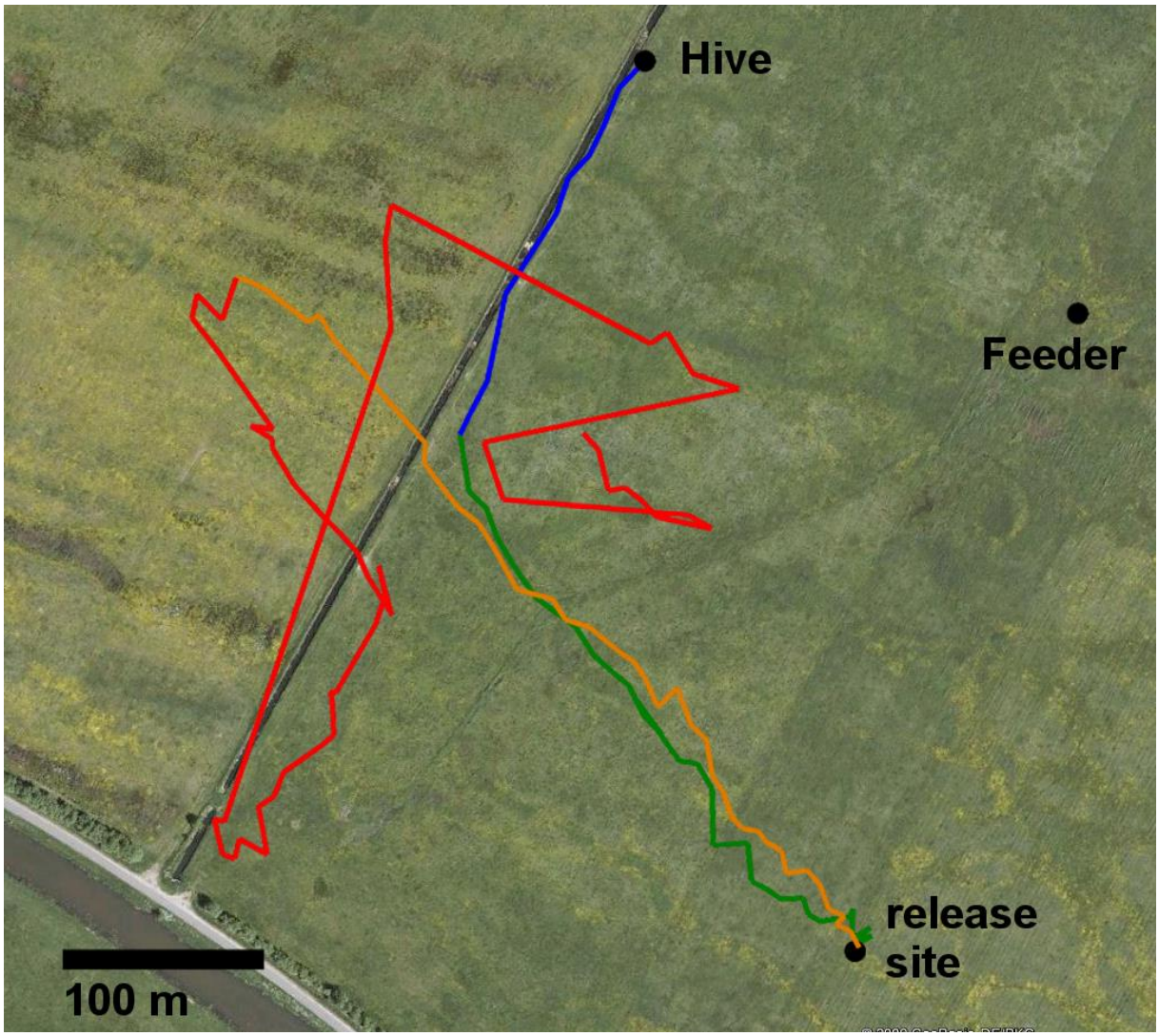

Figure 5.3. Examples of flight paths of two individual bees. Both flight paths start at the release site. A flight path from a control bee is shown in the first green, then blue line. The flight path which is first orange, then red shows the path of a bee treated with thiacloprid. The flight paths are separated in two components. The first component is a straighter path towards west-northwest, shown in green and orange. This is the vector flight component. It resembles the flight vector from the feeder back to the hive, the path that the bee initially assumes to be the correct path. The second component is shown in blue and red and is referred to as the homing flight component. In this component the bee has to find her way back to the hive with the use of navigational cues, other than the vector component. Both bees were released at the same release site and both bees showed a similar flight vector at first. The map was created using Google Earth (Google Inc. 2012). A scale bar is shown for 100 meter.

The following measures were taken:

- release time
- start time of flying
- arrival time at the hive
- flight trace recorded with the harmonic radar

From these measures the following parameters were derived for each bee: departing/not departing bee (if a bee did not depart, was observed sitting in the grass for longer than 30 minutes, or was never seen on the radar then it was classified as non-departing), immediate/delayed departure (if a bee delayed its departure by up to 15 minutes and was then seen on the radar then it was classified as a delayed departure), arriving/non-arriving bee (if a bee was observed by radar but disappeared from the radar and was not seen arriving on the same day then it was classified as non-arriving). In addition, the readings from the radar trace consisted in flight time, flight length, flight speed, directedness of the initial vector flight component and of the homing component. The transition from the vector flight to the homing flight was characterized by an angular turn > 60°, allowing to define the end of the vector flight and the beginning of the homing flight.

Substances:

Three neonicotinoids were tested during the experiment: clothianidin, imidacloprid and thiacloprid (thiacloprid: Bayer Crop Science Deutschland; Monheim, clothianidin and imidacloprid: Sigma Aldrich, Hamburg Germany). All three neonicotinoids were first dissolved in acetone and further diluted in water. This led to final concentrations of the original solvent, acetone, of 0.005% - 0.01%. Finally, all solutions were diluted 1 to 9 with 2 M sugar water. The final concentrations were: clothianidin (0.2µM), imidacloprid (0.6µM and 0.9µM), thiacloprid (0.1mM) leading to doses of 2.5 ng/bee of clothianidin, 7.5 ng/bee, and 11.25 ng/bee of imidacloprid and 1.25µg/bee of thiacloprid. The considerable higher thiacloprid dose was chosen due to the higher resistance of bees to this particular neonicotinoid, as reflected in the higher LD_{50}.Thiacloprid was not tested in 2011. Imidacloprid at 11.25 ng was only tested in 2011; the higher dose was omitted in

2012. This resulted in a lower number of tested individuals for the high imidacloprid dose and the thiacloprid dose. The sucrose solution given to the control bees contained 0.01% acetone, according to the highest concentration of acetone used in the neonicotinoid groups.

We assumed that this low concentration should not have an effect on the bees. To ensure this we compared the data from the first year of this experiment with a parallel experiment concerning the impact of the internal circadian clock of bees (Cheeseman et al. 2012). No difference was found in the number of successful returning bees when released from the same site and returning to the same hive between our bees with acetone in the sugar water (88%) and the bees from Cheeseman et al. (90%).

Harmonic radar tracking:

Bees were tracked with a radar system that was described in earlier publications (Riley et al. 1996, Riley et al. 2005, Menzel et al. 2011). We used a system with a sending unit consisting of 9.4 GHz radar transceiver (Raytheon Marine GmbH, Kiel, NSC 2525/7 XU) combined with a parabolic antenna providing approximately 44 dB. This signal was transmitted to the field while the antenna was rotating with 0.33 Hz. This provided a signal to the transponder on the bee every three seconds.

The transponder consisted of a silver wire with a loop dipole antenna with a Low Barrier Schottky Diode HSCH-5340 of centered inductivity. It was made of a silver wire with a diameter of .3 mm, a length of 11 mm, a weight of 10.5 mg and a loop inductance of 1.3 nH. For this, the silver wire was cut and a loop was created by dragging it around a small metal rod. Then the diode was soldered in a way that connected two sides of the loop. Thereby creating a feedback loop, electrically directed by the diode. When the radar signal was received by the transponder a resonating wave was emitted, containing a harmonic component of the inducted signal. The second harmonic component of the signal (18.8 GHz) was the target for the radar. The receiving unit consisted of an 18.8 GHz parabolic antenna, with a low-noise pre-amplifier directly coupled to a mixer (18.8 GHz oscillator), and a downstream amplifier with a 90 MHz ZF-Filter. A 60 MHz ZF-Signal was used for signal recognition.

This setup allowed to filter out reflected radar signals from the landscape and focused only on the harmonic signal component, produced by the transponder. Thereby we could track a bee that would otherwise be too small to be distinguished from the background via radar tracking.

The range of the harmonic radar was 1 km radius and no bee left the detection range during the experiment. As the transponder has its highest efficiency when the radar waves were perpendicular to the transponder it could happen occasionally that no signal could be received. This can be explained by string winds that shook the radar or when the bee made a turn and the transponder on her back was tilted parallel to the ground. In such a case a surrogate signal was calculated by assuming a straight line between the last and the next radar sweep.

Due to some irregularities in the landscape, the ground was not a perfect plane, it was sometimes difficult to track the last meters around the hive. In this case we assumed the last measurement near the hive as a successful return. If the bee decided not to enter the hive but to continue flying (this only happened in extremely rare cases) its signal was detected after a few meters and the measurements continued.

For better visualization representative examples of flight tracks are attached as supporting information (control_S1, control group; clothianidin_S2, clothianidin treatment; imidacloprid_S3, imidacloprid 0.6 µM treatment, imidacloprid_S4, imidacloprid 0.9 µM treatment; thiacloprid_S5, thiacloprid treatment). These show animated images with the transponder signal moving over time. The x- and y-axis is scaled in meters and the 0/0 coordinate marks the radar position.

Figure 5.4. A resting bee carrying a radar transponder. The transponder had a length of 2.3 cm and a weight of approximately 20 mg. It is glued to the number tag, visible here as a small pink stripe.

Analysis of the flight tracks and statistical analysis:

Each bee was individually tracked, as the radar could not distinguish between the transponder signals. Thereby an important prerequisite was that either only one bee could fly at a time or they had to be spatial separated. Fortunately, the bees either finished their flight before the next bee was released, or their flight paths did not cross. The data of each flight path consisted of x- and y-coordinates, each corresponding to a distinct time point. As described, the radar executed a full rotation every three seconds. Under optimal recording conditions we obtained space coordinates from every three seconds from each flight path. These space-time coordinates were used to reconstruct the flight path of each bee. The signal was not always consistent. There were multiple error sources that could disturb the recording. Following this, we excluded bees with less than 15 data points. This was enough to ensure we did not only record a small part of a full flight. From the obtained space-time coordinates we could calculate the flown distance, changes in the flight pattern and the time necessary to complete the whole

flight or parts of it. The flight speed for sections of interest like vector or homing flight could be calculated by dividing the covered distance by the time necessary for these sections.

In addition to the radar tracking, we recorded manually the time of departure and arrival. Departure time was monitored by the person who released the bee. Arrival time was noted by a person at the hive. When a bee carrying a transponder was seen at the entrance of the hive, one person tried to catch it. The transponder hindered the bees at entering the hive. This allowed an easy catch of the marked bee. Even if a bee managed to enter the hive, the transponder was always visible and we could set a time of arrival. These data allowed us an investigation of homing success, regardless of a good radar recording.

Non- circular statistics were done with Matlab v.R2011b (The MathWorks, Inc., USA). We used Barnard's exact probability test for comparison of arriving and not arriving bees. Data for flight time, speed and length were tested for normal distribution with the Lilliefors test. We found in each variable group at least one treatment group with non-parametric data. Therefore, we used a Kruskal-Wallis multi comparison between the groups with a Scheffe correction to find differences in the groups. This was followed by a group to group comparison using a Wilcoxon Rank sum test.

The circular statistics could not be performed by the same tests as the other data. All results are spread in a circular number space between 0° and 360°, with 0° and 360° being a transition to each other. The comparison of the angles for the different treatments was done with Oriana v4 (Kovach Computing Services, Wales, UK). Angular deviation was calculated with the Watson-Williams F-test while the distribution for angular data between groups was tested with the Mardia-Watson-Wheeler test. All degrees in the angular data were counted clockwise.

5.3. Results

Before looking for the more detailed effects of neonicotinoids on the flight path of bees, a general analysis was conducted to investigate the flight performance and the ability of the bees to return to their home. This was followed by a detailed analysis of the flight paths, divided into the vector flight component and the homing flight component.

5.3.1. Global Analysis

The bees were caught at the feeder and treated with one of the neonicotinoids or the control solution as described earlier. After 1.5 hours they were transferred to the release site and released. Many bees then flew around the release site in small circles, similar to the observed behavior they displayed after the collected sugar solution at the feeder. Most of the bees then flew straight to the west. Following this direction would lead back to the hive if they were still at the feeder where they were caught. Following this path led them to a landmark, a narrow irrigation channel. This channel can be seen on figure 5.3. as a dark strip close to the hive. The location of the expected hive was close to the intersection of the initial flight vector and the irrigation channel. At this point we could observe a change in behavior in most bees. They either made a turn northward, following the irrigation channel, which led them to their hive. Or they at least discontinued their straight flight path and started to change directions more often or fly in circles. This was identified as search behavior, we assumed that the bees were searching for known landmarks and trying to relate those to a way back home. As this point made an easy to recognize marker in the recorded flight paths we decided to separate the paths for further analysis in the initial part, the vector flight and the second part, the homing flight.

Without analyzing the flight paths, we could already make statements regarding the overall performance of the bees. When the bees were released after being caught and relocated to the release site, the time of departure was noted by the experimenter. A second person was also near the hive, waiting for the bee to return. If and when the bee

returned the time was again noted. With this we could state the success rates regarding the ability of the bees to return home. The results are shown in table 5.1. In the first column the different treatment groups are shown, followed by the total number of bees for each group in the second column. These numbers show all bees for which we could note a release event and a return event. We were not able to get an exact time of the arrival at the hive for all bees, due to the fact that some of them did not return during the daily duration of the experiment. However, we were still able to determine if some of these bees returned to the hive, as they were found at the feeding station 1 to 3 days later. Due to the fact that we could expect bees to survive several days without their colony, we assumed that they returned to the hive during a time when no observer was present at the hive entrance and when the radar was not recording, thus showing no sign of a flying marked bee. Hence these bees were also shown as arrived bees in table 5.1.

Table 5.1. Total number of bees used in this experiment per treatment group. Shown are the total number of released bees, including bees for which no analyzable fligth traces exit. The table shows the number of bees that returned succesfully to the hive and the bees that did not. Not arriving bees either were not seen back at the hive or did not start their fligth, as highlighted in the last column. Shown are also starts that were delayed for up to 15 minutes. The data for the delayed starts were only avaiable for the data obtained in 2012.

Treatment group	total number of bees	arrived at the hive	not arrived	not started	delayed start (only 2012 data)
control	**57**	**50**	**7**	**1**	**1**
clothianidin (0.2 µM)	**55**	**43**	**12**	**1**	**2**
imidacloprid (0.6 µM)	**58**	**42**	**16**	**2**	**2**
imidacloprid (0.9 µM)	**19**	**6**	**13**	**2**	**--**
thiacloprid (0.1 mM)	**27**	**12**	**15**	**3**	**8**

We compared the number of successful returns to the hive for each group and found a significant reduction in the number of successfully returning bees in three of the treatment groups. Since some bees did not start, those bees were not included in the calculations for significance for probability of arrival. In the control group, 50 out of 57 (88%) bees arrived successfully at the hive. This number showed that we can expect a

small number of bees not to return to the hive. Similar, 43 out of 55 (78%) of the bees treated with clothianidin 0.2 µM arrived at the hive. A significant reduction in the number of successful returns was noted for the group treated with imidacloprid 0.6 µM with 42 out of 58 (72%, $p < 0.05$; Barnard's exact probability test). A more severe reduction was observed in the group treated with imidacloprid 0.9 µM with 6 out of 19 (32%, $p < 0.00005$; Barnard's exact probability test) and in the group treated with thiacloprid 0.1 mM with 12 out of 27 (44%, $p < 0.005$; Barnard's exact probability test).

An important pretense for the experiments was the assumption that the chosen concentrations had no immediate effect on the bees. Therefore, the portion of not starting bees was tested for each treatment group in comparison to the control group. No significant increase in the number of not starting bees was found in any treatment group. Although the thiacloprid treated group showed significant more delayed starting bees than the control group, with 8 out of 27 bees ($p < 0.05$, Barnard's exact probability test), these bees still managed to fly after up to 15 minutes.

5.3.2. Vector flight

The global analysis already showed a significant influence of some of the used treatment groups. A further analysis of the details of the flight traces had to be done to find an explanation for those differences to the control group and to look for further, more subtle effects. For this the flight traces were separated in the already mentioned components, vector flight and homing flight. Here multiple parameters of the first component, the vector flight, were analyzed.

As described, the vector flight resembled for most of the bees a mainly straight flight from the release site to the intersection of this path with the irrigation channel. Therefore, the length of this vector flight was the first parameter to be analyzed. Figure 5.5. shows boxplots for the five treatment groups with a median length for the control group of 367 m. This is similar for the clothianidin 0.2 µM group with 354 m and for the imidacloprid 0.6 µM group with 380 m. A significant shorter vector flight length could be observed for

the imidacloprid 0.9 µM group with 272 m ($p < 0.05$; rank sum test). Contrary to this the thiacloprid 0.1 mM group showed a significant longer vector flight length with 412 m ($p < 0.05$; rank sum test).

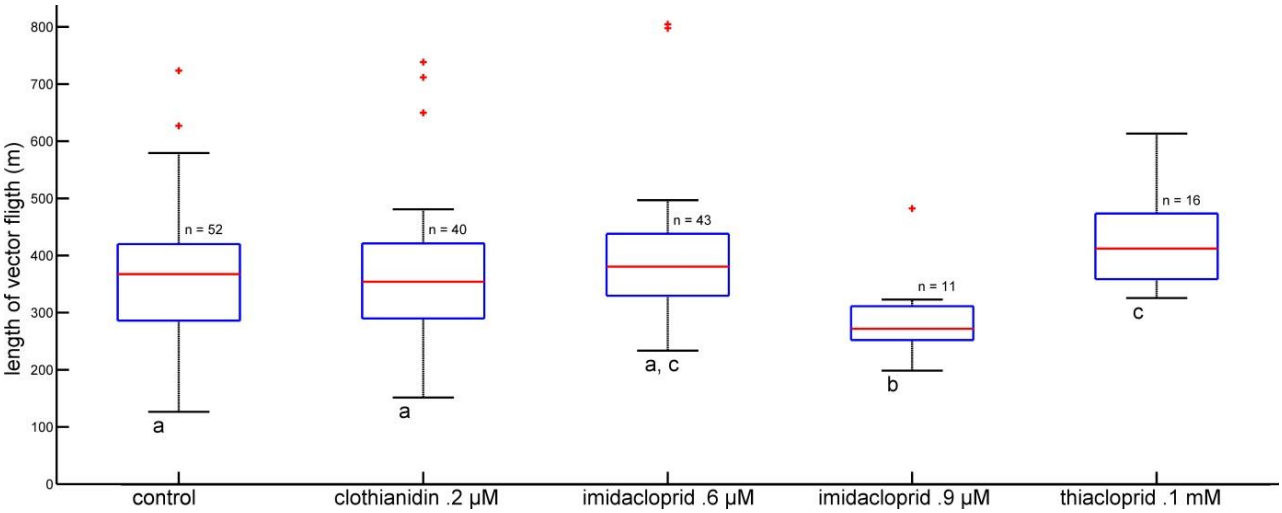

Figure 5.5. Length of vector flights for all treatment groups in meters. Groups are shown as boxplots with the median indicated in red, the edges of the box indicate the 25th and 75th percentiles. Outliers are shown as red crosses. Groups with no significant difference share the same letter under the lower whisker. Animals treated with imidacloprid 0.9 µM performed significant shorter vector flights than those of the control group, the clothianidin treated group, those treated with the lower concentration of imidacloprid, as well as animals treated with thiacloprid. The thiacloprid treatment led to significantly longer vector flights compared to bees from the control group, the clothianidin group and the bees treated with the higher concentration of imidacloprid ($p < 0.05$; rank sum test).

No significant impact of the different neonicotinoids could be found for the duration of the vector flight. 137 s was the median duration of a vector flight for the control group bees. For the bees from the clothianidin 0.2 µM group the median duration was 110 s and 116 s for the bees from the imidacloprid 0.6 µM group. Although no significant results could be found, we found a strong tendency for the imidacloprid 0.9 µM group to complete the vector flight fast with a median 75 s ($p = 0.055$; rank sum test). The thiacloprid group, which flew a longer distance in the vector flight, had a median duration of 174 s ($p = 0.0275$; rank sum test).

We expected the bees to fly along the same direction that they would fly from the feeder to the hive. The angle for this flight path was determined to be 294°. The direct route from the release site to the hive would result in an angle of 343°. Computation of the angle for each vector flight was done by determining the intersection of each flight path with a 200 m radius around the release site. This could easily be done as the flight paths were rather straight at this section of the vector flight. Results for each treatment group are shown as histograms in figure 5.6. Data for each 5° were binned together for better visualization. Almost all bees flew approximately in the expected direction, with the exception of a single bee in the imidacloprid 0.6 µM group. This showed that the bees performed the vector flight according to our anticipations. We expected that they used the path which would lead from the feeder to the hive. The average angle of the vector flight for bees from the control group was 319°. This angle is skewed northwards, which is also closer to the direct route to the hive. Significant differences in the average angle of the vector flight were found in bees from the clothianidin 0.2 µM group with an angle of 311° and in both imidacloprid groups, with an angle of 313° for the 0.6 µM group and 308° µM for the 0.9 µM group ($p < 0.05$; Watson-Williams F-test).

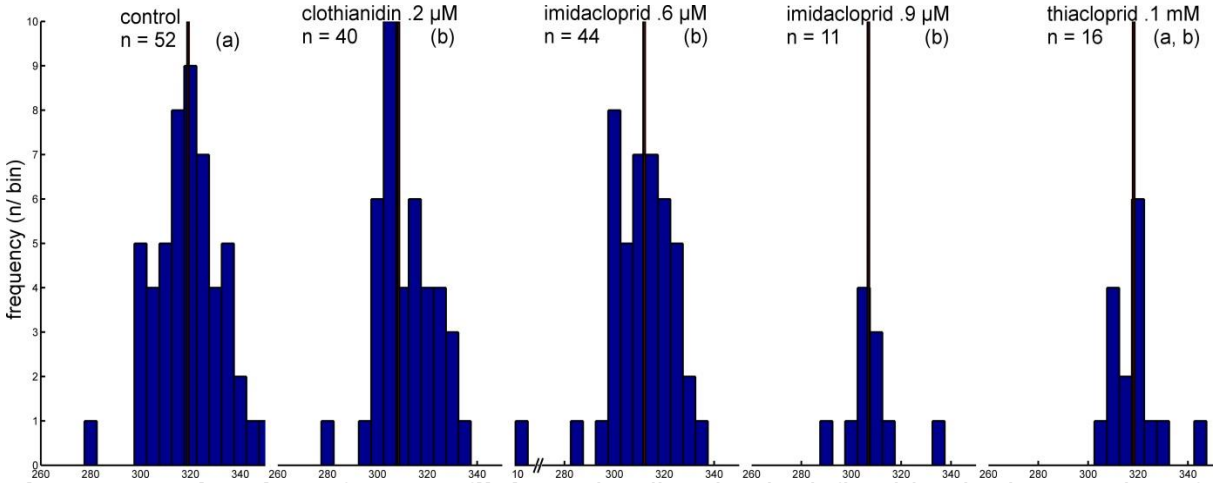

Figure 5.6. Direction of vector flights. The direction is defined by the intersection of the vector flight with a 200 m radius around the release site. The histograms show the measured angels in 5° bins. The x-axes give the angels in degrees clockwise from north. The median angle is marked in red for each group. Significant differences in the direction are indicated by different letters under the group names. Clothianidin treated and both imidacloprid treated groups differ significantly from the control group but not from each other. The thiacloprid treated group did not differ significantly from all other group ($p < 0.05$; Watson-Williams F-test). The direction of the learned route from the feeder to the hive is 294°, and the direct route from the release site to the hive would be 343°. Note that the x-axis is interrupted for the imidacloprid 0.6 µM group as there was one bee flying north-east with a 10.9° angle from north.

The thiacloprid 0.1 mM group showed no significant difference to the control group with an angle of 317°.

The next analyzed parameter was the directedness of the vector flights. When viewing the flight traces (see attached supplemental files) small directional changes were visible after each radar sweep. These were analyzed by looking for the angular changes after each radar sweep, meaning that two consecutive locations were used to draw a line, showing the path that the bee flew in these three seconds. Then we took the next location, drew a line and calculated the angular change between both. This was done for each step. A trace from a bee flying a perfectly straight line would always result in 0° angular deviation, while small changes indicate only small corrections to the flight path, without changing the general course. The data for the tested bees are shown in figure 5.7. as circular histograms. The data were binned to 7.2° (50 segments) changes for better readability. It was already stated that the bees follow the expected vector in a rather straight line. This was confirmed by this analysis. Most of the directional changes

between each radar sweep occurred in all groups close to 0°. Note that a change of 355° is the same deviation as 5°, only counterclockwise. The data are also always relative to the last path that was taken. This means that a bee that has most of the time ~0° directional changes but one event were we could observe a huge change in direction followed by again mostly ~0° changes would not arrive at the expected location but in a completely different location.

The clothianidin 0.2 µM group showed a distribution of directional changes similar to the control group. Most directional changes were close to 0°, indicating a straight flight pattern. Even if the bee changed its course it was most of the time in an angular deviation close to 0°. But a significant difference could be found in other treatment groups. Both imidacloprid treatment groups showed still the prominent bin at 0°, with the imidacloprid 0.9 µM group showing a very strong declination to follow that direction. But those bees also showed changes to their flight path in all directions, including directions between 90° and 270°. This means that the bees performed sharp turns. Taking these factors together, we could determine a significant directional change in the path for the imidacloprid 0.6 µM group ($p < 0.005$; Mardia-Watson-Wheeler test) and for the imidacloprid 0.9 µM group ($p < 0.05$; Mardia-Watson-Wheeler test).

We could not find a significant difference between the angular changes in the flight path from bees of the thiacloprid 0.1 mM and the control group. But it is noteworthy that these bees lack the prominent bin at 0°. The main flight direction was still forward, but the bees changed the direction more often between the radar sweeps.

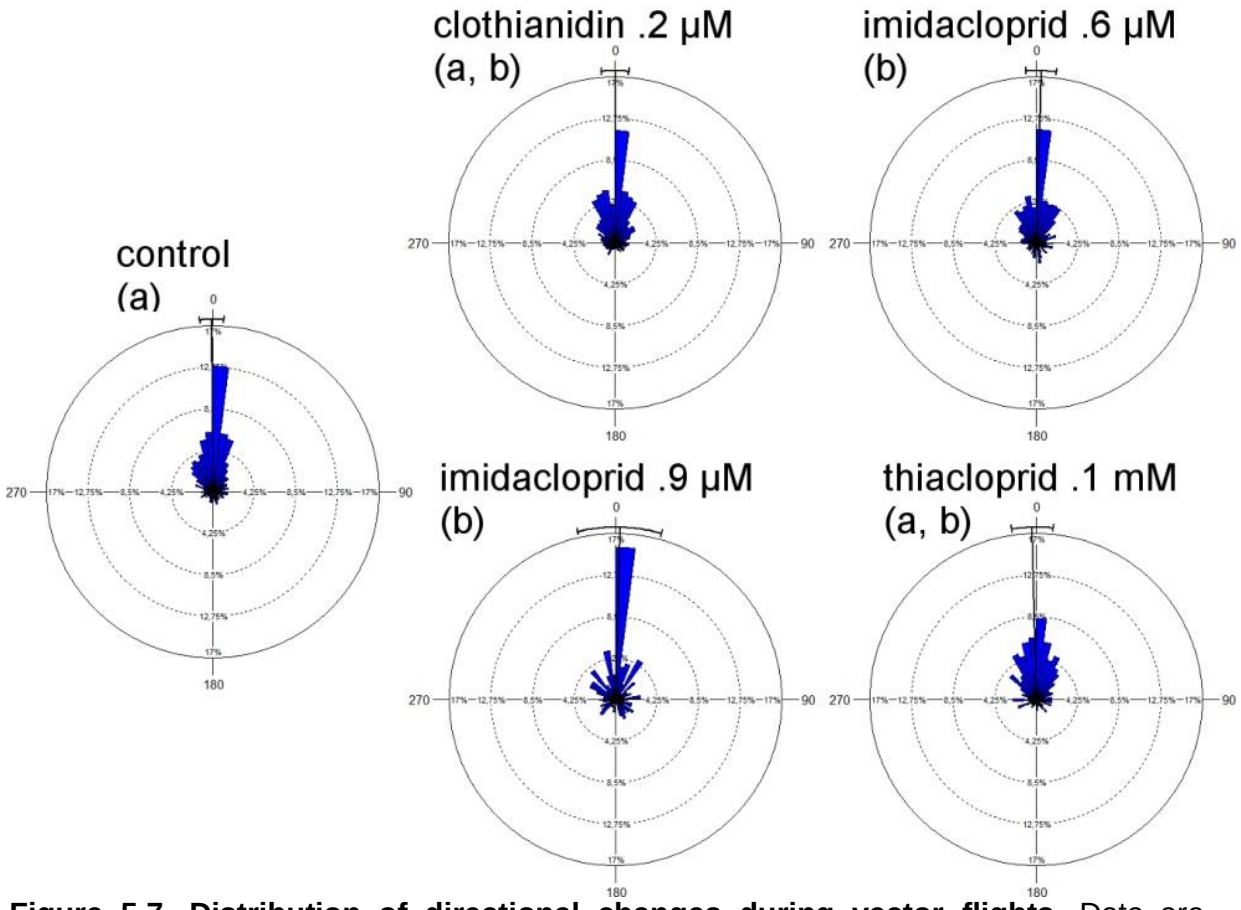

Figure 5.7. Distribution of directional changes during vector flights. Data are grouped in 50 segments (=7.2° each segment). Bars are scaled as percentages from 0% to 17% of the cumulative data. The black line shows the mean direction with standard deviation. Angles are given in relation to the direction of the stretch of flight shortly before, and are not related to a geographic direction (see text). The main component in all groups lies around the 0° direction indicating that the bees flew rather straight. Significant differences between the groups are shown by different letters in the parenthesis. Both imidacloprid treatments (0.6 and 0.9 µM) led to broader distributions of directions and thereby more changes in the flight path as compared to the control group ($p < 0.05$; Mardia-Watson-Wheeler Test).

5.3.3. Homing flight

The transition between vector and homing flight was characterized by a visible change in the flight pattern. The bees discontinued their straight flight path and either flew in circles, a behavior that was assumed to be a search behavior, or they changed the direction with a sharp turn, greater than 60° and continued in that direction for some

time. With the exemption of one control bee all bees showed this behavior. This one bee took the straight path from the release site to the hive.

We classified the bees after the directional changes, as can be seen in table 5.2. The vector flight normally terminated at the already described most prominent landmark, the irrigation channel. Well oriented bees could know that their hive was located next to an irrigation channel. Thereby we expected the bees to follow the channel either northwards or southwards. The shortest way back to the hive would result in a northward course along the irrigation channel and a continuation of this course until the hive is reached. This was characterized as an L-type flight, as the full path resembles an L-form. Examples for the directions are shown in figure 5.8. Note that the directions north and south are adjusted to the course of the irrigation channel. In the first example “north” the turn in the flight path and the point at which the bee determines the way to the hive is visible. In the next example, it is visualized how some bees first turn south, but then turn around and follow the irrigation channel northwards. In the example for “other” directions, it is shown how some bees do not use the irrigation channel as guidance for their flight, but rather cross it or fly back. The last example shows a bee performing circle flight movements at the end of the vector flight. In this example, the circle movements result not in a successful return to the hive.

Most bees chose to follow the irrigation channel in either direction, while some bees performed the search circles. Some bees in both imidacloprid groups showed a different behavior. They flew either east, back to the release site or further west. Some of the thiacloprid treated bees completely refused to continue their flight after not finding their hive at the end of the vector flight. The three bees from the column “other direction” from table 5.2. vanished from the radar at the end of the vector flight. This was interpreted in a way that the bees landed in the grass and stayed there for several hours, as they could not be detected for the rest of the day.

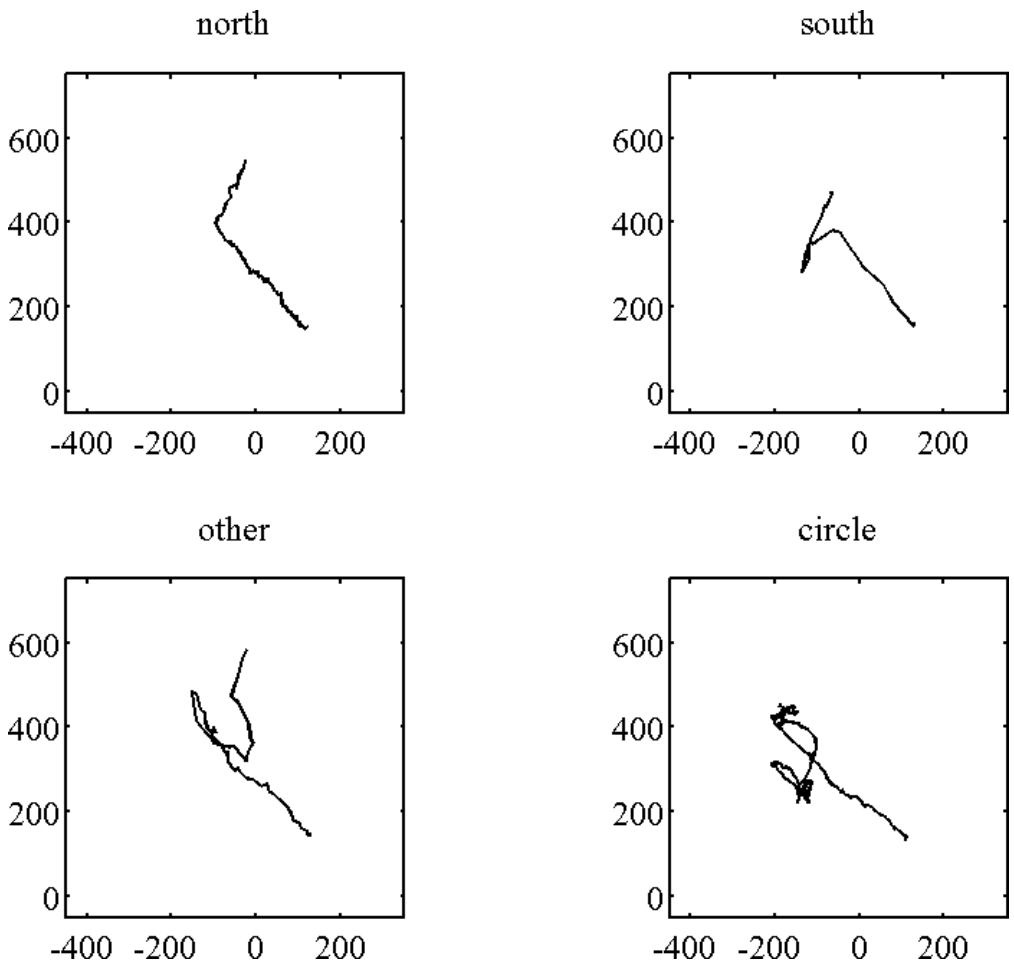

Figure 5.8. Visual examples of the directional categories used to group the bees. Shown are examples of the four directions from table 5.2. The direction indicates where the bee turned after she crossed the irrigation channel and has to find a way back to the hive according to her navigational information.

Many bees performed the L-type flights and no significant difference in this occurrence could be found between the control group and the clothianidin or imidacloprid groups. The thiacloprid 0.2 µM group however had a significant reduction in L-type flights compared to the control group ($p < 0.05$; Barnard's exact probability test), while at the same time displayed a higher probability to choose another direction than northwards.

Table 5.2. Flight direction after the end of the vector flight. The sharp turns (60°) were categorized as leading to a northerly (column north) or southerly (column south) direction along the irrigation channel, or any other direction (e.g. returning to the release site or continuing the vector flight with only a minor correction). Three thiacloprid bees (column other directions) terminated their flight at the end of the vector. (* = only bees flying in a northwards direction could perform an L-type flight)

	flight direction after the end of the vector flight				**percentage of L-type flights**
treatment	north	south	search circle	other directions	of north flying bees*
control (n=48)	31	14	3	0	74%
clothianidin (0.2µM , n=41)	29	12	0	0	62%
imidacloprid (0.6µM , n=41)	31	5	1	4	74%
imidacloprid (0.9µM , n=9)	7	0	0	2	57%
thiacloprid (0.1mM , n=14)	5	5	4	3	60%

In the next step all homing flights were analyzed, regardless if they led to a successful return or not.

First the length in meter was compared between the treatment groups. The visualization of the data can be seen in figure 5.9. Shown are the data for the different treatment groups as boxplots. The median flight distance is marked in red and significant differences between the groups are shown by different letter indices. The median length of the homing flight for bees from the control group was 365 m. Only the bees treated with clothianidin had a significant longer median distance than the control group with 580 m ($p < 0.05$; rank sum test). This was not significantly different from bees from the imidacloprid 0.9 µm group, with a median length of 409 m and from the thiacloprid 0.1 mM group with a median length of 338 m. Bees from the imidacloprid 0.6 µM group had also a significant lower median homing flight length, when compared to the bees from the clothianidin group, but not significantly different to any other group with 318 m.

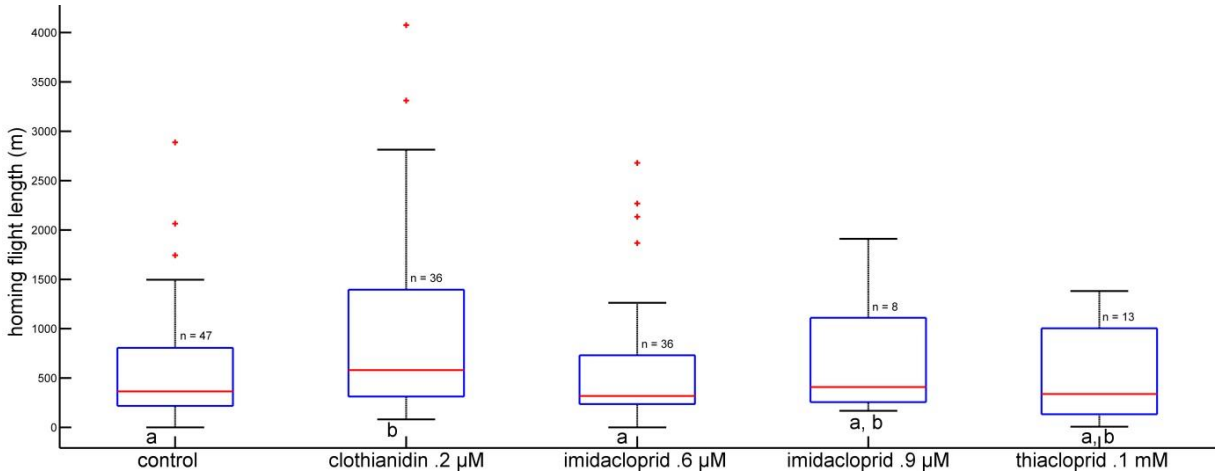

Figure 5.9. Cumulated lengths of flights during the homing phase. The homing phase started at the end of the vector flight as characterized by a turn of >60° during the vector flight and ended when the bee either arrived at the hive or was not recorded with the radar anymore. Groups are shown as boxplots with the median indicated in red, the edges of the box indicate the 25th and 75th percentile. Groups with no significant difference share the same letter under the lower whisker. Only clothianidin treatment resulted in a significantly longer flight during the homing phase, compared to the control group and the imidacloprid 0.6 µM group ($p < 0.05$; rank sum test).

Although only one treatment group showed an increased length of the flight path, more differences could be found when we analyzed the duration of the homing flight. In a case where the bee did not return to the hive we used the last time when the bee was detected by the radar. These data are shown in figure 5.10. in boxplots. The median flight duration per group is shown in red and significant differences between the groups are indicated by different letters. Bees from the clothianidin 0.2 µM group and from the thiacloprid 0.1 mM group took significantly longer for their flight than the control bees or the bees from the imidacloprid 0.6 µM group.

A lot of the bees with a high duration of the homing flight actually suspended their flight and rested for some time in the grass. This was seen in the recordings, as the bees would disappear from the radar and emerge later at the same spot. The most severe cases were found in the thiacloprid 0.1 mM group with six bees that interrupted their flight for at least more than 1500 seconds. But similar cases could be found in the other treatment groups, where bees rested for up to 500 seconds. This was seen for five bees from the control group, three bees from the clothianidin 0.2 µM group and four bees from the imidacloprid 0.6 µM group.

There were also cases where the bees could not be recorded until their arrival at the hive, but which were spotted at the feeder a few days later. For those bees we used the time of last recording. This was found for one control bee which was found one day later, two clothianidin bees and three imidacloprid 0.6 µM bees which also were found one day later and four thiacloprid treated bees from which two were found the next day, one two days later and one three days after the experiment.

Additionally, the flight speed was calculated and the bees treated with thiacloprid had a significantly reduced speed ($p < 0.05$; rank sum test).

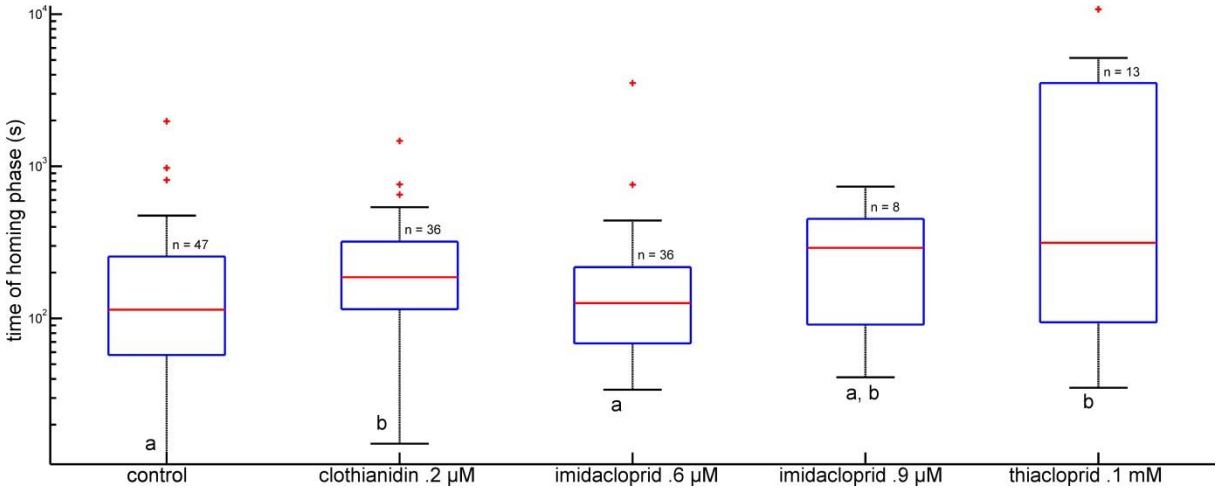

Figure 5.10. Duration of the homing phase. The homing phase started at the end of the vector flight as characterized by a turn of >60° during the vector flight and ended when the bee either arrived at the hive or was not recorded with the radar anymore. Groups are shown as boxplots with the median indicated in red, the edges of the box indicate the 25th and 75th percentile. Note that the y-axis is scaled logarithmically. Significant differences between the groups are shown by different letters at the bottom of each boxplot. Clothianidin 0.2 µM treatment resulted in a longer homing phase as compared to the control group and the imidacloprid 0.6 µM group. The median homing duration of the bees treated with 0.1 mM thiacloprid was significantly longer than the control group ($p < 0.05$; rank sum test).

The last analyzed parameter was the directedness of homing flights, like already done for the vector flight. As shown in figure 5.11. we could find a significant change in the distribution of the directional changes for both imidacloprid treatment groups and the thiacloprid group ($p < 0.05$; Mardia-Watson-Wheeler Test). Like in the significant changes in the vector flight bees in these groups were more prone to strong directional

changes between 90° and 270°. A comparison between vector and homing flight revealed that the control bees performed more directional changes in the homing flight than in the vector flight ($p < 0.05$; Mardia-Watson-Wheeler Test). The same is true for bees from the thiacloprid group. The imidacloprid groups only showed a tendency for a broader distribution, but this was not statistically significant.

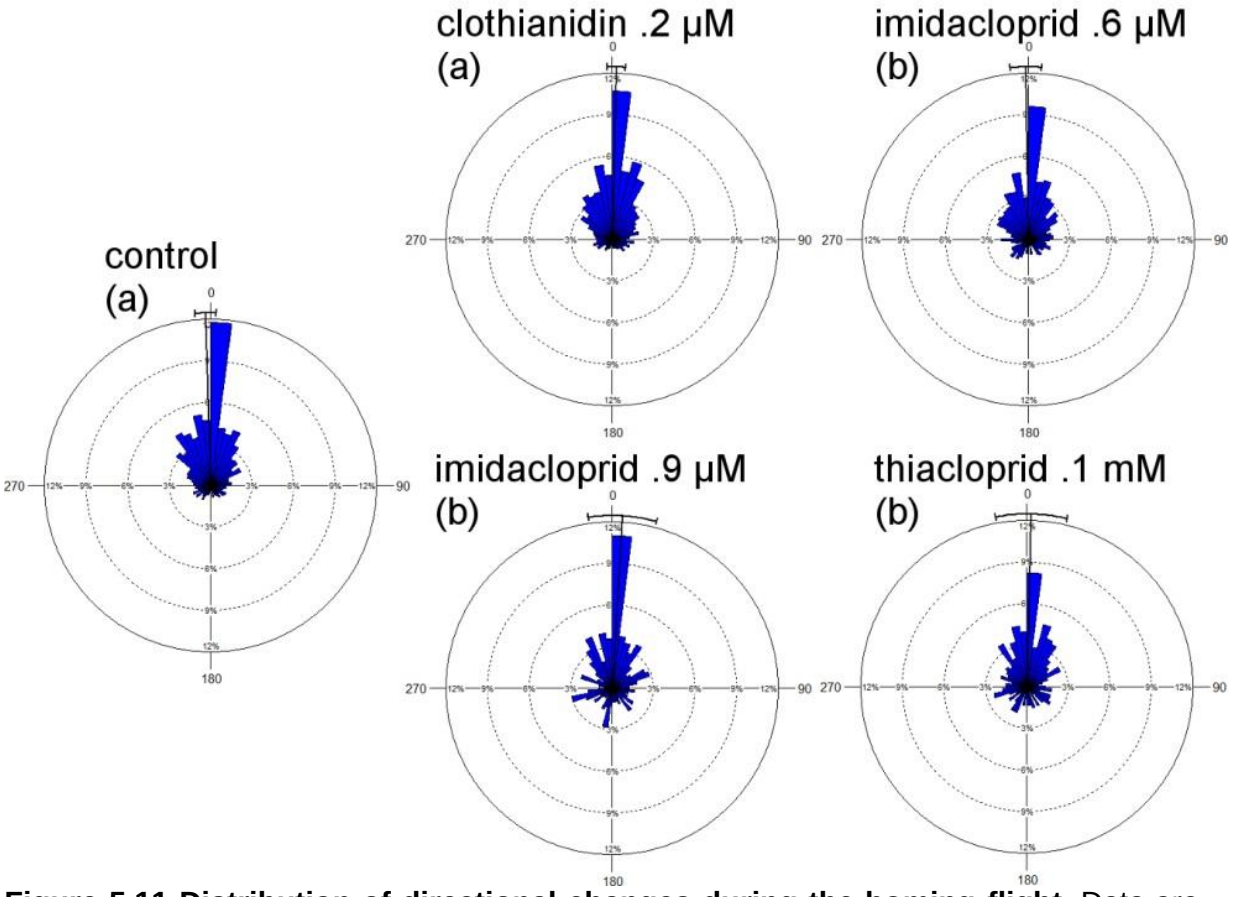

Figure 5.11 Distribution of directional changes during the homing flight. Data are shown in percent and are grouped in 50 segments (=7.2° each segment). Bars are scaled as percentages from 0% to 12% of the cumulative data. The black line shows the mean direction with standard deviation. Like in figure 5.7. all angles are in relation to the direction that the bee already flew, and are not related to a geographic direction. Thus the figure shows the straightness of the bees´ homing flights. Significant differences were found between the control group and the group treated with clothianidin on the one hand and both imidacloprid treated (0.6 and 0.9 µM) and thiacloprid 0.1 mM treated groups on the other hand ($p < 0.05$; Mardia-Watson-Wheeler test). These latter groups showed a broader spread of directions than the control and the clothianidin group, and did not differ from each other.

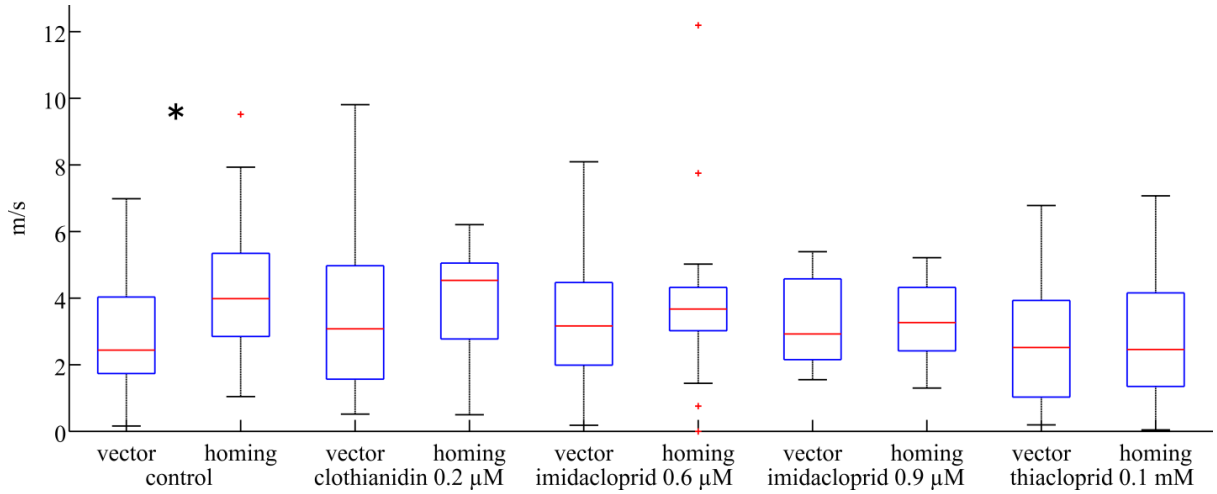

Figure 5.12. Flight speed during vector and homing flight component for bees from all treatment groups. Those speeds were calculated by dividing the flown distance by the time necessary for these sections of the flight. The bees were significantly faster during the homing flight in the control group (***** = $p < 0.05$; rank sum test). No other significant differences were found within each treatment group.

A comparison between flight speed during the vector and the homing flight showed that bees from the control group had a higher speed during the homing flight, as visualized in figure 5.12. The bees had a median speed of 2.44 m/s during the vector flight, which was significantly slower than the 3.98 m/s speed during the homing flight. The clothianidin treatment group showed almost a significant difference with median speed of 3.07 m/s during the vector flight and 4.53 m/s during the homing flight ($p = 0.0634$; rank sum test). The other neonicotinoid groups had no significantly different flight speeds during the two components of the flight. Bees from the imidacloprid 0.6 µM group had a median speed of 3.16 m/s during the vector flight and 3.67 m/s during the homing flight. The values were similar for bees from the 0.9 µM imidacloprid group with 2.92 m/s and 3.26 m/s. Bees from the thiacloprid treatment group were the only bees with an, although not significant, lower median speed. These bees had a speed of 2.52 m/s during the vector flight and 2.45 m/s during the homing flight. For comparison, the average speed for a flying bee under windless conditions was estimated at 8 m/s (von Frisch and Lindauer 1955).

5.4. Conclusion

Radar tracking revealed two components of the flight from honey bees in our experimental design. The components were the initial vector flight and the following homing flight. Without relocating the bees, the vector flight would have taken them from the feeder back to the hive. For this component the sun compass is the dominant guidance factor. This is especially true for the landscape that we chose for this experiment. The lack of visible landmarks in the distance forces the bees to use either local landmarks or the sun compass (Menzel et al. 2005, Menzel et al. 2011, Cheeseman et al. 2012). The local landmarks were in our case the difference in the ground structure. A well oriented bee should be able to recognize the ground structure and recognize that the vector flight did not end at the expected location.

Our results show that, except for a single bee treated with 0.6 µM imidacloprid, all bees performed the vector flight according to our expectations, regardless of their final success at finding the hive.

However, the flight direction for the control animals was skewed north to some degree. Interestingly, all treatment groups followed the expected angle to a higher degree, indicating more dourness for replaying the flight performance they would have applied, if not transported. This may indicate that the control bees recognized the ground structure and adjusted their direction to a more direct path towards the hive, whereas the recognition of this information or the ability to relay this information to the internal map seemed to be disturbed in the treated animals.

Flight speed during vector flights in thiacloprid-treated bees was lower than that of all other groups, indicating either an effect on flight performance or a form of reduced activation of the vector memory. This coincides with the fact that the vector flight was longer for this group. The bees treated with thiacloprid seemed to have difficulties relating the information that the hive was not at the expected location, near a visible landmark (the irrigation channel) and just continued their flight.

When the bees reached the expected location of the hive they had to use the other mentioned navigational mechanism and relate landmark information to their spatial map of their surroundings. For this it was necessary that the bee already explored this location and included it in her memory. In table 5.2. one can see that a high percentage of bees managed to solve this task quickly, resulting in a characteristic L-shaped flight. Examples can be seen in figure 5.8. The bees which decided to fly southwards were not necessarily negatively influenced and chose a wrong direction. The experimental setup excluded major landmarks which would have helped the bees to orientate. But they have to rely on local landmarks. The irrigation channel is such a landmark, but when the bee crossed it and did not find her hive at the expected location it is not given that her current information are sufficient to decide if the hive lies northwards or southwards along the channel. Thereby the occasionally occurring decision to fly southwards might not be an effect of a treatment, but a result of the experimental design. This is especially true, when the bee turned around after some time to fly northwards. Here we can assume that she acquired additional navigational information to correct her course.

Only bees from the treatment groups decided to fly east or westwards at the end of the vector flight. These directions can’t be related to a landmark that indicated the way back to the hive, as not a single control bee showed this behavior. Nevertheless, there were a number of bees in all groups that decided to fly southwards. This is easily explained, as the hive is located at the irrigation channel, but the bee can’t be sure if the correct location is north- or southwards by locating the channel. Additional information is needed to relate the position at the end of the vector flight to the hive. This information may be less visible than the irrigation channel.

Overall we could see that the bees were straighter in their flight path during the vector flight. As this component is not relying on the cognitive mechanism of recognition of landmarks and remembering the learned map, one could imply that it is easier to complete the vector flight. This may result in a relative more insecure flight pattern during the homing flight, when compared to the vector flight.

The bees treated with imidacloprid performed the vector flight more akin to the expected angle, while showing more changes in flight directions during the homing flight. Both can be connected to a decreased ability to perceive or process navigational information. If these mechanisms are inhibited, then there is less distraction to follow the vector information. And similar there are less information available during the homing flight to assess the correct path to the hive.

Similar effects could be found in bees treated with thiacloprid, but the disruption of the navigational capabilities of the bees seemed more severe in the homing flight. The directional changes seemed random, or at least not directly linked to a navigational cue obvious to the experimenter.

We had over all groups a certain number of bees that did not manage to return to the hive, but this was only significant for three treatment groups. However, we can conclude that the experimental procedure can expose the animals to some stress that can reduce the probability of a successful return. Nevertheless, both imidacloprid and the thiacloprid treatments led to a significantly reduced probability to return home. As all those bees performed their vector flight with only slight differences to the control group, the difficult part seemed to be the homing flight.

The clothianidin treatment did not reduce the probability of a successful return, but those bees took a significantly longer time for their homing flight. Only the bees in the thiacloprid group also differed in this way from the control group. The bees treated with clothianidin were able to return to the hive, whereas a substantial number of thiacloprid treated bees did not return to the hive. This leads to the conclusion that the effects of clothianidin might subside faster. Those effects seem to be well noticeable, as the bees from this group also differed in the direction of the vector flight when compared to the control group. But as the clothianidin treated bees arrived with a success similar to the control group those influences on the navigational capabilities are easily overlooked.

The doses of neonicotinoids applied here (imidacloprid 7.5 or 11.25 ng/bee, clothianidin 2.5 ng/bee, thiacloprid 1.25 µg/bee) were selected on the finding that the treated bees were able to fly 90 minutes after starting to imbibe the solution, and to depart from the

release site without obvious changes of their flight behavior. Thus, our study comprises a behavioral-toxicological approach and not an eco-toxicological approach. Nevertheless, it will be interesting to compare the doses used here with those used by other authors on the basis of estimates about the doses of the respective neonicotinoids expected to be taken up by bees in an agricultural environment. Our doses of imidacloprid and clothianidin were close to the highest doses tested by Schneider (Schneider et al. 2012) and Henry (Henry et al. 2012). Furthermore, bumble bees which had to forage from pollen containing 6µg/kg and sugar water containing 0.7µg/l imidacloprid showed significant depressing effects on several parameters of their natural development, such as queen production and growth rate (Whitehorn et al. 2012). The authors reported that the doses were selected on the basis of findings in the agricultural conditions. Gill et al. exposed bumblebees to two pesticides (neonicotinoid and pyrethroid) at concentrations that could approximate field-level exposure and detected impaired natural foraging behavior and worker mortality leading to significant reductions in brood development and colony success (Gill et al. 2012). Clothianidin of 10 ppb is often exceeded in pollen carried back by foragers, and a value of 88 ppb has been measured (Guez 2013). Our used concentration can be converted to 50 ppb clothianidin, which places in this range. It has been estimated that nectar collected by bees on oil rape flowers whose seeds were treated with imidacloprid contains on average (with very large variance) about 10 ppb which is approximately 30 times less than the lower doses of imidacloprid used in our study (Blacquiere et al. 2012, Cresswell 2011). Thus, 30 foraging trips of bees to such oil rape flowers combined with full absorption of the collected nectar would lead to a similar dose as in our study under the assumptions that the pesticides are fully absorbed and are not metabolized substantially. Thus, the doses in our study and those of Henry et al. and Schneider et al. can be considered to reflect a worse case as compared to those taken up by an individual bee during one foraging trip (see also EFSA Journal 201210(6) 2752). Although the debate about the relevance of the doses in behavioral-toxicological studies for the evaluation of environmental hazards through neonicotinoids is not settled (Henry and Decourtye 2013, Guez 2013) it is obvious that the doses in these studies are not far from what can be expected for bees foraging on the flowers of treated plants.

To explain the impairments of normal navigation in the different treatment groups we have to look where these substances act on the bees.

As described earlier the neonicotinoids activate the nAChR in the honey bee brain. Those receptors can be found in almost all neuropils of the brain, as shown by anti-nAChR staining (Bicker 1999). This means that uptake of a neonicotinoid can interfere with the cholinergic synaptic transmission in all these brain regions. Normally we can expect neonicotinoids to act as agonists to the receptor, leading to activation and thereby an ion current. However, this current can be lower or higher than after activation by the natural agonist acetylcholine. Also the main mechanism to end this activation, cleavage of acetylcholine by the acetylcholinesterase, can't be used to end the activation triggered by a neonicotinoid.

The mushroom body, which is heavily reliant on cholinergic signals, is particularly relevant for integration of multimodal information and the formation and learning of memory as well as memory retrieval (Menzel 2012). Neuropils, responsible for the primary uptake of sensory information, are also dependent on correct processing of cholinergic transmitted signals. The same is true for the excitatory neurons which relay the processed navigational decisions to the muscles to achieve a change in flight direction.

Thereby it can be concluded that uptake of non-lethal doses disturbs the usual brain chemistry and interference of cholinergic signal transmission can best explain the findings.

6. General conclusion

The experiments in this work were designed to investigate the effects of neonicotinoids on the honey bee. The focus was on non-lethal effects, about doses that don't kill the bees but can have subliminal effects which might not be immediately apparent. The design ensured that the neonicotinoid exposure was the main influence which separated treated bees from the control animals.

Multiple alterations of behavior could be noted, following the different treatments. But an important distinction must be made between the used substances. All neonicotinoids have the same main target site, the nicotinic acetylcholine receptor. But their physiological impact can lead to the complete opposite observable behavior. An overall trend for the experiments described in this dissertation was, that clothianidin led to a more active, although not necessary always targeted behavior. Imidacloprid and thiacloprid treated bees showed more often reduced activity, up to lethargic behavior in the case of the chronic thiacloprid feeding.

The reasons for these differences can be found in the reactions which the substances cause in the nervous system. The nervous system has been described thoroughly and it is known that acetylcholine plays the mayor role in excitatory signal propagation in bees. Neonicotinoids have a high binding affinity to the insect nAChR (Shimomura et al. 2006). It was already shown that imidacloprid is only a partial agonist of the nAChR in bees (Déglise 2002) as well in *Drosophila* (Brown et al. 2006). I described in this thesis first experiments that suggest thiacloprid to be a partial agonist for nAChRs in bees, similar to the findings for imidacloprid. Clothianidin in contrast was described as a full agonist in *Drosophila*, meaning that it elicits a current greater than the current after the activation through acetylcholine (Brown et al. 2006). If neonicotinoids activate the NAChR, but elicit a stronger or weaker ion current through the ion channel, then this disturbs the controlled neuronal function. In order to compute input from sensory cells or from memory retrieval, it is important the neuronal weight of information is balanced. The transfer of a signal from one neuron to another relies on transmitter, which should have a predictable effect on the perceiving receptor. The receiving cell can mostly only

influence the receptors which receive the signal, not the transmitter. Thereby an agonist that has an effect which is not comparable to the natural agonists disturbs the balance between excitatory and inhibitory transmission, which can result in an unanticipated result, after these signals were then cleared against each other. For example, a high affinity agonist like clothianidin should be able to overwrite the inhibitory effect of a GABA – signal, as this would otherwise block the excitatory effect of an acetylcholine signal.

The findings are foremost explained by effects of the neonicotinoids on neurons, specifically processing interneurons. Although there are no reports for actions of neonicotinoids against muscles, we cannot exclude the possibility of this potential target site for the neonicotinoids. A further possibility is an action on other, non-nervous tissue, like the intestines which come in contact with the substances after ingestion. It is also possible that the bees realize they ingested a substance that affects their nervous system. In this case the observed effects could be partly due to reactions against a perceived poisoning, which can trigger behavioral responses, such as the regurgitation of water, or that the bees chose to stay away from the hive to prevent a possible infection of others (McDonnell et al. 2013). Similar, if a nurse bee perceives an infection or another problem with brood, the bee can kill the developing bee to stop the spreading of a possible infection (Review: Cremer et al. 2007). It has also been shown that neonicotinoids can boost the expression levels of immune-related genes (Derecka et al. 2013). Therefore, although the neuronal effects can be expected to have the biggest impact on the behavior, non-neuronal effects do also occur and can disturb the bees and the hives.

Important talking points in the discussion about neonicotinoids are effects which are overlooked because the hive is seemingly not affected. Application of imidacloprid, clothianidin or thiacloprid interfered with the navigation of honey bees. It did not prevent the majority of bees from returning home eventually. But I could show in this thesis that specific parts of the skills, necessary for navigation, are impaired. The active and recently acquired navigation memory which would have brought the animals back to the hive (vector memory) is less compromised and appears even more stereotypical than in

control bees, because control bees tend to correct the displacement already during the vector flight. The second phase (homing) is impaired in treated bees reducing the probability of arriving at the hive, performing the correct turn at a salient landscape structure, and following a straight flight towards the hive. Since the homing phase in catch-and-release experiments documents the ability of the animal to activate a remote memory acquired during the exploratory orientation flights of a young bee and possibly during foraging flights before training to the feeder, we conclude that sub lethal doses of the three neonicotinoids tested either block the retrieval of a remote memory or alter this form of navigation memory. The findings on navigational effects after application of clothianidin were well visible in several of the observed parameters and seemed almost as severe as the thiacloprid treatment. But only the thiacloprid treated bees showed a significant reduction in probability to return home. Thereby clothianidin treatment seems not as severe for the individual bee. This highlights the rather selective and highly relevant impairment of the foraging behavior of bees. This experiment highlights another important point regarding bees. Unlike solitary insects, they rely heavily on each other

The here discussed results can reinforce existing reservations about the application of neonicotinoids in plant protection (Henry et al. 2012, El Hassani et al. 2008, Decourtye et al. 2003). The question stands how a hive would be affected if a large number of bees are influenced in a way that does not result in an absence of these bees. This question is partly answered by the flight room experiments. Here a small, although not significant, reduction in the collected sugar solution was observed. Also the treatment with 8.876 mg/kg thiacloprid had a severe impact on the brood nest size and prevented the development of pupae from the eggs.

Although neonicotinoids are not deadly in low doses, they still exert stress on the worker bees. Experiments like the ones described in this thesis focus on specific targets in the ecosystem of the bee, ranging from food over social interaction to weather conditions. Interactions between multiple parameters have been described and found. The ingestion of imidacloprid seems to have a negative effect on bees infested with a pathogen (Alaux et al. 2010).

The effects on bees are just a side effect of the usage of pesticides. The main targets are pest insects. But when confronted with these substances, selection for resistances can occur. In pest insects this already thought to have happened. An example for this is the Colorado potato beetle *Leptinotarsa decemlineata (*Szendrei et al. 2011). A method to avoid resistance is to alternate between different treatments. Interchanging of neonicotinoids seems however not sufficient to avoid the development of resistances, as the mode of action is too similar between the substances (Mota-Sanchez et al. 2006). We cannot assume that bees can adapt to pesticides to the same degree. Reproduction is partly under human control with targeted breeding. A beekeeper can also help a hive which would otherwise collapse under the ecological pressure. This can allow hives which are susceptible to neonicotinoids to endure and also to produce queens and drones. For pest insects such an inclusion of vulnerable individuals cannot be expected.

A rather current discussion point in the field of neonicotinoids is the question if very low quantities exist that may have a more severe effect than higher, but not lethal quantities. The reasoning is as follows. Although we can assume that bees are not able to detect neonicotinoids directly in the food (Kessler et al. 2015), they experience behavioral changes due to the neural effects of neonicotinoids. This may lead to an activation of detoxification mechanisms. For this the animals have to be able to detect the toxic qualities of these substances in some way. Quantities may exist at which the bees can be affected but which are not sufficient to activate the detoxification mechanisms. This may lead to two sub lethal concentration spans that show bigger effects. First a higher concentration span, where the detoxification mechanisms are not able to compensate the neurological effects. This span would cover most of the concentrations used in this thesis, as these concentrations are the most obvious targets for sub lethal effects. The other concentration span would be at much lower quantities and would not trigger the detoxification mechanisms. A normal dose-response essay would possible not cover these concentrations and assume them to be ineffective. The chronic feeding described in chapter 4 might affect bees in this way.

Metabolites of neonicotinoids after digestion and detoxification are another problem when trying to assess the implications of a certain neonicotinoid for bees.

Metabolization can occur in the insects which consumed it, but also in the plants which are treated. This is especially true for neonicotinoids which are distributed by seed coating. In this case the substances enter the plant trough the roots and are absorbed and distributed through the, primarily young and growing, plant body. This can also lead to the presence of a toxic substance, in some cases another neonicotinoid in a very low concentration, although this substance was never added to the targeted organism (Nauen et al. 2003).

All the mentioned points make it difficult to construct a direct relationship between only one neonicotinoid and the effects on a bee. It is always important to consider the environment in which the effects are discussed in regards to other stressors, such as other harmful chemicals (e.g. acaricides, fungicides), pathogens (e.g. bacteria, virus or parasites) or a challenging landscape (e.g. few landmarks, short flowering period).

Several effects of neonicotinoids on the behavior of honey bees have been found in this dissertation. The described findings help the understanding of the implications of this widely used class of insecticides. As part of an EU – supported project they can help to find guidelines for their proper usage. Additionally, they offer insight in how a cholinergic agent alters individual and social behavior in the bee.

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8. List of Figures

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11. Erklärung

Ich erkläre hiermit, dass ich mich bisher keiner Doktorprüfung im Mathematisch-Naturwissenschaftlichen Bereich unterzogen habe.

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Versicherung

Ich erkläre hiermit, dass ich die vorgelegte Dissertation über

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selbständig angefertigt und mich anderer Hilfsmittel als der in ihr angegebenen nicht bedient habe, insbesondere, dass alle Entlehnungen aus anderen Schriften mit Angabe der betreffenden Schrift gekennzeichnet sind.
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