Neonicotinoid insecticides impair foraging behavior, navigation, learning, and memory in honey bees (*Apis mellifera*)

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LÉA TISON
from Bordeaux, France

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1st Reviewer: Prof. Dr. Dr. h.c. Randolf Menzel

2nd Reviewer: Prof. Dr. Hans-Joachim Pflüger

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To rirou

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Declaration

I hereby declare that the work presented in this thesis has been conducted independently and without inappropriate support. All sources of information are referenced. I hereby declare that this thesis has not been submitted either in the same or a different form to this or any other university for a degree.

Contents

This dissertation includes the following manuscripts:

Manuscript 1

Tison, L.; Hahn, M. L.; Holtz, S.; Rößner, A.; Greggers, U.; Bischoff, G. and Menzel, R. (2016). **Honey bees' behavior is impaired by chronic exposure to the neonicotinoid thiacloprid in the field**. *Environ. Sci. Technol*. 50, 7218−7227. http://dx.doi.org/10.1021/acs.est.6b02658

Author's contribution: L.T, M-L.H, U.G and R.M performed the field experiments. U.G performed and analyzed the electric fields recordings. L.T, S.H and A.R designed and performed the choice experiments and interpreted the results. G.B and L.T did the pesticide residues analysis and interpreted the results. L.T and M-L.H analyzed the foraging and navigation data, L.T and R.M designed the study, interpreted the results and wrote the manuscript.

The section "Perspectives" presents preliminary results of a project in collaboration with Dr. Tim Landgraf, which will be published together with additional data on honey bee navigation.

Manuscript 2

Tison, L.; Adeoye, A.; Kalkan, Ö.; Holtz, S.; Irmisch, N. S. and Menzel, R. **Effects of sublethal doses of thiacloprid and its formulation Calypso® on the learning and memory performances of honey bees.**

(A shorter version of this manuscript is currently under review by *J. Exp. Biol*)

Author contributions: L.T, S.H, A.A, Ö.K, and N.S.I performed the sucrose responsiveness and conditioning experiments. L.T and G.B performed the residue analysis. L.T and R.M designed the experiments. L.T analyzed the data, interpreted the results and wrote the manuscript.

Manuscript 3

Tison, L.; Púčiková, V.; Rößner, A.; Gerschewski, S. and Menzel, R. **Detrimental effects of clothianidin revealed in field and laboratory studies.**

(In prep research article)

Author contributions: L.T and V.P performed the field experiments and analyzed the pesticide residues. L.T, A.R and S.G performed the PER experiments. L.T and R.M designed the experiments. L.T analyzed the data and wrote the manuscript.

The section "Perspectives" presents on going analysis involving the participation of Aron Dür and Dr. Tim Landgraf. Depending on the outcome, these results will be published together with the results presented in Manuscript 3 or compiled in another research article or short communication.

General Summary

The decline of pollinators worldwide is of growing concern because of the essential role they have in our ecosystem, agriculture and economy. Both biotic and abiotic factors have been implicated as possible contributors to their decline; however, the potential role(s) of commonly-used neonicotinoid insecticides has emerged as particularly concerning in the last decade.

I chose to focus my research on two substances: thiacloprid, a widely used cyano-substituted neonicotinoid thought to be less toxic to honey bees and of which use has increased in the last years, and clothianidin, a nitro-substituted neonicotinoid mostly applied as seed-treatment and currently subject to a moratorium in the EU.

Honey bees (*Apis mellifera carnica*) were chronically exposed to field-realistic concentrations of thiacloprid or clothianidin in the field for several weeks. Foraging behavior, homing success, navigation performance, and social communication were impaired by thiacloprid and residue levels increased both in the foragers and the nest mates over time. Exposure to clothianidin also impaired the foraging span, foraging behavior and recruitment abilities of honey bees in the field but the homing success was not impaired. For both substances, we showed that the effects observed in the field were not due to a repellent taste.

Furthermore, we applied a laboratory standard procedure, the Proboscis Extension Response (PER) conditioning, in order to assess which processes, acquisition, memory consolidation and/or memory retrieval were compromised after bees were fed either with clothianidin, thiacloprid or the formulation of thiacloprid named Calypso® at 3 different sublethal doses. Extinction and generalization tests were performed to determine whether bees respond to a learned stimulus, and how selectively. We showed that thiacloprid, as active substance and as formulation, poses a substantial risk to honey bees by disrupting learning and memory functions. The consolidation and the retrieval of memory was also impaired in the case of bees exposed acutely to sublethal doses of clothianidin.

Using both field and laboratory studies, we could show that commonly used neonicotinoids are strong candidates for the observed decline in efficiency of pollinators´ populations. Evidence that sublethal doses of thiacloprid, its formulation Calypso® and clothianidin are having such negative effects on honey bees at field-realistic concentrations raises important and challenging questions for agricultural management.

Zusammenfassung

Der weltweite Rückgang von Bestäubern ist von großer Bedeutung aufgrund ihrer wichtigen Rolle für die Ökosysteme, den Ackerbau und die Wirtschaft. Sowohl biotische als auch abiotische Faktoren werden als potentielle Ursachen für diesen Rückgang betrachtet. In den letzten zehn Jahren hat sich auch eine mögliche Rolle von Neonikotinoiden, den üblich verwendeten Insektiziden, herausgestellt.
Für meine Forschung habe ich zwei Substanzen gewählt: Thiacloprid, ein weit verbreitetes cyano-substituiertes Neonikotinoid, das als wenig toxisch für die Honigbienen eingeschätzt wird und dessen Verwendung in den letzten Jahren rasch gestiegen ist; und Clothianidin, ein nitro-substituiertes Neonikotinoid, das meistens in der Samenbehandlung eingesetzt wird, und für das aktuell ein möglicher Zulassungsstopp in der EU geprüft wird.

Honigbienen (*Apis mellifera carnica*) wurden über mehrere Wochen chronisch feldrealistischen Konzentrationen von Thiacloprid oder Clothianidin ausgesetzt. Die Futtersuche, erfolgreiche Nest- Rückkehr, Navigationsleistung und intraspezifische Kommunikation wurden durch Thiacloprid beeinträchtigt und seine Rückstände haben in Sammlerinnen sowie in Stockbienen im Laufe des Versuchs zugenommen. Auch die Behandlung mit Clothianidin hat die Futtersuche und die Rekrutierungsfähigkeit der Sammlerinnen beeinträchtigt und ihre Lebenserwartung verkürzt. Die Nest-Rückkehr wurde durch Clothianidin nicht beeinflusst. Wir haben für beide Substanzen nachgewiesen, dass der beobachtete Effekt nicht mit dem repellenten Geschmack zusammenhängt.

Des Weiteren wurde ein standardisierter Versuch unter Laborbedingungen, die Proboscis Extension Response (PER) Konditionierung, durchgeführt, um zu erforschen, welche Prozesse der Gedächtnisbildung (der Erwerb, die Konsolidierung und/oder der Abruf des Gedächtnisses) durch die Aufnahme von Clothianidin, Thiacloprid oder einer Thiacloprid-Mixtur Calypso® in drei verschiedenen subletalen Konzentrationen beeinträchtigt werden. Extinktions- und Generalisierungsversuche wurden durchgeführt um zu bestimmen, ob und wie selektiv die Bienen auf einen gelernten Stimulus reagieren. Wir zeigen dass Thiacloprid, als reine aktive Substanz sowie in Form einer Mixtur ein erhebliches Risiko für Honigbienen, durch eine Störung der Lern- und Gedächtnisfunktion, darstellt. Die Konsolidierung und der Abruf des Gedächtnisses wurden auch nach der Aufnahme von subletalen Dosen von Clothianidin negativ beeinflusst.

Durch unsere Feld- und Laborversuche wurde gezeigt, dass üblich verwendete Neonikotinoide einen starken Einfluss auf die beobachtete Abnahme der Leistung von Bestäuberpopulationen

haben. Die nachgewiesene negative Wirkung der feldrealistischen subletalen Konzentrationen von Clothianidin, Thiacloprid und seiner Mixtur Calypso® auf die Honigbienen, wirft eine wichtige und herausfordernde Frage für das Agrarmanagement auf.

General Introduction

As our environment changes, honey bees have recently become an emblem of its fragility. Amongst chemical pollution, global warming, and intensive agriculture, the honey bee appears as the innocent victim of these worrisome subjects. Its destiny is the witness of Nature's disorder, more and more dominated by human technology. In the face of human's pretentious habit of knowing and controlling everything, the honey bee appears as a fragile creature, symbol of the vulnerability of Nature, subject to the dictates of humans. Nowadays fears for the future are projected onto honey bees, an ancient philosophical and mystical insect and symbol of the greatness and beauty of Nature. In ancient Greek mythology, Aristaeus, son of Apollo and Cyrene, was the first professional beekeeper. When Aristaeus was born, he was taken by Hermes who fed him nectar and ambrosia. As a child, the nymphs taught him how to care for bees and "cultivate" bee hives. He later passed on his knowledge to humanity and was known by humans as Mellisseus. One day his bees got sick and disappeared; suddenly all of his hives were empty. Aristaues became desperate; especially as he did not understand the reason of his misery. The gravity of the situation was well beyond him. Today, in the real world, bees are disappearing and mankind is responsible. The aim of my thesis is to better understand some of the mechanisms behind the decline of honey bees and pollinators in general. To accomplish this, I investigated the effects of chronic and sublethal concentrations of two neonicotinoids on the foraging behavior, navigation, communication, learning, and memory in honey bees.

The importance of pollinators

Pollinators are tightly linked to human well-being through their service to our ecosystem and agriculture. They are responsible of the ecosystem health maintenance, diversity and the reproduction of wild plants. They are securing a reliable and diverse seed and food supply and producing honey and other beekeeping products (Potts *et al.*, 2016). Pollination can be achieved by wind and water, but the large majority of the global cultivated and wild plants depend on pollination by animals. Animal pollination directly affects the yield and/or quality of approximately 75% of globally important crop types (Potts *et al.*, 2016) and up-to 35% of global production comes from crops depending on them (Klein *et al*, 2007). Bees remain the most economically valuable pollinators of crop monocultures worldwide, visiting more than 90% of the main 107 global crop types (Klein *et al.*, 2007). The honey bees, *Apis mellifera* is the most commonly managed bee in the world, and the yields of some fruit, seed and nut crops would decrease by more than 90% without these pollinators (Southwick and Southwick, 1992). Wild pollinators also contribute greatly to global crop production (Garibaldi *et al.*, 2013).

Pollinators' populations are declining and habbitat loss and homogenization, parasites and pathogens, invasive species, climate change, and pollutants have been identified as past and current threats to pollinators.

There has been increased interest from science (Lautenbach *et al.*, 2012), the public and policymakers and yet no clear management responses. Restoring and maintaining pollinators and their diversity is highly important for agriculture (Gill *et al.*, 2016) but focusing conservation only on the services delivered by pollinators is not wise, and the sole argument for conservation of our biodiversity should be motivating enough (Kleijn *et al.*, 2015).

Honey bees' behavior in the field

Bees travel from one location to another in order to reach a particular goal appropriate to their current motivation. They are guided by their memory of the goal location and the path leading to it, helped by the presence of landmarks. Some components of their memory may be innate, but most result from individual experience. At about three weeks old, before becoming foragers, honey bees take several orientation flights, exploring and learning the environment which will be later essential for successful foraging and homing flights (Capaldi *et al.*, 2000; Degen *et al.*, 2015). Foraging honey bees are usually flying straight trajectories between specific locations like food sources and the hive. Bees travelling along a route many times can learn landscape features and use them to navigate (Menzel *et al.*, 2006; Menzel, 2012). During the flight, bees are using a visual odometer which allows them to estimate the flown distance, sun light and polarized-light compass to adjust the direction of the flight (Wehner *et al.*, 1985). If the sun compass is not available, bees can retrieve flight directions from landmarks (Menzel *et al.*, 2006; Menzel, 2012). Nectar containing sugar is the primary source of energy for their vital functions and for the survival of the entire colony. Honey bees return back to their hive after every foraging trip to empty their crop from the collected nectar. Once they are back inside the colony, they are capable of communicating the location of a food source (its direction and distance) as well as its quality to other forager bees by performing the waggle dance (von Frisch 1967).

The use of neonicotinoid insecticides

The presence of honey bees in the environment depends on biotic factors like pathogens (e.g. *Nosema cereanae*, several type of viruses), parasites (e.g. *Varroa destructor*), availability of resources due to habitat fragmentation and loss, and also on abiotic factors like climate change and pollutants (Decourtye and Devillers, 2010). The recent loss of pollinator populations can be attributed to a lot of different factors but it has already been admitted that the major use of pesticides for crop protection may contribute their decline (Brittain and Potts, 2011). Widely used for the management of insect pests, the neonicotinoids insecticides are a

relatively new class of insecticides designed in the 80s and represented by imidacloprid, acetamiprid, clothianidin, thiamethoxam, thiacloprid, dinotefuran and nitenpyram. They are now some of the most popular and widely used insecticides (van der Sluijs *et al.,* 2013; Simon-Delso *et al.,* 2014). Neonicotinoids can be found everywhere. They are used in field crops, orchards, parks, landscapes, backyard gardens, ornamentals, lawns, pets, and in structural pest control. The majority of all neonicotinoid applications are delivered as seed/soil treatments, representing 80% of the worldwide insecticide seed treatment market and 24% of the total insecticide market in 2008 (Jeschke *et al.,* 2011). But they can also be applied as foliar sprays to above-ground plants or as trunk injections to trees. Neonicotinoids are highly water soluble and due to their systemic action, they are taken-up in the entire plant via the xylem, providing a long period of protection against a large number of insect pests. But only about 2-20% of a seed treatment is actually absorbed by the plant. The rest of the substance is either blown into the air (dust drifts), remains in soil, and/or finally washed away into ground and surface water (Goulson, 2013; Krupke *et al.*, 2012).

Neonicotinoids are especially effective against sucking pests such as aphids, whiteflies, planthoppers, and thrips (Elbert *et al.,* 2008). Because of their high toxicity to insect pests and relatively low toxicity to vertebrates, neonicotinoids and fipronil (also systemic) are supposed to act specifically on insect pests while reducing impacts on non-target organisms (Tomizawa and Casida, 2003, 2005). In the last decade, concerns associated with the widespread and increasing use of these synthetic pesticides have raised because of their effects on non-target pollinators and other invertebrates as well as their persistence in soils (Bonmatin *et al.*, 2015; Goulson, 2013).

Honey bees' exposure to neonicotinoids

Honey bee foragers can be exposed to these substances by two main exposure routes: contact and oral exposure. They can be affected by pesticides during the spreading of chemical products or the sowing of treated seeds and they can also be exposed orally or by contact to pesticides by drinking contaminated water or by foraging nectar and pollen on treated plants (Krupke *et al.*, 2012). Acute intoxication sources for bees via contaminated guttation droplets containing high level of neonicotinoid residues (Girolami *et al.,* 2009; Tapparo *et al.,* 2012) and direct exposure of flying bees to dusts emitted by the drilling machine during sowing of treated seeds (Girolami *et al,.* 2012; Krupke *et al.,* 2012; Tapparo *et al.,* 2012) have been identified.

In addition to their acute toxicity at high doses, exposure to neonicotinoids can also result in serious sublethal effects to non-target insects exposed chronically to low doses. Indeed, due to their systemic properties, neonicotinoids can be found in the nectar and pollen of treated and untreated crops and flowers for a long time after the spraying or sowing of these susbstances (Botìas *et al.*, 2015). Small concentrations of neonics are found in both pollen and

nectar usually between < 1 and 8 parts per billion (ppb) in nectar and between <1 and 50 ppb in pollen. Much higher concentrations occur when neonics are sprayed onto plants. Coktails of numerous pesticides including neonicotinoids are found in apiaries, treated fields, soils and in the wildfllowers from field margins (Mullin *et al.*, 2010; Botias *et al.*, 2015; David *et al.*, 2016) with expected consequences for non-target insects (Botias *et al.*, 2016).

Sublethal toxicity to bees is the most likely exposure scenario in the field and has impacts on the physiology and behavior of an individual without directly causing its death (Desneux *et al*, 2007). Indeed, concentrations detected in pollen and nectar from seed-treated crops with neonicotinoids are generally too low to cause immediate bee death from acute poisoning but they lead to long-term exposure of bees foraging on treated crops.

The honey bee brain, target of neonicotinoids

Neonicotinoids are agonists of the post-synaptic nicotinic acetylcholine receptor (nAChR) which are normally activated by the neurotransmitter acetylcholine. They permanently bind to nAChRs, activating them and the passage of nerve impulses via depolarization, causing muscle cramps and paralysis (Tomizawa and Casida, 2005; Brown *et al.*, 2006; Déglise *et al.*, 2002). The normal signal transmission via the nAChR is thus disturbed either by continuous synaptic stimulation or by blocking the binding of the normal transmitter acetylcholine. Acetylcholine being the most abundant excitatory transmitter in the insect central nervous system, it is not surprising that sublethal doses of neonicotinoid compromise behavior and cognitive abilities in honey bees (Belzunces *et al.*, 2013). The nAChR are expressed at the input site of the mushroom bodies of honey bees (Déglise *et al.*, 2002) and mushroom bodies are essential for mediating high order brain functions such as multisensory integration, learning, memory, and spatial orientation (Heisenberg, 2003; Menzel, 2012; Zars, 2000). The 150,000 or so mushroom body intrinsic neurons synapse onto a few hundred extrinsic neurons (Rybak and Menzel, 1993), and it is at these synapses that the value of a learned stimulus combinations is encoded (Menzel, 2012). Palmer *et al.* (2013) showed that the neonicotinoids clothianidin and imidacloprid at concentrations < 10nM had a depolarizing effect on the nAChRs in the Kenyon cells, principal neuronal components of the mushroom bodies (Szyszka *et al.*, 2005). Also, sublethal dosage of imidacloprid reduced the microglomerular density of honey bee mushroom bodies (Peng and Yang, 2016). Another study has shown changes in olfactory coding already in the antennal lobes following acute exposure to imidacloprid (Andrione *et al.*, 2016).

Neonicotinoids are thought to cause irreversible and accumulative damage to the central nervous system of insects (Tennekes, 2010). And yet, honey bees' efficacy in bringing back pollen and nectar to the hive and communicating the food sources locations is indispensable

to the well-being of a colony. Effective learning, memory, navigation and communication systems are necessary in honey bee foraging activity and colony survival.

The impact of neonicotinoids on honey bees

Toxicity tests of neonicotinoids before release on the market evaluated only the short term and lethal effects of these products on worker honey bees. Research in ecotoxicology is developing new approaches of risk assessment by studying long term sublethal effects, effects on larvae, queens and cognitive faculties like learning, navigation and communication. Sublethal doses of neonicotinoids were shown to compromise a large range of behaviors in bees such as learning and memory (Decourtye *et al.*, 2004, 2005; Stanley *et al.*, 2015; Williamson *et al.*, 2013; Piiroinen and Goulson, 2016), foraging behavior (Bortolotti *et al.*, 2003; Colin *et al.*, 2004; Schneider *et al.*, 2012; Yang *et al.*, 2008; Tison *et al.*, 2016), communication (Eiri and Nieh, 2012; Tison *et al.*, 2016), homing success and navigation (Henry *et al.*, 2012; Fischer *et al.*, 2014; Tison *et al.*, 2016), colony development and fitness (Henry *et al.*, 2015; Williams *et al.*, 2015; Wu-Smart and Spivak, 2016). Given the increasing evidence that these systemic insecticides pose serious risk of impacts on some non-target organisms (EFSA, 2012, Pisa *et al.*, 2015; Bonmatin *et al.*, 2015), the European commission has decided in 2013 to suspend the use of clothianidin, imidacloprid and thiamethoxam.

Some synergism effects between neonicotinoids and other pesticides have also been revealed in honey bees, sometimes increasing the toxicity by up to 560-fold (Iwasa *et al.*, 2004; Schmuck *et al.*, 2003). The effects of pesticide mixtures on honey bees are very hard to quantify even if exposure to cocktails of pesticides is a much more realistic exposure in field conditions (Mullin *et al.*, 2010, 2016; Botìas *et al.*, 2015; David *et al.*, 2016). Also, in contrast to acute effects, no standardized protocol exists for measuring chronic effects on individual bees in field or semi-field conditions (Pisa *et al.*, 2015). Depending on the species studied and pesticide used, field studies have described conflicting results (Godfray *et al.*, 2014). The value of tests on single animals has been questioned as evidence for negative effects on managed honey bees at the colony scale has not always been clear (van der Sluijs *et al.*, 2015; Godfray *et al.*, 2014; Pisa *et al.*, 2015; Rundlöf *et al.*, 2015; Henry *et al.*, 2015).

In the first manuscript and third manuscript, my colleagues and I studied the effects of a chronic exposure to the neonicotinoids thiacloprid and clothianidin on honey bees' behavior in the field.

Studies on the impact of neonicotinoids on homing success started when beekeepers noticed huge bee losses in colonies located close to crops treated with systemic insecticides. Honey bees were not dying in the hive nor around it, they just "disappeared". These observations (now known as the *Colony Collapse Disorder*) led to the hypothesis that bees failed to find

back to the hive after foraging on pollen and nectar contaminated with sublethal doses of neonicotinoids. In 2012, the study from Henry *et al.* showed that honey bees exposed to acute sublethal doses of the neonicotinoid thiamethoxam failed to return to their hive after being released at an unknown location. In 2014, Fischer *et al.* showed that thiacloprid, a neonicotinoid considered less toxic than others in its family due to its high LD50 (Iwasa *et al.*, 2004; Pisa *et al.*, 2015), had even more drastic effects on the navigation of honey bees than the other neonicotinoids tested. The use of the harmonic radar technology allow us to see the homing trajectories of control and treated bees exposed chronically to sublethal doses of thiacloprid in the field and thus understand better the reason of their homing failure. Negative effects of acute intoxications with other neonicotinoids on foraging behavior were revealed in field studies (Yang *et al.*, 2008; Schneider *et al.*, 2012). Also, in 2012, Eiri and Nieh found that sublethal doses of imidacloprid affect honey bee sucrose responsiveness and decrease waggle dancing. Chronic and sublethal exposure to neonicotinoids is the most likely exposure scenario in the field (Simon-Delso *et al.*, 2014; Krupke *et al.*, 2012) but no field study to our knowledge has yet studied the effects of a chronic exposure to field-realistic concentrations of neonicotinoids or pesticides in general on honey bees' foraging behavior, navigation, and recruitment abilities via dance communication. By studying the effects of a chronic exposure to sublethal concentrations of thiacloprid and clothianidin on such large range of behaviors in field conditions, the studies presented in the first and third manuscripts are aiming to fill these gaps of knowledge.

In the second and third manuscripts, we investigated the effects of thiacloprid and clothianidin on honey bees sucrose responsiveness, learning, and memory abilities.
Sugar is one of the most important stimuli for honey bees, because it is their main source of carbohydrates, resulting in foraging and recruitment behavioral responses. In addition, it is a reinforcement stimulus for instrumental and operant associative learning (Scheiner *et al.*, 2004). When stimulating the gustatory receptors set on the tarsae, antennae, or mouth parts with nectar or sucrose solution, honey bees show a Proboscis Extension Response (PER), leading to the uptake of nectar and the memorization of the floral odours (Decourtye *et al.*, 2004). Each bee has an individual threshold, a specific sucrose concentration for which it will show a PER. With laboratory and semi-field experiments, we investigated whether the sucrose responsiveness of bees exposed to sublethal doses of thiacloprid, Calypso® (a thiacloprid-based formulation) or clothianidin was impaired.

Associative learning and memory formation in honey bees can be studied in the laboratory with a robust paradigm: the PER conditioning (Takeda, 1961; Bitterman *et al.*, 1983; Felsenberg *et al.*, 2011). Harnessed bees learn to associate a conditioned stimulus (CS) with a rewarding unconditioned stimulus (US) presented a few seconds after the CS. In the olfactory

conditioning, the odour represents the CS and sucrose the US. During conditioning, the initially neutral CS becomes associated with the US and subsequently elicits a response (conditioned response), previously elicited only by the US. After conditioning, bees respond with proboscis extension to the CS which are not followed by the US. The neural bases for olfactory learning and memory processes are distributed across several areas such as antennal lobes and mushroom bodies in the honey bee brain. Efficient learning and memory plays an important role in many aspects of honey bee behavior, including the recognition of nestmates, the ability to locate and relocate floral resources and their quality, efficiently collect nectar and pollen, and navigate accurately when foraging in the field (Menzel and Müller, 1996; Menzel *et al.*, 2006; Dukas *et al.*, 2008). Honey bees have sophisticated sensory systems, including well-developed learning and memory capacities. Any disruption in olfactory learning and memory may result in a negative impact on their natural behavior (Raine and Chittka, 2008; Farooqui, 2013). Using the PER conditioning paradigm we studied the effects of thiacloprid, the formulation Calypso®, and clothianidin on the learning and memory performances of honey bees. By acutely intoxicating bees with different sublethal doses at different timings we studied the effects of these substances on the acquisition function and on the different phases of the memory.

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Chapter 1

Honey bees' behavior is impaired by chronic exposure to the neonicotinoid thiacloprid in the field

Léa Tison[1*], Marie-Luise Hahn[1], Sophie Holtz[1], Alexander Rößner[1], Uwe Greggers[1], Gabriela Bischoff[2] and Randolf Menzel[1].

[1] Free University Berlin, Institute for Biology-Neurobiology, D-14195 Berlin, Germany;
[2]Julius Kühn-Institut, Institute for Bee Protection, D-14195 Berlin, Germany.

Corresponding author: E-mail: lea.tison@gmail.com.

Author's contribution: L.T, M-L.H, U.G and R.M performed the field experiments. U.G performed and analyzed the electric fields recordings. L.T, S.H and A.R designed and performed the choice experiments and interpreted the results. G.B and L.T did the pesticide residues analysis and interpreted the results. L.T and M-L.H analyzed the foraging and navigation data, L.T and R.M designed the study, interpreted the results and wrote the manuscript.

Abstract

The decline of pollinators worldwide is of growing concern and has been related to the use of plant protecting chemicals. Most studies have focused on three neonicotinoid insecticides, clothianidin, imidacloprid and thiamethoxam, currently subject to a moratorium in the EU. Here we focus on thiacloprid, a widely used cyano-substituted neonicotinoid thought to be less toxic to honey bees and of which use has increased in the last years. Honey bees *(Apis mellifera carnica)* were exposed chronically to thiacloprid in the field for several weeks at a sublethal concentration. Foraging behavior, homing success, navigation performance, and social communication were impaired, and thiacloprid residue levels increased both in the foragers and the nest mates over time. The effects observed in the field were not due to a repellent taste of the substance. For the first time, we present the necessary data for the risk evaluation of thiacloprid taken up chronically by honey bees in field conditions.

Introduction

Bees, including honey bees, bumble bees and solitary bees represent the most prominent group of pollinators worldwide and contribute largely to agriculture as 35 % of the food crop production depends on them (Klein *et al.*, 2007). The recent loss of pollinator populations can be attributed to multiple factors such as habitat loss and fragmentation, colony management, bee pests and parasites, and additional environmental and anthropogenic elements. Doubtlessly the use of pesticides for crop protection contributes to the loss of pollinator abundance both at the species level and the quantity of a particular species (Brittain and Potts, 2011; Rundlöf *et al.*, 2015; Whitehorn *et al.*, 2012). It has also become evident that neonicotinoids (and other insecticides like fipronil) play a crucial role as the promoters of pathogen and parasite infections that effectively drive colony losses (Brandt *et al.*, 2016; Christen *et al.*, 2016; Sanchez-Bayo and Desneux, 2015). Thanks to their systemic properties, neonicotinoids are present in the pollen and nectar and will thus be continuously collected by pollinators for as long as flowering persists. They are agonists of nicotinic acetylcholine receptors (nAChR) which are normally activated by the neurotransmitter acetylcholine (Liu *et al.*, 2006). Nicotinic synaptic transmission is a major component of neural integration in the circuits related to sensory integration and functions related to the mushroom bodies, mediating multisensory integration, learning, and memory formation (Heisenberg, 2003; Menzel, 2012). Neonicotinoids negatively affect the mushroom bodies' physiology (Peng and Yang, 2016) and function (Palmer *et al.*, 2013) in honey bees. It was already proven that some neonicotinoids compromise olfactory learning (Williamson and Wright, 2013) as well as the ability of worker bees to forage and to communicate (Desneux *et al.*, 2007; Eiri and Nieh, 2012; Henry *et al.*, 2012; Schneider *et al.*, 2012). The toxicity of sublethal doses is also expected to be reinforced over time (Tennekes, 2010; Tennekes and Sanchez-Bayo, 2011). However, a detailed analysis of the chronic exposure to thiacloprid on foraging, navigation, and social communication is lacking.

The cyano-substituted neonicotinoid thiacloprid is declared less toxic to bees than nitro-substituted compounds like imidacloprid and thiamethoxam (EFSA, 2012a; EFSA, 2012b; Iwasa *et al.*, 2004; Pisa *et al.*, 2015). The formulations based on thiacloprid are registered and sold in more than 70 countries worldwide (FAO) and act against sucking and chewing pest insects of more than 50 crops (Elbert *et al.*, 2008; Simon-Delso *et al.*, 2014). The formulations based on thiacloprid are used in the field for spraying treatment at application rates much higher than for the 3 neonicotinoids suspended in Europe (EFSA, 2012b; Poquet *et al.*, 2014). These formulations are allowed to be sprayed during flowering because less damage to pollinators is expected. Thiacloprid is also used in a maize seed treatment since the withdrawal of clothianidin and thiamethoxam on maize across Europe in 2013.

Toxicity tests performed by the company at the time before releasing thiacloprid on the market evaluated only the short term and lethal effects on worker honey bees. In contrast to acute effects, no standardized protocol exists for measuring chronic effects on individual bees under semi natural conditions (van der Sluijs *et al.*, 2013). The value of tests on single animals has been questioned because a whole colony may be more robust to pesticide exposure (Henry *et al.*, 2015). However, honey bees are acting as single animals during foraging; they need to adjust their behavior to the changing availability of food sources, return to the colony for survival, deliver the collected food and communicate with other foragers. Therefore, testing single foraging honey bees represents best conditions faced by honey bee foragers and other insect pollinators in nature. A few lab studies have shown that chronic exposure to sublethal doses of thiacloprid affects honey bees' sensitivity to the gut pathogen *Nosema cerenae* (Doublet *et al.*, 2014; Retschnig *et al.*, 2014; Vidau *et al.*, 2011) and a field study has shown that navigation is compromised when thiacloprid was given as a single acute dose (Fischer *et al.*, 2014). Chronic and sublethal exposure to the substance is the most likely exposure scenario in the field (Simon-Delso *et al.*, 2014; Krupke *et al.*, 2012) but no field study to our knowledge has yet uncovered any specific behavioral effect of such condition of exposure. In our experiments honey bee foragers were exposed chronically for several weeks in the field to a concentration similar or lower to those used in previous chronic exposure studies with thiacloprid (Doublet *et al.*, 2014; Retschnig *et al.*, 2014; Vidau *et al.*, 2011). The concentration of thiacloprid in the contaminated sucrose solutions was 5.4 ng μl^{-1} whereas the concentration of thiacloprid in the formulation Calypso® directly sprayed on plants and flowers at a distance of 30 to 40 cm is 150 ng/µl.

Since most of the collected sucrose solution will be deposited by the forager inside the hive, and only a small proportion will be taken up and metabolized by the bee during its return flight from the feeder to the hive, only a small amount of thiacloprid will reach the brain and interfere with nicotinic synaptic transmission.

We found that a chronic exposure to thiacloprid significantly impaired honey bees' foraging behavior, communication, and navigation. The substance increased in the foragers over time affecting also the animals indirectly exposed in the colony. We found no avoidance of or preference to the substance, supporting the idea that a neural impairment was responsible for affecting the honey bees' abilities to forage, communicate, and navigate rather than a repelling effect.

Material and methods

Preparation of the solutions

Stock solution: 10 mg thiacloprid ([3-[(6-chloro-3-pyridinyl) methyl]-2-thiazolidinylidene] cyanamide, Sigma-Aldrich Pestanal) diluted in 1 mL acetone (≥99.9 %, Sigma-Aldrich) plus 39 mL distilled water leading to a concentration of 0.25 g L^{-1}. Acetone was chosen as the solvent following the EPPO guidelines (EPPO, 1992). The final concentration of acetone (0.05 %) in the contaminated sucrose solutions was shown to not have an effect on honey bee navigation (Fischer *et al.*, 2014). The thiacloprid sucrose solutions used in the field (0.02 mM, 4.5 ppm) as well as for the taste and choice experiments (0.025 mM, 5 ppm) were freshly made every morning from the stock solution. The concentration of thiacloprid at the treated feeder was always the same regardless of the sucrose solution concentration. The concentration of the solutions used were confirmed by LC-MS/MS (Methods S1).

Field experimental design

The experimental area is a highly structured agricultural landscape (trees and bushes, pathways, creek, grass fields, etc) nearby Großseelheim, Germany. Two colonies housed in two observation hives (W.Seip, Bienenzuchtgerätefabrik) were put up on two opposite sides of a cabin at the western border of the experimental area (50°48'51.9"N). Each colony of *Apis mellifera carnica* was equipped with one comb of sealed brood plus newborn bees and one comb of food (Deutsch Normal Mass combs) originating from the same honey bee colony. The queens were kindly provided by the Bieneninstitut Kirchhain, they derived from selected breeder colonies of the carnica breeding population of the institute. They were open mated and aged 1 year old. Sister queens were used in an attempt to keep the genetic difference among the honey bee individuals from each colony at a low level.

Training to the feeders

Two feeders (F1 and F2) were established 350 meters northeast and 340 southeast respectively and were separated by an angle of 90° as seen from the cabin. The release site (RS) was located 780 meters east of the cabin. A group of foragers from each of the two colonies was trained to its respective feeder and marked individually with number tags. The origin of each newly marked bee from the two colonies was controlled at the respective hive entrance. In Experiment 1, one group of bees (treated group) foraged during 19 days on a sucrose solution containing thiacloprid (4.5 ppm), and the other group (control group) foraged over the same time at a feeder containing only sucrose solution. In Experiment 2, the control hive became the treated hive and the treated hive was removed and replaced by a new control hive. The feeders´ locations were exchanged between Experiment 1 and 2 in order to exclude any possible landscape effect related to the feeders' position. In Experiment 2, the two groups

of foragers were feeding at their respective feeder during 29 days. Each feeder was placed in a little wooden box to allow counting the entrances and exits of foragers with a retro-reflective sensor (Baumer GmbH). The total number and the identity of bees visiting their feeder throughout each day was known as well as the amount of sucrose solution consumed at both feeders. The concentration of the sucrose solution at each feeder was adjusted during the day in order to regulate the traffic at the feeder (25 - 40 bees) following evaluation by the experimenter of the number of trained foragers visiting the feeder. Dance recruitment was induced 19 times on 19 different days (time: 1500 - 1700 hours) by first halving the sucrose concentration at both feeders for one hour and then increasing it twofold for another hour.

Homing experiment

Colonies were settled in the field for at least a week before the homing experiments started. After a certain number of days foraging at the feeders, single bees were caught on their departure at their respective feeder and transferred into a glass vial after they had freely drunk either a 1 M sucrose solution (control bees) or a 1 M sucrose solution containing 4.5 ppm thiacloprid (treated bees). They were kept in the dark for 45 min while they were transported to the release site. Then a transponder was fixed to thorax and the bee was released (time: 1100 - 1700 hours, temperature: 17-30°C, wind < 15 km h^{-1}). No release was made when the sky was evaluated too cloudy or totally overcast, nor when it was raining. Care was taken that the number of control and treated bees released every day were evenly distributed and it was ensured that each bee was released only once. The radar was shut down not before 120 min after the last bee was released if the bee was not yet back to its hive. Since none of the bees that did not return to the hive after being released was seen at the feeder or at the hive entrance on the same or the following days, we assume that they died in the field. The method used for tracking bees with a harmonic radar system has been described before (Menzel *et al.*, 2011; Riley *et al.*, 1996; Scheiner *et al.*, 2013). The transponders were built by ourselves following the procedure from Riley *et al.* (1996), their attachment and carrying by the bees do not alter honey bees' flight behavior (Degen *et al.*, 2015; Capaldi *et al.*, 2000). The flights of the released bees carrying a transponder were monitored using the radar system over a distance of up to 900 meters radius and at a temporal resolution of 1/3 Hertz[37].

Electric field recordings

The electric fields emitted by dancing bees (Greggers *et al.*, 2013) consist of low-frequency (movements of the abdomen, 16 Hz on average) and high-frequency (buzzing of the wings, 230 Hz) components synchronization, leading to an average of three to seven electric pulses per waggle. The distance from the hive to a feeding site is encoded in the number of waggle runs and 1 sec is known to represent a distance of about 1 km (von Frisch, 1967). The feeders were located 350 meters northeast (F1) and 340 southeast (F2) of the hives and since very few natural food sources existed in the experimental area and none of

them were present at the same distance as the feeders, the distinction between dances from trained and untrained foraging bees was possible. Electric field measurements were performed at the same time on both sides of the lower comb in the control and treated hives using 4 copper wires with a silver coating positioned in the dance area (12 cm^2 covered), connected on each side to a stereo amplifier (USB - Soundbox 7.1, Conrad electronics SE) with a sample rate of 44.1 KHz. Each amplifier was connected to a laptop and the software Presonus Studio One (version 2.4) was used for saving the data as wave files. We recorded in total 340 hours of electric fields on 32 different days (average of 2.67 hours per day).

Thiacloprid residues analysis

Bees were caught at their feeder after foraging for a certain number of days and after they had filled their crop with a 1 M sucrose solution contaminated or not. They were then kept in the dark for 45 minutes before being killed by chilling and put into a -20° C deep-freezer. We also collected unmarked forager bees at the entrance of the treated and control hives when flying out on a foraging trip in order to assess the in-hive contamination of foragers not visiting the feeders but exposed indirectly to thiacloprid inside the hive via the stored food.

Collected bees were cut into 3 parts: head, thorax, and abdomen and ten samples from the same groups of bees were pooled and weighed. The samples were homogenized with a disperser and centrifuged (10 minutes at 3000 U min^{-1}). Then 4 ml of the extract were removed and dried in a metal block thermostat with a nitrogen blow device. 950 µl of a water-methanol mixture (1:1, v/v) and 50 µl of an internal standard solution containing imidacloprid D4 (50 pg $µl^{-1}$) were added and the residual extract dissolved using an ultrasonic liquid mixer and put into a freezer (-18°C) over night. On the next day, the samples were filtered cold (syringe filter 0.2 µm) and further diluted in the case of high concentrations before proceeding with the identification and quantification of thiacloprid by LC-MS/MS. The LC-MS/MS system used was a UltiMate® 3000 RS HPLC (Dionex Corporation, Sunnyvale, USA) coupled to an hybrid stage quadrupole/linear ion trap mass spectrometer Q TRAP® 5500 (AB SCIEX, Framingham, USA) equipped with an electrospray ionization (ESI) source. Thiacloprid was identified by its retention time and two Multiple Reaction Monitoring (MRM) transitions. The residues in the samples were measured using matrix standards (concentrations: 0.5, 1, 5, 10, 25, 50 pg $µl^{-1}$). The value given for each sample represents the average of double-injections. The method was validated on the basis of the recoveries obtained for fortified samples. Control samples of bee body parts were fortified with thiacloprid at the fortification level of 0.010 mg kg^{-1}. For quantification (internal standard method) and the estimation of recoveries, the limit of detection (LOD) and quantification (LOQ) control blank matrix standards were prepared with the concentration levels: 0.05, 0.1, 0.5, 1, 5, 10, 25, 50 and 100 pg $µl^{-1}$ in methanol/water (1/1, v/v). See Table S3 for the recovery, the limit of detection and quantification.

Frozen samples of contaminated sucrose solutions were sampled and analyzed by LC-MS/MS. Limit of detection (LOD) = 0.05 pg $\mu l^{-1}$and limit of quantification (LOQ) = 0.1 pg μl^{-1}.

Repellent effect

PER experiment

The Proboscis Extension Response (PER) was used to sample hungry bees' sensitivity to varying concentrations of sucrose (Bitterman *et al.*, 1983; Page *et al.*, 1998) containing or not thiacloprid (5 ppm). Honey bees were captured at 1400 hours when leaving the hive, immobilized by chilling, and mounted in small brass tubes which restrained body movements but allowed the antennae and the mouthparts to move freely (Bitterman *et al.*, 1983). One hour later they were tested in the laboratory by touching both antennae with a droplet of ascending concentrations of sugar concentrations (dry sugar diluted in tap water + 0.05 % acetone, 0.1 %, 0.3 %, 1 %, 3 %, 10 %, 30 % and 50 %, w/v). Only the bees which showed a PER for the 50 % sugar concentration were considered as the non-responders (control: 1/74, treated: 3/74) were considered physically unable to extend their proboscis.

Choice experiment

In May, a group of bees was trained to a training/feeding platform located about 30 meters from the hive. The platform was composed of a yellow background and 10 blue squares randomly distributed and containing a mini-feeder from which the bees could freely drink a 1 M sucrose solution. The test platform contained only 6 mini-feeders. During testing of single bees three feeders contained 8 µl of a 1 M control sucrose solution each and the other three 8 µl of a 1 M sucrose solution with thiacloprid (5 ppm) each. The positions of the control and treated mini-feeders were randomly allocated on the platform. The number of feeders drunk and the time a bee took to drink at each of the 6 feeders was recorded. At the end of the test the bee was killed and the same test was repeated with a new naive bee.

Flight tracks and statistical analysis

From the x/y coordinates collected by the radar, the length and duration of the flight from the first to the last signal was calculated. The x/y-coordinates were fitted into a google map scaled in meters using CorelDraw.X5. The criteria used to categorize the different flight parameters were arbitrarily defined. A "vector flight" was considered as such when fitting into an angle of 45° as seen from the release site (± 22.5° each side of the feeder-hive vector direction, F1: 313°, F2: 222°) and had a minimal length of 200 m. The angle of a vector component is the angle between the crossing point of the vector track with the 200 m circle around the release and the direction towards north. The criterion "pass close to F" and "Return to RS" was attributed respectively to bees getting closer than 100 m from their feeder or from the release site during their flight.

The electric field data were transformed to SMR files, preliminary filtered in Spike 2 (version 8.03) and further analyzed using custom-made programs written in Visual Basic 2013 (Microsoft). An amount of 6 ± 2 waggles per run (about 360 ± 120 meters) was used as a criteria to select the dances indicating the location of the feeders. If the number of waggles per run was exceeding this range, the waggle runs were attributed to the “other bees” group.

For the statistical analysis of the data, we used R and Prism 5 and 6. The normality of the data was tested using the D'Agostino-Pearson omnibus test. If the data were normally distributed, we used a paired/unpaired t.test or an analysis of variances with Tukey’s post-hoc tests. Otherwise non-parametric tests were performed (Mann-Whitney test, Wilcoxon signed rank test). The Fischer’s Exact Test was used to compare proportions. . For the PER data we performed a mixed effects logistic regression in R (lme4 package) with “Bee” and “Date” as random effects to account for the difference between individuals and the date. This was followed by Overall Likelihood Ratio Tests and Tukey’s post-hoc tests (multcomb package). The Wheeler-Watson test was used to calculate the angular distribution of the vector components. The survival analysis was conducted using censored Kaplan Meier Log-Rank in R and the influence of multiple variables was investigated using a Cox-regression model. The numbers of bees tested for each experiment and test groups are indicated in the legends of the figures and in the text.

Results

Honey bees' foraging behavior and dance communication are compromised

The total foraging span of honey bees foraging at the control feeder was significantly longer than the foraging span of honey bees foraging at the treated feeder (Table 1, Kruskal-Wallis, $P < 0.0001$). Control bees foraged at their feeder on average 0.78 days longer than treated bees ("Total", Table 1). The significance was different between the groups according to the Experiment.

Table 1: Foraging span in days of the trained honey bees at the control or treated feeder

	Experiment 1	Experiment 2	Total [§]
Control	5.21 ± 0.32 *(n = 67)* [*] [a]	4.19 ± 0.24 *(n = 72)* [a]	4.68 ± 0.20 *(n = 139)*
Treated	4.7 ± 0.22 *(n = 79)* [a]	3.34 ± 0.14 *(n = 111)* [*] [b]	3.91 ± 0.13 *(n = 190)*

Numbers shown are means (days foraging) ± s.e.m.

[§] Mann-Whitney, $P < 0.01$

[*] The control group in Exp. 1 and the treated group in Exp. 2 correspond to the same colony, as the control colony in Exp. 1 became the treated colony in Exp. 2 and continued to forage at the same feeder (F1).

Different letters indicate significant differences (post-hoc tests with Bonferroni correction): a-b (Exp.2), $P < 0.05$, a-b (Treated), $P < 0.001$, a-b (F1), $P < 0.001$.

Sucrose consumption at the control and treated feeder was significantly different in both experiments (Paired t-test, $P < 0.0001$). Control bees consumed 1.7 times more sugar solution per day than treated bees (Table S1). The average amount of thiacloprid collected per bee and per day at the treated feeder was estimated at 12118 ± 900 ng in Experiment 1 and 10990 ± 833 ng in Experiment 2 (Table S1). Treated bees performed on average 1.8 times and 1.4 times less foraging trips per day than control bees in Experiment 1 and 2 respectively. On one trip, we estimate that a bee collected on average 216 ng of thiacloprid (40 µl of solution). The total amount of thiacloprid metabolized by a bee per day during the return flights to the hive ranges between 141 and 212 ng (Table S1). This calculation is based on the data related by Rortais *et al.* (2005) that a bee needs 8 - 12 mg of sugar per hour to fly (Rortais *et al.*, 2005; Balderrama *et al.*, 1992) and on our measurements (treated bees collected on average 1 M sucrose solution and flew on average 2 minutes from the feeder to the hive).

The reduced sugar consumption is linked to a reduced visitation rates of foragers at the contaminated feeder. Indeed, treated bees visited their feeder significantly less frequently than the control bees and higher sucrose concentrations were needed at the contaminated feeder in order to keep the bees visiting the feeder (Fig. 1A). The median sucrose concentration used for regular foraging was 0.5 M at the control feeder and 1 M at the treated feeder. Recruitment

of foragers via the waggle dance was induced by raising the sucrose concentration at the feeder (von Frisch, 1967). First the sucrose concentration at both feeders was reduced to halve of the current concentration for one hour, then it was increased twofold for another hour. Sucrose concentrations as high as 2 M during dance induction did not significantly increase the traffic at the treated feeder (ANOVA, $F_{3,72}$ = 14.01, P < 0.0001), whereas a median concentration of 1 M increased significantly the number of visits at the control feeder (p < 0.05, Fig. 1B).

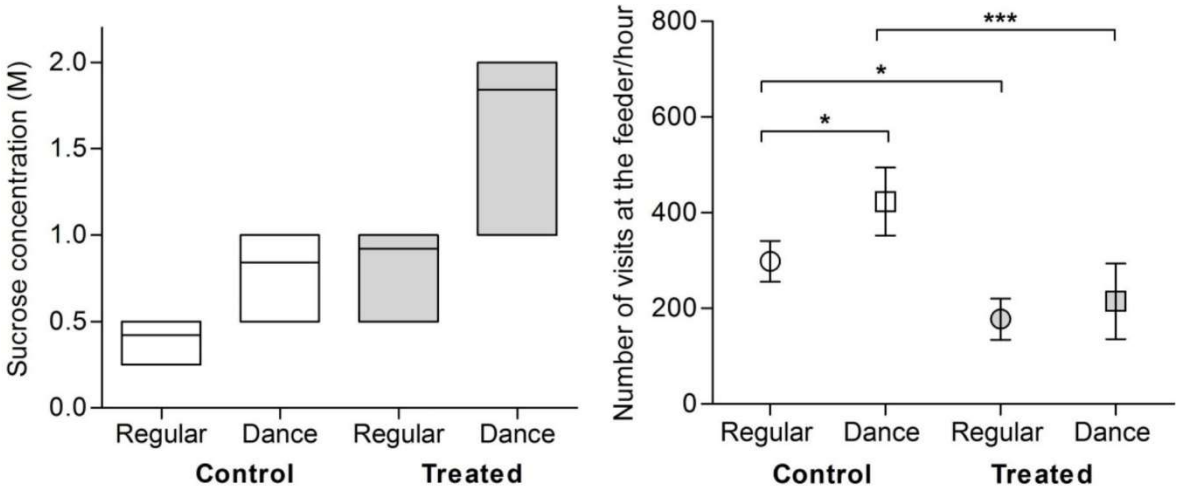

Figure 1. Required sucrose concentrations and foraging activity at the control and treated feeders. A. Sucrose concentrations used in order to keep a similar number of foragers coming regularly to the control and treated feeders and to induce dances. Lower sucrose concentrations were required for control bees than for treated bees. **B**. Mean (± 95 % confidence limits) number of visits per hour recorded on the same days (n = 19) at both feeders during regular foraging (circles) and during dance induction (squares). The foraging behavior of the treated bees (filled marks) as well as their ability to recruit new untrained foragers are significantly reduced (ANOVA, $F_{3,72}$ = 14.01, P < 0.0001 and Tukey post-hoc tests). *P < 0.05, **P < 0.01, *** P < 0.001.

Reduced recruitment at the feeder could indicate less waggle dances or compromised dance performance. Therefore, we monitored and estimated the number of waggle runs performed by the dancing bees in both colonies, taking advantage of the fact that waggle dances can be measured by the temporal modulation of the electrostatic field emanating from the dancing bee (Greggers *et al.*, 2013). The number of waggles performed by the bees trained to the control feeder was significantly higher than those of the bees trained to the contaminated feeder (Fig. 2, Wilcoxon signed rank test, P < 0.0001) although the sucrose concentration during dance induction was higher at the contaminated feeder (Fig. 1A). Indeed, honey bees foraging at the control feeder performed on average 3.2 times more waggles per hour than

honey bees foraging at the treated feeder. The reduced dance activity of treated bees explains the lower foraging activity at the contaminated feeder.

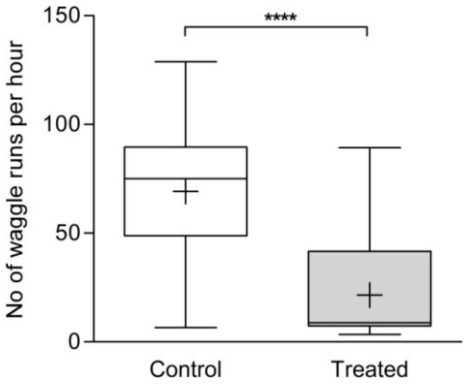

Figure 2. Number of waggles runs performed by the trained bees from the control and treated feeders. The number of waggles runs per hour was obtained from electrostatic field recordings performed on the same days in both hives (n days = 32). The mean number of waggles runs per hour is represented with a cross in the box-plots, it was found significantly higher for the bees foraging at the control feeder than for the bees foraging at the contaminated feeder (Wilcoxon signed rank test, $p < 0.0001$).

We also differentiated dances for feeders and dances to unknown natural food sources on the basis of the number of waggle runs as indicators of distance to the respective food source (Greggers *et al.*, 2013; von Frisch, 1967). We found significantly lower dance activity advertising for natural food sources in the treated colony (Fig. S1) indicating that the accumulation of thiacloprid inside the colony also affected bees that did not forage at the contaminated feeder but were on contaminated stored food.

No repellent effect of thiacloprid.

One explanation for lower foraging activity found in treated bees could be an aversive taste of the substance in contaminated sucrose solution. In the laboratory experiment, we tested the proboscis extension response (PER) of hungry foragers to water and 7 different sucrose concentrations (0.1 %, 0.3 %, 1 %, 3 %, 10 %, 30 % and 50 % w/v) containing thiacloprid (5 ppm) or not (Fig. 3). No difference was found in the PER of bees stimulated either with the control sucrose solutions or the contaminated sucrose solutions (logistic regression with random effects “Bee” and “Date”, Sugar concentration x Treatment: $\chi_6^2 = 2.5224$, $P= 0.866$). The results of the Tukey’s post-hoc tests between the control and treated groups for each of the different sucrose concentrations tested can be found in Table S2.

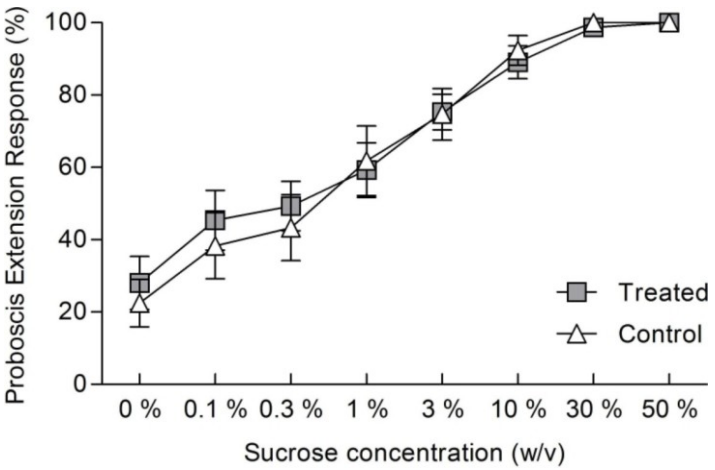

Figure 3: Proboscis Extension Response (PER) to different sucrose concentrations containing 5 ppm thiacloprid (treated) or not (control). N control = 73. N treated = 71. No difference was found between the two groups (logistic regression with random effects, Sugar conc x Treatment: $\chi_6^2 = 2.5224$, P= 0.866).

In the free flight experiment, 45 bees had to choose between feeders containing a 1 M sucrose solution with or without thiacloprid (5 ppm). No significant difference was found in the visitation rate of the bees to the control (64 %) and contaminated (65 %) feeders (n=135 feeders, Fischer Exact test, P = 0.8989). The average (± s.e.m.) drinking time per bee and feeder was 6.88 ± 0.27 sec at the control feeders, and 7.37 ± 0.36 sec at the contaminated feeders (no significant difference, Mann Whitney, P = 0.5578). These results rule out the possibility that thiacloprid has a repellent taste for honey bees.

Thiacloprid residue levels increase in foragers.

The amount of thiacloprid in bees foraging at the contaminated feeders in Experiment 1 and 2 was analyzed by LC-MS/MS. Fig. 4 shows how it accumulated in different body parts over time. The amount of thiacloprid residues found in bees can be seen as the status of intoxication at the moment a bee is released with a transponder after foraging chronically during 2, 3 or 4 days at the contaminated feeder.

The length of exposure of the foragers at the contaminated feeder as well as the amount of thiacloprid collected is related to the amount of residues found in the bees (Fig. 4, Table S3). The more foraging trips honey bees performed to the treated feeder in a certain number of days, the higher was the cumulated amount of contaminated sucrose solution collected and the higher was the amount of thiacloprid residue found in the bees. Only a fraction of the cumulated total amount of thiacloprid collected by the bees at the feeder will be metabolized and most of this uptake will happen during their return flights from the feeder to the hive. This

fraction was found very close to the amount of thiacloprid residues found in bees after a defined number of days foraging at the contaminated feeder (Table S3).

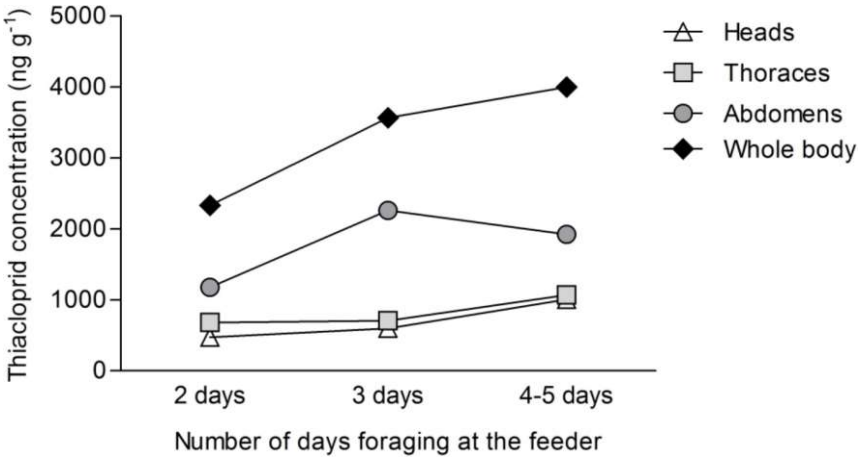

Figure 4. Accumulation of thiacloprid residue in heads, thoraces, abdomens and in the whole body (representing the sum of the measurements) of honey bees foraging at the contaminated feeder over time. Honey bee foragers were collected at the end of 2, 3 or 4 days of foraging after they had filled their crop at the feeder containing thiacloprid (4.5 ppm). 10 bees per foraging group.

In-hive contamination was assessed by collecting unmarked forager bees at the entrance of the treated hive when flying out on foraging trip. Thiacloprid was found in these bees but at much lower amounts than in the foragers trained to the contaminated feeder (Table S3). Indeed, these foragers did not visit the contaminated feeder but they were exposed to thiacloprid inside the hive via the food collected and stored by the foragers visiting the contaminated feeder. Since their waggle dance activity was significantly reduced (Fig. S1) even these low levels of thiacloprid impaired social communication.

Honey bees' homing success and navigation performance are impaired.

Navigation requires the integration of multisensory cues and the retrieval of appropriate memory about the landscape structure. We tested navigation abilities of the bees trained to feeder 1 and 2 during the Experiments 1 and 2. We found that treated bees returned to their hive at a significantly lower proportion than control bees (Fig. 5, homing success: control 91.76 %, treated 76 %, Fischer Exact Test, $P < 0.01$). Based on the crop-emptying measurements by Fournier *et al.* (2014) we calculated that the foragers released with a transponder could have assimilated in 45 min up to 7 µl and thus 38 ng thiacloprid in addition of the residues already assimilated over *n* days foraging at the feeder. This value is a higher estimate because the amount of assimilated sucrose during the 45 minute waiting time may well be much lower depending on the activity of the waiting bee (Rothe, 1989). In any case the partial acute treatment component involved in the navigation experiments adds to the chronic effect.

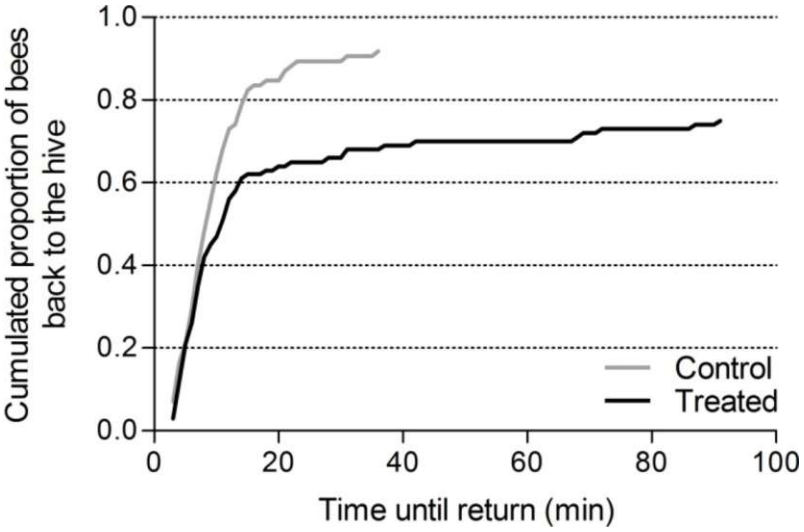

Figure 5. Probability of homing success as a function of time until return. Treated honey bees returned to their hive at a significantly lower proportion than control bees ($n_{treated}$ = 100, 76 % return; $n_{control}$ = 85, 91.76 % return, Fischer Exact Test, P < 0.01). The origin of the temporal axis represents the moment of release.

A survival analysis was conducted on the data and a significant influence of thiacloprid on honey bee homing success was found (Kaplan Meier Log Rank test, $\chi_1^2 = 12.9$, P < 0.001). For the survival analysis, a flight duration of 120 min was settled for bees that flew out of the radar range and did not come back within the radar range or to the hive during this time. The flight duration of all other bees was the flight time in minutes from the release site to the hive or from the release site to a point inside of the radar range where the signal was lost. The influence of multiple variables was tested in a cox-regression model (Table 2). The variable "Treatment" shows a significant negative effect on honey bee survival. The hazard rate of the treated bees, representing the likelihood of returning to the hive, is almost half the hazard rate of the control bees. The period during which the experiment was performed ("Experiment"), the number of days a bee foraged at its feeder before being released ("Time foraging"), as well as the number of days from the first day of the experiment until a bee was released ("Time exposure") had no significant effect on honey bee homing abilities. The duration of the exposure had no effect possibly because 45 % of the treated bees individually released foraged at the contaminated feeder for less than 3 days. The temperature at the release time did not seem to play a role in the ability of honey bees to come back to their hive. At their release, 76.5 % of the control honey bees and 61 % of the treated honey bees waited for a short time at the release site before starting to fly. This waiting time ("Time before flying") was not different between the control and the treated bees (mean ± s.e.m control = 3.17 ± 0.33 min,

treated = 4.53 ± 0.69 min, Mann Whitney, P = 0.5067) and had no influence on the homing success (Table 2).

Table 2: Summary of the Cox regression model

Variables	Model 1				Model 2			
	Regr. coef	exp (coef) *	Z	P	Regr. coef	exp (coef) *	Z	P
Treatment	-0.5772	0.5614	-3.408	**0.000656**	-0.5866	0.5562	-3.505	**0.000456**
Experiment	-0.3728	0.6887	-1.563	0.117983	-0.2864	0.7510	-1.745	0.080899
Time foraging ǂ	-0.0351	0.9654	-0.674	0.500248				
Time exposure §	-0.0136	0.9864	-0.838	0.402182				
Temperature	-0.0079	0.9921	-0.238	0.811991				
Time before flying	0.0173	1.0174	1.133	0.257266				
	Rsquare: 0.091 (max possible= 0.999), Likelihood Ratio Test: 17.71 on 6 df, P=0.007007				*Rsquare: 0.08 (max possible= 0.999), Likelihood Ratio Test: 15.52 on 2 df, P=0.0004268*			

A backward selection on the AIC was performed on Model 1 in order to obtain Model 2

Values in bold indicate significant differences

*exp (coef) = Hazard ratio

ǂ **Time foraging** is the time in days during which a bee is foraging at its feeder before being released

§ **Time exposure** is the time in days from the first day of the experiment until the day the bee is released

During the flight, 9 pauses were recorded in the control group and 24 in the treated group with a maximum of 3 pauses per bee (Table S5). The probability of making a pause during the return flight to the hive was not found significantly different between the control (13 %) and treated groups (24 %, Fischer Exact test, P = 0.0617). However, the mean (± s.e.m.) pause duration was higher for the treated bees (20.13 ± 5.28 min) than for the control bees (5.29 ± 2.12) but not significantly different between the two groups (Mann Whitney, P = 0.0974) possibly because of the limited number of cases and the large variance. The duration of the pause was deleted from the total flight duration in order to calculate an accurate flight speed (Tables S4 and S5). The total flight duration including pauses was however considered for every other analysis. If we take out the duration of the pauses from the total flight duration of the concerned bees and run the survival analysis again, the variable “Treatment” remains significant (Kaplan Meier Log Rank test, χ_1^2 = 8.8, P < 0.01; cox regression Model 1: P = 0.00435) and none of the other variables tested before become significant.

Among the bees returning to their respective hives, no significant difference was found between the flight duration of control and treated bees (Table S4, median control = 7.8 min,

treated = 7.4 min, Mann Whitney, P = 0.5741), and no significant difference was found in the distance flown (Median control = 2032 m, treated = 1908 m, Mann Whitney, P = 0.4778). However, the treated bees flew significantly slower than the control bees (Table S4, mean ± s.e.m., speed treated = 4.32 ± 0.13 m s^{-1}, control = 4.78 ± 0.15 m s^{-1}, Unpaired t-test, P < 0. 05).

In a catch and release situation like in the test performed here, bees usually fly first along a vector they would have taken if they were departing from the feeder in direction to the hive (vector flight) (Menzel *et al.*, 2005). Then they usually search for some time before flying back to the hive rather straightly. The proportion of vector flights performed did not differ between the control (*n* = 55, 71 %) and treated (*n* = 57, 76 %) bees which returned to their hive (Table S5, Fischer Exact test = 0.4703). There was a difference in the duration of the vector component between the control bees in Experiment 1 and 2 (P < 0.05). Also, control bees from Experiment 2 flew the vector component faster than control bees from Experiment 1 and treated bees from Experiment 2 (P < 0.01 and P < 0.05 respectively). Since these bees foraged at different feeding locations the effect indicates a site specific component. Therefore, we compared the parameters of the flights of control and treated bees separately for the two training sites, and found no differences with respect to the duration, length and the spatial distribution of the vector component (Table S5). The homing flight was considered as the flight component from the end of the vector to the hive. No difference was found in the length, duration, or speed of the homing flight between control and treated bees (Table S5). However, we found that more control bees returned less than 100 m from their release site at least once during their search flight (Fisher Exact test, P < 0.05) indicating their ability to remember where they were released and use this location to start over the homing flight. Also, significantly more control bees flew less than 100 meters close to their feeder (Fisher Exact test, P < 0.01) before heading to the hive indicating the use of known landmarks for a successful homing. Indeed, all the bees which passed close to their feeder flew directly back to the hive from the feeder.

The bees which did not return to the hive performed different kinds of flight trajectories before getting lost (Fig. 6). None of the control bees got lost out of the radar range whereas 9 treated bees out of 20 were lost bees in experiment 2 and flew in the opposite direction of the hive, left the radar range and did not return within the range or to the hive. Interestingly, some treated bees (Fig. 6 c) terminated their flights at the end of the vector component. These bees did not initiate search flights or homing flights and did not arrive at the hive.

Figure 6. Flight trajectories of the non-returning bees. Map data provided by: Google Earth and GeoBasis - DE BKG. The figures show the flight trajectories of individual bees, each in a different color within a group (**a, b, c** and **d**). The trained route of the bees released at the release site (RS) is represented with a red line between the hive (H) and the feeders (F1 and F2). In Experiment 1, F1 was the feeder of the control bees and F2 the feeder of the treated bees. In Experiment 2 the situation was reversed (F1: treated bees, F2: control bees). The circle (black dashed line) represents the edge of the radar range (900 m from the radar). Bees leaving the radar range and then returning into it are marked with a black arrow directed to the East (leaving the range) or to the West (returning into the radar range) respectively. A square at the beginning of each flight track marks the first radar signal, and the triangle at the end of the flight marks the last radar signal. See Table S4 for the number of bees lost within each group.

Discussion

Our study documents important sublethal effects of a low concentration (4.5 ppm) of thiacloprid taken up chronically by foraging bees. We found that higher-order functions like navigation according to a learned landscape memory, motivation to forage and to communicate in a social context were compromised.

Honey bees visiting a feeder containing thiacloprid foraged over shorter periods of time probably because they died earlier than the control bees. This result is not surprising, since a 10-day exposure to a sublethal concentration of another neonicotinoid, thiamethoxam, reduced honey bees' life span by 41 % (Oliveira *et al.*, 2013). Exposure to pesticide residues in brood comb was also shown to shorten adult longevity (Wu *et al.*, 2011). Overexpression of the vitellogenin transcript in the honey bee brains could be one of the molecular indicators for the alteration in foraging activity and accelerated aging upon neonicotinoid exposure (Christen *et al.*, 2016). Previous studies also demonstrated a reduced foraging activity of honey bees on sucrose solutions contaminated with thiacloprid (Schmuck *et al.*, 2003), imidacloprid (Eiri and Nieh, 2012; Colin *et al.*, 2004; Yang *et al.*, 2008), or clothianidin (Schneider *et al.*, 2012). These effects could be explained by a prolonged stay inside the hive before returning to the feeder (Schneider *et al.*, 2012). We found that if occurring, a prolonged stay inside the hive was not used for dance communication, as dance activity was highly affected by a chronic uptake of thiacloprid, as already shown with imidacloprid (Eiri and Nieh, 2012).

We tried to compensate for the reduced foraging activity by increasing the sucrose concentration at the contaminated feeder, but the reduced dance activity could not be totally compensated for even though very high sucrose concentrations were applied during the dance induction periods. Thiacloprid increased the minimum sucrose concentration that honey bee foragers are willing to gather at the feeder as was found for imidacloprid (Eiri and Nieh, 2012). Since increasing sucrose concentration could partially compensate for the reduced foraging activity observed at the contaminated feeder, it is most likely that thiacloprid did not alter the sensory or motor components of foraging but rather the motivation to forage. The results on dance performance point in the same direction. Pollination would be disturbed because of a reduced visitation of the flower by bees (van der Sluijs *et al.*, 2013) leading to less flowers pollinated and thus reduced yields for farmers. In addition, honey bee colonies may suffer from a reduced food inflow, making them more susceptible to other disturbances (weather conditions, additional pesticides intoxication, parasites and pathogens).

Several studies reported low toxicity of thiacloprid (EFSA, 2012a). Laurino *et al.* (2011) reported that acute uptake of thiacloprid (144 ppm) appeared to be not dangerous unless the

honey bees were starved. It was thus suggested that thiacloprid acts as a repellent leading to reduced uptake and thus to lower toxicity. Here we disprove this hypothesis, documenting that thiacloprid does not have a repellent effect on honey bees. Furthermore, we show drastic effects on honey bee behavior for a concentration 32 times lower than the one used by Laurino *et al.* The results of our field study, especially the impairment of the foraging behavior and social communication, cannot be related to an avoidance of the substance, corroborating recent findings with other neonicotinoids (Kessler *et al.*, 2015).

The chronic exposure to thiacloprid lead to an accumulation over time in both the honey bee foraging at the contaminated feeder as well as in bees of the same colony via a contamination of the stored food. The estimated amount of thiacloprid metabolized by a foraging honey bee can be estimated by the energy supply necessary to perform the return trips from the feeder to the hive assuming that all energy for the return flight is taken up from the collected sucrose solution. Applying a concentration of 5.4 ng/µl at the feeder, we calculated that a foraging bee collected on average 216 ng of thiacloprid (40 µl of solution) on one trip (80 times less than the acute oral $LD50^{(48h)}$ of 17320 ng a.s per bee). Based on the data about metabolic rates in flying bees (Rortais *et al.*, 2005; Balderrama *et al.*, 1992) the bee will metabolize only 0.53 - 0.8 µl of the sucrose solution and thus incorporates 2.86 - 4.32 ng thiacloprid while flying back to the hive from the feeder (2 min return flight, 1 M sucrose solution). In natural conditions, foraging bees can be exposed to different concentrations of the substance in nectar. Pohorecka *et al.* (2012) report data on thiacloprid residues in nectar from flowers, combs and in honey up to 208.8 ng/g. The amount of the substance a bee will metabolize when foraging on nectar sources contaminated with 208.8 ng/g (0.25 ng/µl) thiacloprid depends on the distance from the food source to the hive, the flight time during foraging, the motivational state (Balderrama *et al.*, 1992) and the reward rate (Balderrama *et al.*, 1992; Fournier *et al.*, 2014). If a bee performs a 20 minutes foraging flight and forages on a 50 % nectar concentration, we can estimate that it will metabolize rather similar amounts of thiacloprid (2.6 - 4 ng) as in our study." Furthermore, we estimated an amount of metabolized thiacloprid between 141 and 212 ng per day and per bee foraging at the contaminated feeder. The lower range of this estimation, which is the most probable, is not far from the daily consumption and thus exposure of 112.1 ± 4.4 ng per bee and per day measured by Vidau *et al.* (2011) in his experiment.

Homing flight performance has been considered by the EFSA as a relevant criterion for measuring sublethal effects in free-ranging pollinators (EFSA, 2012b). Indeed, in order to perform a successful homing flight, a bee has to use its sensory, motor and cognitive functions for successful foraging trips. We showed here that the sensory and motor functions are not

compromised but rather specifically their cognitive abilities, such as retrieval of spatial memory about the landscape and motivation to forage and communicate. The homing success of the foragers exposed to thiacloprid was impaired, supporting previous findings on the effects of thiacloprid, imidacloprid, clothianidin (Fischer *et al.*, 2014) and thiamethoxam (Henry *et al.*, 2012; Henry *et al.*, 2015). Honey bee colonies are behaving like a 'superorganism'[58] and a sufficient number of honey bees in each class is needed to perform the various and different tasks in order to keep the information flow going and to adapt efficiently to changing environmental conditions (Khoury *et al.*, 2011). High forager death rates can induce a shift in the age that honey bees are starting to forage (Herb *et al.*, 2012) and a change in the relative proportions of worker brood versus drone brood production (Henry *et al.*, 2015) which might affect the fitness of the colony (Khoury *et al.*, 2011).

The radar tracking method applied here allows identification of which components of navigational tasks necessary for successfully return to the hive are compromised. The catch and release test exposes the bee to the condition of localizing itself after being released at an unexpected place within the area around the hive which it had explored during its orientation flights (Degen *et al.*, 2015). Treated bees were more frequently lost than control bees, particularly during the initial part of their homing flight. Treated bees also had a higher probability to start their flight by taking a wrong direction, and they had a tendency to interrupt their flights towards the hive, indicating their inability to recall their memory and locate themselves. Our results also corroborates previous findings (Fischer *et al.*, 2014) that the vector flight of bees acutely treated with thiacloprid was not altered, indicating an uncompromised application of the recently learned vector memory if it is retrieved. Homing, however, requires the activation of a remote memory acquired during exploratory orientation flights and the recognition of landmarks as indicators for the route towards the hive from an unexpected location. The flight trajectories recorded in the Fischer *et al.* study and here strongly indicate a loss of memory retrieval that differs from the recently learned route flight. Neonicotinoids affect predominantly higher-order cognitive functions of the bee brain that are related to the integrative properties of the mushroom bodies. These structures are known to be essential for across sensory integration, learning, and memory formation (Heisenberg, 2003; Menzel, 2012) and they require functional nicotinic acetylcholine synaptic transmission both at their input site and their output site. It is thus likely that neonicotinoids at low level doses interfere predominantly with mushroom body functions (Peng and Yang, 2016; Palmer *et al.*, 2013).

Moreover, thiacloprid is often used together with other pesticides in mixtures (Mullin *et al.*, 2010) and some synergism effect between thiacloprid and ergosterol biosynthesis inhibiting fungicides has already been observed in honey bees, increasing the toxicity by up to 560-fold

(Iwasa *et al.*, 2004; Schmuck *et al.*, 2003). For Mullin *et al.* (2015) “the formulation and not just the dose makes the poison”. Future studies should concentrate their efforts on investigating the effects of neonicotinoids not only as active substances but also as formulations. It should also be noted that the risk of neonicotinoids to bumble bees or solitary bees is about two to three times as high as for honey bees, due to the different sensitivity among the species (Sanchez-Bayo and Goka, 2014).

Dramatic consequences on honey bees and more generally pollinators chronically exposed to very low concentrations of thiacloprid are thus to be expected. Therefore, thiacloprid cannot be considered a less harmful neonicotinoid. Our results also demonstrate how important it is to include field test procedures directed towards chronic exposure to sublethal doses of these pesticides and how essential it is to test a large range of possible behavioral effects of a substance before commercializing it.

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Supplementary Information content

Number of waggle runs performed by bees foraging at food sources other than the feeders (Fig. S1), sucrose consumption at the feeders and estimated amounts of thiacloprid collected and metabolized (Table S1), Tuckey's post-hoc tests of the Proboscis Extension Response experiment (Table S2), pesticide residues analysis of honey bees directly and indirectly exposed to thiacloprid (Table S3), flight data of honey bees returned to the hive (Table S4), detailed flight parameters of honey bees returned to the hive (Table S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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Supporting Information

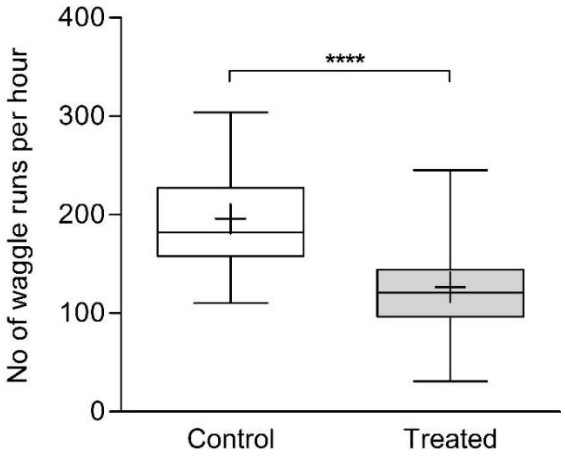

Figure S1. Number of waggle runs performed by bees foraging at other food sources. The number of waggle runs per hour was obtained from electrostatic field recordings performed on the same days (*n* = 32 days) in both hives. The mean is represented by a cross in the box-plots and it was found significantly higher for the bees foraging at the control feeder than for the bees foraging at the contaminated feeder (Paired t.test, $p < 0.0001$).

Table S1. Sucrose consumption at the feeders and estimated amounts of thiacloprid collected and metabolized.

	duration feeder open (min)	total consump -tion /day (ml)	No. of bees at the feeder	estimated sucrose collected /bee /day (ml)	estimated amount collected /bee /day (ng)	estimated No. of trips /bee /day	cumulated duration of return flights /bee /day (hours) *	estimated amount metabolized /bee /day (ng) **
Experiment 1, *n* = 19 days								
Control	463 ± 22	122.36 ± 8.56 [a]	33 ± 3	3.79 ± 0.26 [a]		85.56 ± 7.37	2.852	
Treated	458 ± 24	74.21 ± 6.38 [b]	34 ± 2	2.23 ± 0.16 [b]	12118 ± 900	48.78 ± 4.27	1.626	140.93 - 211.39
Experiment 2, *n* = 29 days								
Control	432 ± 23	93.13 ± 7.20 [c]	28 ± 1	3.35 ± 0.26 [a]		68.60 ± 6.73	2.286	
Treated	436 ± 22	54.57 ± 6.09 [d]	26 ± 2	2.03 ± 0.15 [b]	10990 ± 833	48.97 ± 4.66	1.632	141.45 - 212.17

Numbers shown are means ± s.e.m.

Different letters indicate significant differences. a-b and c-d: Paired t-test, $P < 0.0001$; a-c: Unpaired t-test, $P = 0.01$; b-d: Unpaired t-test, $P < 0.05$.

* based on a 2 min return flight duration from the feeder to the hive

** based on the estimations from Rortais *et al.* (2005) that a bee needs 8 - 12 mg of sugar per hour to fly and that treated bees are always collecting a 1 M sucrose solution (median of the sucrose solution concentrations used at the treated feeder)

Table S2. Tukey's post-hoc tests of the Proboscis Extension Response experiment

Sugar conc x Treatment	Estimate	Std. Error	z value	Pr (>|z|)
C 0 % - T 0 %	-1.1057	0.8479	-1.304	0.710
C 0.1 % - T 0.1 %	-1.2637	0.7881	-1.603	0.488
C 0.3 % - T 0.3 %	-0.9949	0.7815	-1.273	0.732
C 1 % - T 1 %	-0.4814	0.7877	-0.611	0.992
C 3 % - T 3 %	-0.7714	0.8315	-0.928	0.925
C 10 % - T 10 %	-0.5509	1.0527	-0.523	0.997
C 30 % - T 30 %	14.4338	2247.7280	0.006	1.000

No difference was found in the PER of the bees tested with different sucrose solution concentrations contaminated or not with thiacloprid (5 ppm).

Table S3. Pesticide residues analysis of honey bees directly and indirectly exposed to thiacloprid.

	days of exposure at feeder / days of exposure since start of Exp (hive)	sample weight (mg)	thiacloprid residues (ng/g)				(ng/bee) whole body *	estimated cumulated amount collected at the feeder (ng/bee) **	estimated cumulated amount metabolized by the bees (ng/bee) ***
			head	thorax	abdomen	whole body			
Treated feeder	2 days[1]	924	473	682.52	1175.05	2330.44	**215.33**	29612	**285** - 427
	3 days[2]	1076	596.36	705.79	2262.15	3564.30	**383.52**	45981	**508** - 762
	4 days[1]	853	1006.25	1067.98	1923.08	3997.31	**340.97**	34886	**441** - 661
Control feeder	*not known*[1]	810 ± 83	0.93 ± 0.93	0.74 ± 0.21	< LOQ	1.66 ± 1.15	0.13		
	not known[1]	870	n.n.	< LOQ	< LOQ	< LOQ	< LOQ		
	not known[2]	911	n.n. ‡	1.9	n.n.	1.9	0.17		
Treated hive	1 day[1]	880	n.n.	0.78	< LOQ	0.78	0.07		
	15 days[1]	717	6.34	7.13	25.91	39.37	2.82		
	18 days[2]	964	39.69	32.13	44.40	116.23	11.20		
	32 days[2]	871	39.40	11.71	80.25	131.36	11.44		
Control hive	1 day[1]	852 ± 12	n.n.	0.86 ± 0.23	< LOQ	0.86 ± 0.23	0.073		
	17 days[2]	923	n.n.	n.n.	n.n.	n.n.	n.n.		
	32 days[2]	812	n.n.	n.n.	n.n.	n.n.	n.n.		
LOD §			*0.6*	*0.16*	*0.25*				
LOQ §			*1.25*	*0.31*	*1.25*				
Recovery (% RSD) *n =5*			*92 % (5 %)*	*75 % (17 %)*	*94 % (5.4 %)*				

Each sample (line in table) consists of 10 bees. The mean ± s.e.m. is shown if $n_{samples} = 2$, otherwise $n_{samples} = 1$.

‡ n.n = not detectable

§ LOD, limit of detection (3 times background noise); LOQ, limit of quantification (10 times background noise)

[1,2] Experiment (1 or 2) during which the bees were collected for analysis

* calculated from the weight of the 10 bees analyzed

** estimated from the cumulated amount of thiacloprid collected by a bee at the feeder during the 2, 3, or 4 days before being collected for residues analysis.

*** based on a 2 min return flight duration from the feeder to the hive and the cumulated number of trips performed by the bees in the 2 ($n_{trips} = 98$), 3 ($n_{trips} = 176$) or 4 days ($n_{trips} = 153$) before being collected for residue analysis. From the estimations of Rortais *et al.* (2005) that a bee needs 8 - 12 mg of sugar per hour to fly and that treated bees collected a 1 M sucrose solution (median of the sucrose solution concentrations used at the treated feeder) we could estimate the range of the cumulated amount of thiacloprid metabolized by the bees in *n* days of foraging.

Table S4. Flight data of honey bees returned to the hive.

Group	Feeder	No. of bees returned /total	Mean duration ± s.e.m. (min)	Median duration	Mean distance ± s.e.m. (m)	Median distance	Mean speed ± s.e.m. (m sec^{-1}) *
Experiment 1							
Control	F1	34/35	9.4 ± 1.2	6.78	2063 ± 160.5	1978	4.5 ± 0.2
Treated	F2	36/41	18.3 ± 3.8	9.9	2867 ± 466.8	1961	4.2 ± 0.1
Experiment 2							
Control	F2	44/50	8.9 ± 0.8	8.6	2543 ± 226.4	2144	5 ± 0.2
Treated	F1	39/59	12.9 ± 2.8	7.1	2052 ± 219.8	1644	4.4 ± 0.2
Total (Exp.1 + Exp.2)							
Control		78/85	9.1 ± 0.7	7.8	2334 ± 147.3	2032	4.8 ± 0.2
Treated		75/100	14.5 ± 2.3	7.4	2443 ± 254.2	1908	4.3 ± 0.1

No difference between the control and treated groups regarding the duration of the flight and the distance flown from the release site to the hive (Mann-Whitney tests)

* Control bees flew in total significantly slower than treated bees (Unpaired t-test, $P < 0.05$).The largest difference in speed occurred between the control and treated bees which foraged at F2 (Unpaired t-test, $P < 0.01$)

Table S5. Detailed flight parameters of honey bees returned to the hive.

	Control			Treated		
	Exp.1 (*n* = 34)	Exp.2 (*n* = 44)	Exp1+2 (*n* = 78)	Exp.1 (*n* = 36)	Exp.2 (*n* = 39)	Exp.1+2 (*n* = 75)
Feeder	F1	F2	F1+F2	F2	F1	F1+F2
Vector flight *(n bees)*	26	29	55	26	31	57
Duration (sec) *	94.93 ± 10.21	67.83 ± 11.34	80.41 ± 7.85	89.72 ± 14.20	106.8 ± 20.30	99 ± 12.75
Distance (m)	303.2 ± 16.38	279.7 ± 14.95	290.6 ± 11.05	302.3 ± 14.77	265.6 ± 17.54	282.3 ± 11.83
Speed (m sec^{-1}) ǂ	3.9 ± 0.31	5.56 ± 0.38	4.79 ± 0.27	4.45 ± 0.38	4.06 ± 0.35	4.25 ± 0.25
Angular distribution (°)	233 ± 9.25	308.7 ± 2.24	273.6 ± 5.29	307.3 ± 1.93	232.5 ± 1.36	266.6 ± 5.10
Homing flight *(n bees)*	26	29	55	26	31	57
Duration (sec)	507.7 ± 87.3	523.4 ± 61.71	516.1 ± 51.83	979.2 ± 273.7	717.7 ± 211.3	837 ± 169
Distance (m)	1823 ± 193.9	2413 ± 257.4	2139 ± 168	2267 ± 269.8	1858 ± 252.4	2045 ± 184.7
Speed (m sec^{-1})	4.49 ± 0.28	4.86 ± 0.24	4.69 ± 0.18	3.79 ± 0.27	4.40 ± 0.28	4.18 ± 0.20
Return to RS (*n* bees) §	4	18	22	6	4	10
Pass close to F (*n* bees) ¥	9	7	16	3	1	4
No of pauses	4	5	9	12	12	24
Duration of pauses	6.96 ± 4.92	3.96 ± 0.84	5.29 ± 2.12	15.80 ± 5.68	24.47 ± 9.01	20.13 ± 5.28

The duration, distance, speed, and angles distribution values are expressed as mean ± s.e.m.

* Significant difference in the duration of the vector component (Kruskal Wallis, P = 0.0241, post-hoc tests with Bonferroni correction, Control Exp.1 vs Control Exp.2, P < 0.05)

ǂ Significant difference in the speed of the vector component (One-way ANOVA, $F_{3,440.9} = 4.497$, P = 0.0051, Tukey post-hoc tests Control Exp.1 vs Control Exp.2, P < 0.01 and Control Exp.2 vs Treated Exp.2, P < 0.05).

§ Significant difference between the number of control and treated bees returning to the release site (RS) during their flight in total (Fisher Exact test, P < 0.05), in Exp.2 (Fisher Exact test, P < 0.01) and between the 2 control groups in Exp.1 (Fisher Exact test, P < 0.01).

¥ A significantly greater amount of control bees compared to treated bees flew less than 100 meters from their feeder (Exp.1+2, Fisher Exact test, P < 0.01).

Chapter 1 - Perspectives

The data collected with the harmonic radar in 2013 are interesting in many regards. A detailed analysis of the navigation data was however not included in the above manuscript (Tison *et al.*, 2016) as further analysis of navigation patterns was not relevant for a general article about the effects of thiacloprid on honey bees' behavior. Furthermore, the help of bio-informaticians was needed in order to develop efficient tools for analyzing such data. A numeric framework will ease the analysis and establish formal and objective definitions to it. The scripts used in this study were written with Matlab and Python. Together with Dr. Tim Landgraf and his students, we work on developing tools for extracting and analyzing navigational information from flight tracks. The data presented here are only preliminary results, on-going analysis and hypothesis.

Interpolation of flights

The first step of this work was the interpolation of the flights. Although the radar is supposed to measure the position of a flying bee every 3 seconds, time gaps of more than 10 seconds between two measurements sometimes occured. The quality of the data collected in the field depends on different factors such as the radar itself and its precision, the weather conditions, the presence of objects in the field which could disturb or block the radar beam, the position of a bee (flying too low down, sitting), etc.

The first step before interpolation was to make sure that each data point from a track had a unique timestamp. If this was not the case, both data points for this timestamp were deleted and replaced by a single data point averaging the coordinated of the two deleted ones.

The next step was the segmentation. For this, we had to distinguish between short, long plausible and long unplausible gaps in the recorded flight track. Short gaps correspond to a gap in the flight track shorter than 4 data points (= 12 seconds). Long gaps are larger than 3 data points. Long gaps can be plausible if they correspond to a bee flying at the minimum speed of 0.2 m/s. This way, we made sure that the bee flew more or less straight during the time when coordinates were missing. If this condition was not fulfilled (unplausible gap), the original segment was split into two segments separate by a gap (Figure A).

In order to work with more or less constant time series, surrogate data points (timestamps) were used. A surrogate data point was inserted at time x + 3 seconds if the difference between point x and y was greater than 4 seconds. The coordinate corresponding to a surrogate point was written as NaN.

The last step of the interpolation was the Kalman filter. The algorithm is guessing the next data point from what it has experienced before. To improve the quality of the interpolation of the

time gaps, the data are interpolated backward and forward and then the mean of those two interpolations is set as the final interpolation. Figure A shows an example of the defined flight segments and the resulting interpolated flight track.

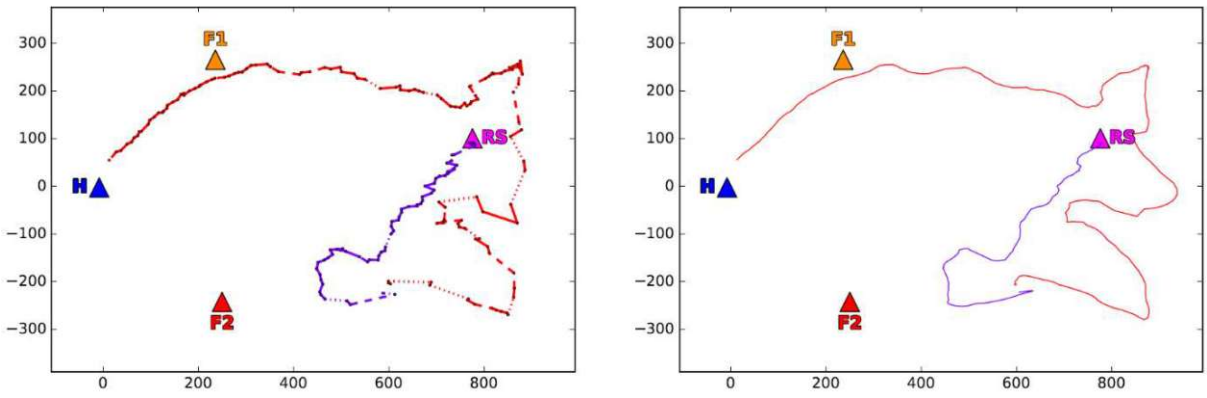

Figure A. Example of a flight from a control bee trained to F1 before and after interpolation. H: Hive, RS: Release Site, F1: Feeder 1 and F2: Feeder 2. Two different segments separated by a gap in the data ("unplausible gap") were defined according to the velocity of the bee (purple and red segments). The raw data are the black points on the left image. Dashed lines represent gaps in the data (large dashes: short gaps, small dashes: large gaps). The flight track after interpolation is displayed on the right image. Source: Colin Thomas.

Linear and search segments

The next step of the analysis was to subsegment each flight segment in two kind of subsegments: linear and search segments. Linear subsegments are attributed to straight lines of more than 80 m with no turning angle of more than 80°. The linear flight also as to stay below a certain error threshold in its linearity. These criteria can always be adjusted depending on what we want to look for. The hypothesis behind this categorization is that the bee knows its current location and where it is heading to.

The algorithm used to separate linear subsegments from search subsegments is a divide and conquer algorithm which finds the longest linear subsegment and iterates on the previous and following part of the segment. All subsegments remaining after linear subsegments have been extracted are defined as search subsegments. The hypothesis of search segments is that the bee does not know where it is and try to locate itself by flying circles or zigzagging.

Figure B shows a flight before and after the subsegmentation.

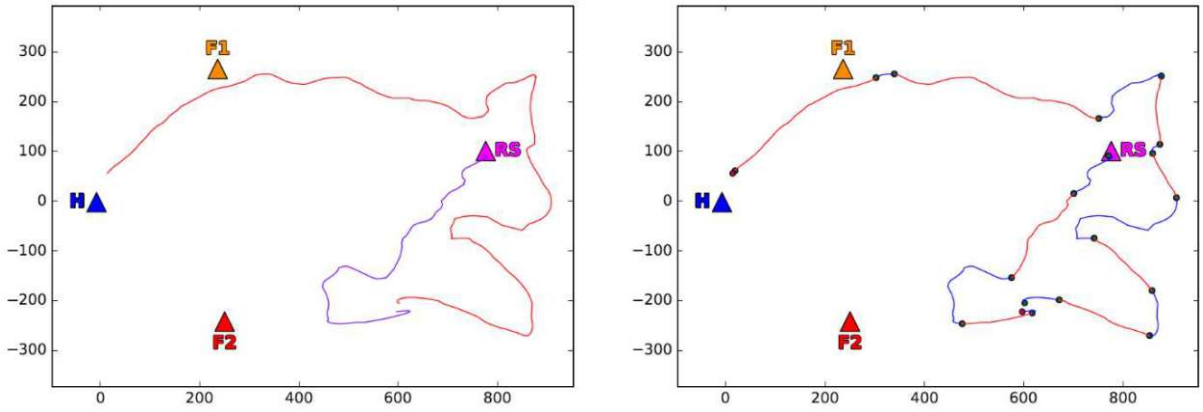

Figure B. Example of an interpolated flight from a control bee trained to F1 before and after subsegmentation. H: Hive, RS: Release Site, F1: Feeder 1 and F2: Feeder 2. Left image: interpolated flight. Right image: the linear (red) and search (blue) subsegments. Black points are the transition points. Source: Colin Thomas.

From this analysis can be extracted the number of linear and search subsegments. We could also extract the total number of transitions (how often a bee changes its direction given a certain threshold). These “transition points”, when bees switch from one behavior to another (or switch direction between two linear subsegments) are of particular interest as they reflect in-flight decision-making. We would expect a rather similar distribution of linear and search subsegments according to the feeders bees were trained to (F1/F2) and/or maybe to the treatment they received (control/treated).

More precise definitions of linear and search subsegments could be established as for example, long curved trajectories are not classified as linear but they don’t indicate a searching behavior either.

Vector and homing flight segments

When released at an unknown location after being caught at their feeder (feeder 1 or 2), bees usually start their flight by performing a linear vector flight, representing the path they would have taken from the feeder in order to come back to the hive. Fig. A / B displays a flight of a bee performing a vector flight. To define vector flight segments we used the same criteria as in the Tison *et al.* (2016) article. A vector flight segment was defined as the first linear subsegment of a bee flight (starting within a radius of 80 m from the release site), heading in the same direction as the direction feeder-hive (according to the feeder it was trained to) within a 45° window. To be define as a vector flight, the subsegment had to be minimum 200 m long. The first 200 m of the vector fligths from the control and treated bees are displayed in Fig. C.

Figure C. Extracted vector flight segments: Vector segments of control (left image) and treated (right image) bees released. North-East directed vectors belong to bees trained at feeder 2 (yellow point), South-West directed vectors to bees trained at feeder 1 (grren point).

We considered a homing flight segment the last long linear segment ending at the hive. The hypothesis is that a bee performing a homing flight segment as fully located or relocated itself at the beginning of the homing segment. A bee performed a homing flight segment if it flew a linear subsegment (minimum 80 m straight flight) ending within a radius of 80 m from the hive. The extracted homing flight segments from all bees are showed in Fig. D.

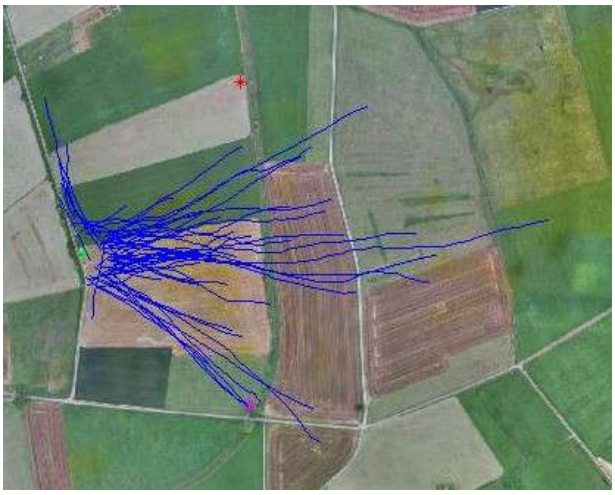

Figure D. Extracted homing flight segments. Are represented the homing segments of all bees released: control and treated bees trained to feeder 1 (red cross) or 2 (pink cross) irrespectively).

From this kind of analysis can be extracted information such as the number of vector or homing flight segments according to the treatment and to the feeding location, the angular deviation between the vector flight segment and the feeder-hive segment, and the angular

deviation between the actual homing flight segment and the direct homing segment (from starting location of the homing segment to the hive.

Relationships between ground structure and flight behavior

Figures E, F, G, and H are showing the interpolated flights plotted on a map according to the treatment and/or feeding location of bees. All figures have the same scale. The distance between the hive (green point) and the release site (red point) is 780 meters.

The flight tracks allow us to see differences between control and treated bees but also differences related to the feeding location (Feeder 1 or 2). Some flight patterns were already extracted and quantified manually in the Tison *et al.* (2016) article (Table S5) but the aim with this automated track anaylsis will be to extract flight patterns automatically, saving some time and avoiding bias.

Different flight patterns could be revealed according to the feeding location. The flight tracks from Fig. E (control) and F (treated) are similar to each other as bees were trained to the same feeding location (Feeder 1). The same kind of similarities were observed between Fig. G and H corresponding to Feeder 2.

Bees which were foraging at Feeder 1 usually flew South-West (vector flight) and then, interestingly, seem to search a lot on and/or around the road located in the middle of the experimental field. They reach the hive (homing flight segment) with a narrower angle than bees trained to feeder 2. Bees which were foraging at Feeder 2 are showing very different flight patterns. They usually performed first a North-West directed vector and searched more often at the end of the vector flight or around the release site (returning more often to the release site). They reached the hive from a much broader angle than bees from feeder 1.

We know already that the position of the feeders had an effect on the flight patterns of the released bees. The design of the experiment allow us to differentiate landscape/feeding location effects from treatment effects. The different flight patterns informations are currently being analyzed. Comparing the data obtained manually (Table S5, Tison *et al*., 2016) and automatically allow us to validate the tool and adjust it according to our criteria and hypothesis. From this analysis can be extracted: the distance flown by the bees, the flight duration, the average velocity, the use of different landmarks and landscape structures such as the release site, the feeder, the roads (middle pathway and hive pathway), etc.

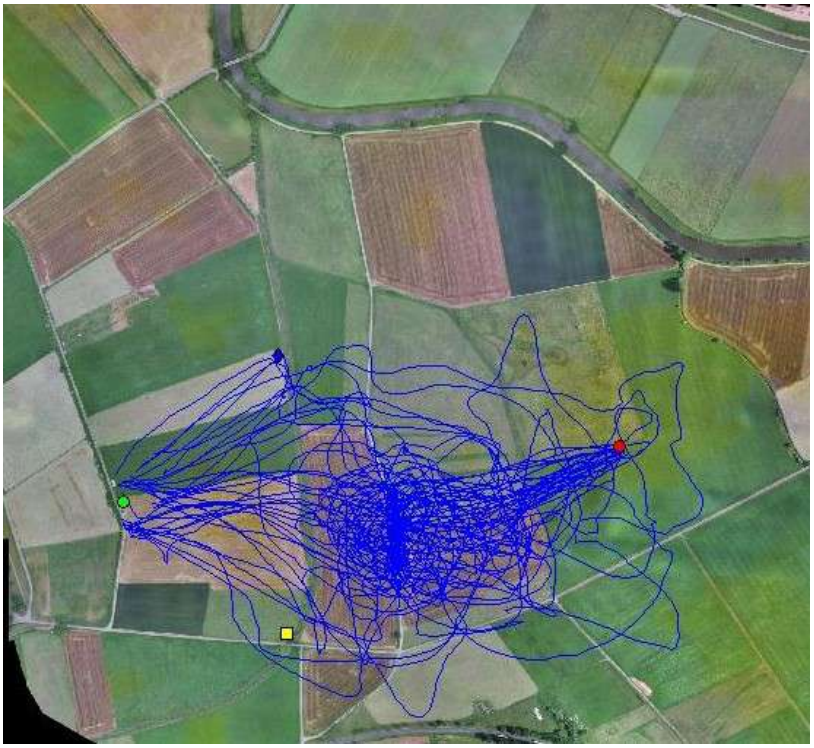

Figure E. Interpolated flight tracks of <u>control bees</u> trained to <u>feeder 1</u> (experiment 1).
Green point = Hive, Red point = Release Site (distance H-RS = 780 m). Yellow square = Feeder 2, Blue diamond = Feeder 1.

Figure F. Interpolated flight tracks of <u>treated bees</u> trained to <u>feeder 1</u> (experiment 2).
Green point = Hive, Red point = Release Site (distance H-RS = 780 m). Yellow square = Feeder 2, Blue diamond = Feeder 1.

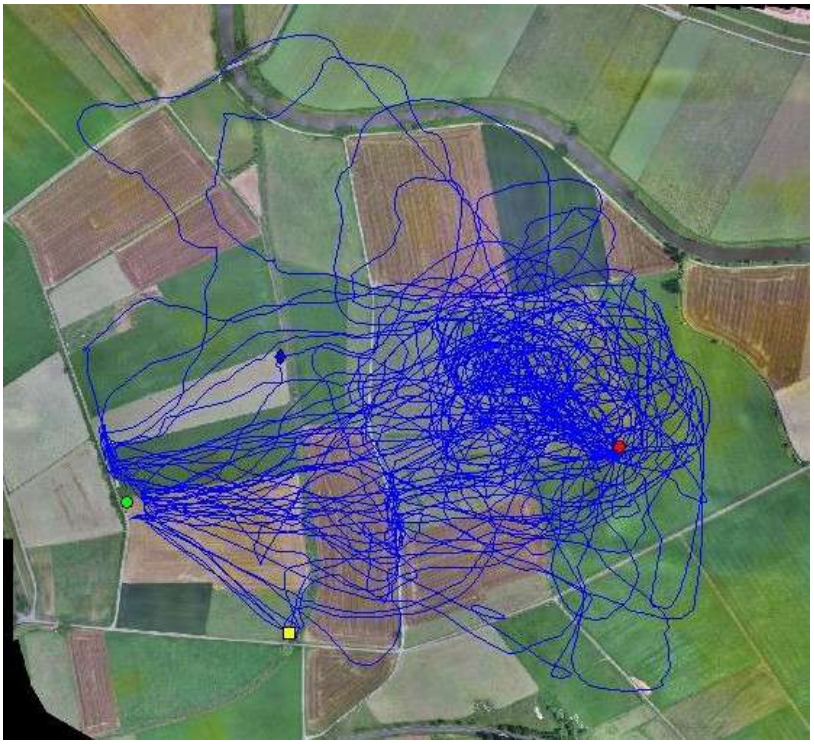

Figure G. Interpolated flight tracks of control bees trained to feeder 2 (experiment 2).
Green point = Hive, Red point = Release Site (distance H-RS = 780 m). Yellow square = Feeder 2, Blue diamond = Feeder 1.

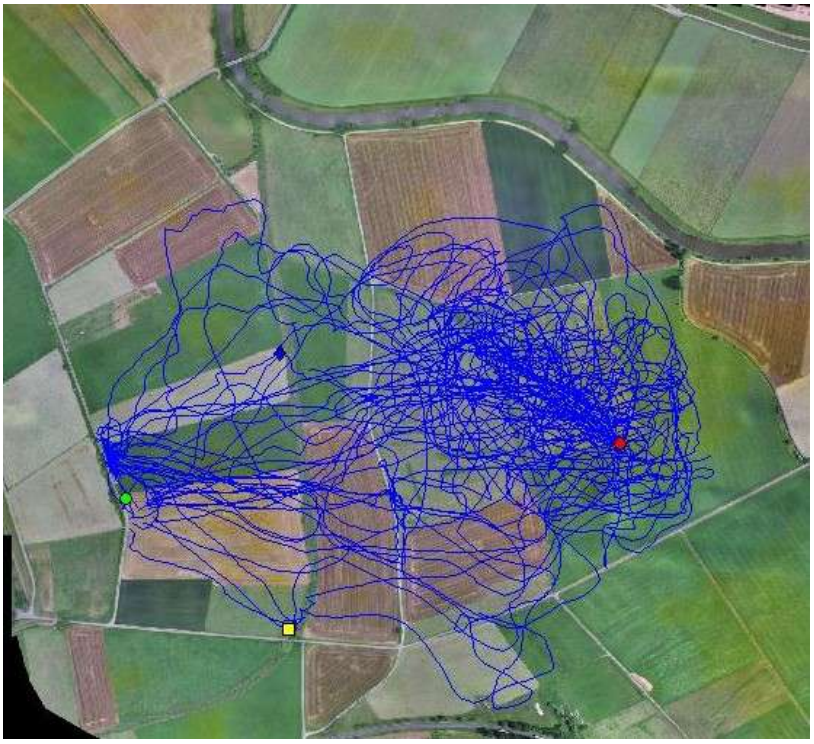

Figure H. Interpolated flight tracks of treated bees trained to feeder 2 (experiment 1).
Green point = Hive, Red point = Release Site (distance H-RS = 780 m). Yellow square = Feeder 2, Blue diamond = Feeder 1.

Trajectories following a specific ground structure such as a road or a river are of particular interest to us. Bees trained to feeder 1 (Fig. E and F) especially, tend to follow a remarkable landmark, the road in the middle of the experimental area. The recognition of this extended landmark by the bees clearly depends on their feeding loaction. It also seems that bees foraging at feeder 2 (Fig. G and H) tend to arrive at the hive from the North after flying on the hive pathway. These types of homing flights (using the hive pathway) were named “L-shaped flights” and are currently being analyzed.
The flight of bees following elongated landmarks such as roads will be deeply studied in order to understand the influence of the feeding (trained) location and ground structure on the honey bee recognition of landmarks and decision making.

Another step of this project will be to understand why bees would decide to fly a linear or a search flight (or simply to fly in one direction or the other) according to what they would see of the ground structure.
In order to measure similarities between two views, the original map image is transformed, then views are built from subimages of the transformed maps, and finally, image similarity functions are applied to the subimages.
The 2D map of the experimental field was obtained by drone photography in June 2016 (see Fig. I). This map is in RGB colors, but as the flights were acquired between June and September 2013, the colors may not have been exactly the same than in 2016.
As we are then more interested in the structures of the landscape (such as roads, rivers, trees, field borders...) which are time invariant as in the colors, two image transformations have been applied to the map image. First, the map was turned into grayscale by taking the mean of the RGB levels (see Fig. I, left image). The difference between fields was lowered but still present. Thus, a canny filter was applied on the grayscale map resulting in an “edges map” (see Fig. I, right image). This image represents the basic structure of the landscape. Two fields planted with different crops could not be distinguished anymore but fields borders of similar colors could not be always retrieve.
To represent the view of a bee at a certain point in its trajectory, the map image was first rotated in direction of where the bee is heading and then the bee view defined as a rectangular subimage from the rotated map. A view is then a matrix of pixels.
We will use similarity functions to compare the views of subimages. The higher the Pearson Correlation correlation is, the more similar two views are.

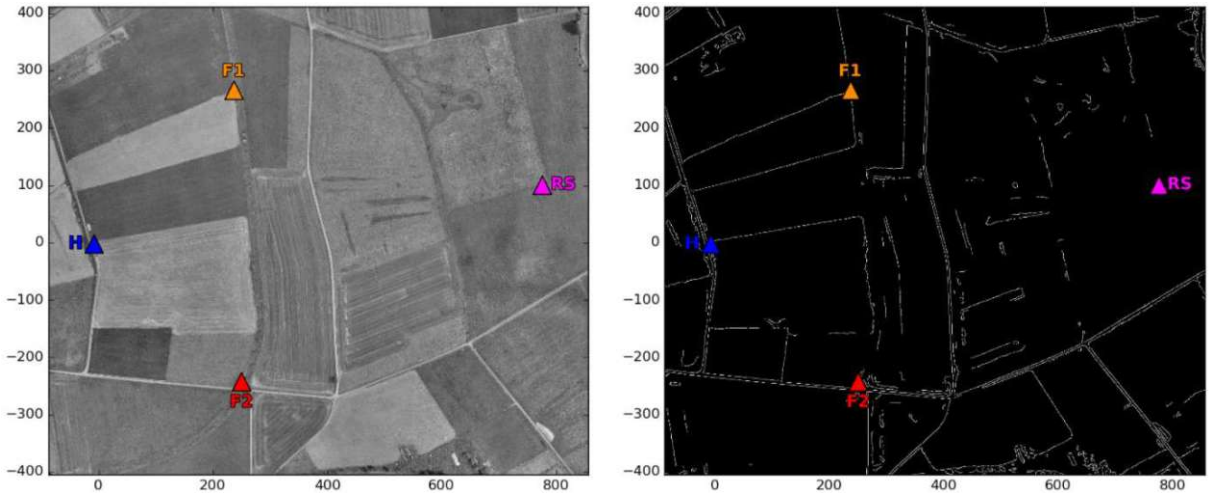

Figure. I. Left image: grayscale map: Right image: Edges map. Source: Colin Thomas

One of the many hypotheses we want to test by using this tool is whether the transition points between linear and search subsegments are influenced by the ground structure. In order to test this hypothesis, the similarity between the current view and the previous view is computed for every point of the trajectory. If the hypothesis reveals itself true, an off-peak at the beginning or at the end of a subsegment is expected. This would indicate a sudden change in the ground structure, maybe influencing the bee's decision to change its behavior.
Another interesting hypothesis will be to check if the end of a vector flight segment can be related to a difference in the ground structure or to a sudden dismilarity between the actual ground structure and the expected ground structure (corresponding to the arrival at the hive).
After extracting patterns of landmarks from the maps, we could compare whether specific patterns tend to appear more when bees are flying linear subsegments. The hypothesis is that linear flights tend to follow road and field edges more consistently than search flights. This would indicate that bees remember and use patterns such as elongated landmarks to orientate. We would then expect specific ground structures in areas of the map corresponding to a linear-linear transition or to a search-linear transition, assuming that a bee is able to locate itself with the help of extended landmarks. However, we would not expect specific landmarks (only grass fields for example) in areas of the map corresponding to a linear-search transition, assuming that a bee starts searching because it is not able to locate itself as everything around looks the same.

We expect very promising results from this analysis. The tool developed will be used with other flight tracks collected with the harmonic radar. The outcome of these analyses will be published in a peer-reviewed scientific journal.

Chapter 2

Effects of sublethal doses of thiacloprid and its formulation Calypso® on the learning and memory performances of honey bees

Léa Tison, Sophie Holtz, Amy Adeoye, Önder Kalkan, Nina Sophie Irmisch, and Randolf Menzel

Free University Berlin, Institute for Biology-Neurobiology, D-14195 Berlin, Germany

Corresponding author: E-mail: lea.tison@gmail.com.

Author contributions:
L.T, S.H, A.A, Ö.K, and N.S.I performed the sucrose responsiveness and conditioning experiments. L.T performed the residue analysis. L.T and R.M designed the experiments. L.T analyzed the data, interpreted the results and wrote the manuscript.

Abstract

Learning and memory play a central role in behavior and communication of foraging bees. We already showed that chronic uptake of the neonicotinoid thiacloprid affects honey bees' behavior in the field. Foraging behavior, homing success, navigation performance, and social communication were impaired. Thiacloprid collected at a feeding site at low doses accumulates in foragers over time. Here we applied a laboratory standard procedure, the Proboscis Extension Response (PER) conditioning, in order to assess which processes, acquisition, memory consolidation and/or memory retrieval were compromised after bees were fed with thiacloprid or a formulation containing thiacloprid named Calypso® at 3 different sublethal doses. Extinction and generalization tests were performed to determine whether bees respond to a learned stimulus, and how selectively. For the first time, we show that thiacloprid, as active substance and as formulation, poses a substantial risk to honey bees by disrupting learning and memory functions. These data support and specify the data collected in the field.

Introduction

Bees are the predominant and economically the most significant group of pollinators worldwide. Over the last decades, the number of pollinators has declined steadily. The abundance of pollinators in the environment is influenced by biotic factors (predators, pathogens, parasites, competitors, availability of resources) and abiotic factors (climate, pollutants). Although the putative causes of the recent decline in pollinators are still under investigation, it has been revealed that the extensive use of pesticides against pest insects for crop protection has contributed to the loss of many pollinators (Brittain & Potts, 2011; Rundlöf *et al.*, 2015).

Pesticides are substances widely used throughout the world to kill, repel, or control plants or animals considered as pests. Depending on their type, dose, and persistence in the environment, they can have an impact on non-target species such as beneficial arthropods like pollinators (Farooqui, 2013). Neonicotinoids are systemic insecticides, making the entire plant, including the nectar and pollen, toxic to the pest insects but also to honey bees. Thiacloprid, like the other neonicotinoids, acts as an agonist to the insect nicotinic acetylcholine receptor (nAChR). In the insect brain this receptor is predominantly abundant in the neuropil regions of the central nervous system (Tomizawa and Casida, 2005). At least two types of such receptors have been described in the honey bee brain: the α-bungarotoxin (α- BGT)-sensitive and the α-BGT-insensitive receptor (Gauthier *et al.* 2006). These receptors are involved in tactile and olfactory sensation, learning and memory (Cano Lonzano *et al.* 1996; Cano Lozano *et al.* 2001; Dacher *et al.* 2005), two essential functions for foraging behavior.

Sugar is one of the most important appetitive stimulus for honey bees, because it is their main source of carbohydrates, controlling feeding behavior, foraging, and recruitment during social communication. In addition, it serves as a reinforcing stimulus for instrumental and operant associative learning (Hammer and Menzel, 1995). When stimulating the gustatory receptors set on the tarsae, antennae, or mouth parts with nectar or sucrose solution, hungry honey bees show a Proboscis Extension Response (PER), leading to the uptake of nectar and the association of odors or other stimuli received by the antennae. In odor PER conditioning the odor represents the conditioned stimulus (CS) and sucrose the unconditioned stimulus (US). During conditioning, the initially neutral CS becomes associated with the US and subsequently elicits a response, which was previously elicited only by the US (Bitterman *et al.* 1983).

Memory formation after PER conditioning and during natural foraging follows sequential and parallel consolidation processes leading to short-, mid-, and long-term memory each transition characterized by specific training requirements, time dependences, and molecular reaction cascades (Menzel, 1999; Müller, 2002; Menzel, 2012). Particularly important in our

context is the distinction between short-term and long-term memory induction, the first one resulting from a single conditioning trial, the second from multiple temporarily spaced conditioning trials (Menzel and Manz, 2001). Multiple molecular, cellular, and network properties are involved in olfactory learning and memory formation (Menzel, 2012). The mushroom bodies (MB) play a particularly important role, and the nAChR at their olfactory input sites are known to converge with the pathway transmitting the appetitive US.

Olfactory memory plays an important role in many aspects of honey bee behavior, including recognition of nestmates, foraging, food preferences, social communication, and navigation (Menzel *et al.*, 2005; Menzel and Müller, 1996). Any disruption in olfactory learning and memory may result in a negative impact on their foraging performance (Farooqui, 2013).

The PER assay can be used for estimating sublethal effects of pesticides in bees and it has been used in a number of studies investigating pesticide effects in honey bees and bumble bees (Decourtye *et al.*, 2005; Williamson and Wright, 2013). Negative effects of imidacloprid were observed on odor coding (Andrione *et al.*, 2016), olfactory learning (Decourtye *et al.*, 2004a) and memory (Decourtye *et al.*, 2004b; Williamson and Wright, 2013). Palmer *et al.* (2013) showed that imidacloprid and clothianidin negatively affect the learning ability of honey bees and postulate that an exposure to multiple cholinergic pesticides causes enhanced neurotoxity. Stanley *et al.* (2015) showed that thiamethoxam impaired learning and memory in bumble bees. These three substances are the most studied neonicotinoids and actions were already taken in Europe to suspend them (EFSA, 2012a, EFSA, 2012b). Thiacloprid, thought to be less toxic to honey bees (Iwasa *et al.*, 2004), however, was not studied in this respect, despite the fact that it has been used increasingly in the last years.

Here we chose to study thiacloprid as active substance diluted in sucrose, and Calypso®, a ready-to-spray formulation containing thiacloprid, used against sucking and chewing insect pests on a large number of plants, flowers, fruit trees, and vegetables. It is sold in garden shops without restrictions, to be sprayed directly on plants or flowers, and also during the flowering season because it has been declared safe to bees. Calypso® safety data sheet (Bayer CropScience Safety Data Sheet) cites 1,2-benzisothiazol-3(2H)-one as a hazardous component of the formulation in addition to the active substance thiacloprid. This substance is active against bacteria and fungi and for in-can preservation of pesticide emulsions (DOW, Product Safety Assessment). One can imagine other components of the formulation, probably declared ‘inerts’ as the specific ingredients that make up spray adjuvants are hidden under the cloak of ‘trade secrets’ and ‘proprietary information’ and are therefore usually not disclosed (Mullin *et al.*, 2015; Cox and Surgan, 2016). Co-formulants and supplemental adjuvants often enhance the pesticidal efficacy as well as inadvertently the non-target effects of the active ingredient after application (Holloway *et al.*, 1994; Holloway *et al.*, 1998). Numerous studies

have found that pesticide active ingredients elicit very different physiological effects on non-target organisms when combined with their formulation ingredients (Surgan *et al.*, 2010).

In our most recent study we showed that thiacloprid taken up chronically with sucrose solution impaired foraging behavior, navigation, and communication of honey bees trained to feeders under field conditions (Tison *et al.*, 2016). In the following experiments, we used the olfactory PER conditioning paradigm to investigate the effects of thiacloprid active substance and formulation (Calypso®) on learning, memory formation, and memory retrieval. We tested different concentrations, all of which were significantly lower than the Lethal Dose 50, and for the first time, we showed that both tested forms of thiacloprid pose a substantial risk to honey bees by disrupting their learning and memory functions.

Methods

Sampling and Preparation of Bees for Olfactory Conditioning

Summer honey bees were collected at 2 p.m. from hives located in the garden of the Institute of Neurobiology of the Free University of Berlin. Bees were collected with a Plexiglas pyramid on their outbound flight at the hive entrance. The bees were then transferred one by one from the pyramid into ventilated glass vials and cooled on ice until immobile (about 3 minutes). Then they were harnessed individually in tubes that allowed free movements of the mouthparts and antennae (Matsumoto *et al.*, 2012). At 4 p.m. the bees were fed to satiation with a 30 % (w/v) sucrose solution and put in a dark and humid box in a 20° C room until the next morning.

Thiacloprid and Calypso® solutions

Bees were given a range of field realistic thiacloprid or Calypso® doses based on previous published results (Tison *et al.*, 2016). Thiacloprid (Pestanal, 98 % purity, Sigma Aldrich) was dissolved in acetone (≥99.9%, Sigma-Aldrich) and distilled water in order to obtain a 0.5 M (126.36 ng. μl^{-1}) stock solution. Acetone was chosen as the solvent following the EPPO guidelines (OEPP guideline). The control group was fed sucrose solution without acetone as we demonstrated in the sucrose responsiveness experiments that acetone had no effect on sucrose perception (Fig. 1) nor on behavior in bees (Fischer *et al.*, 2014). Calypso® stock solution was directly taken from the Calypso® "ready to spray" bottle ("Schädlingsfrei Calypso® Perfekt AF) for which the thiacloprid concentration is 150 ng/µl. Calypso® safety sheet (but with thiacloprid at 480 g L^{-1}) cites 1,2-benzisothiazol-3(2H)-one) as a hazardous component of the formulation (>= 0.01 – <= 0.05 %) in addition to the active substance thiacloprid (40.40 %). Other chemicals of the formulation, if any, are unknown.

Thiacloprid and Calypso® stock solutions were then diluted in order to obtain 3 concentrations (50, 5, and 0.5 ng/µl) in a 30 % (w/v) sucrose solution. Each bee was fed with 4 µl of feeding solution (sucrose solution only or sucrose solution with Calypso® or thiacloprid). We chose to give the pesticide to the bees in sucrose solutions rather than in pollen because it allowed us to more easily control the dose and the route of exposure. The total amounts of thiacloprid fed to the bees were 69 ng in a first set of experiments and 200 ng, 20 ng or 2 ng per bee in a second set. The amounts of Calypso® solutions ingested were 120, 12 and 1.2 ng in a first experiment and 200, 20 and 2 ng per bee in a second experiment. The feeding solution was delivered orally using a multipette at different timings during the behavioral experiments. Bees were fed only once with the pesticide, either 1 hour before the first conditioning trial (Intox Group I), 5 hours after conditioning (Intox Group II) or 23 hours after conditioning (= 1 hour before the memory test, Intox Group III). When a group of bees received pesticide at a given

time, the corresponding control group received the same amount of sucrose solution (4 µl) but without pesticide.

The control and treated groups were always tested blindly and simultaneously as well the different doses. In the second experiment, all doses and all intox groups were tested blindly and simultaneously.

At the end of the memory test of the second set of experiments, honey bees from each of the 12 groups were collected and analyzed with LC/MS-MS for thiacloprid content as described below. Thiacloprid and Calypso® stock solutions and feeding sucrose solutions were also analyzed for thiacloprid content.

Honey bee mortality was assessed throughout the experiment. They were recorded as dead when no movement of the antennae or the abdomen was visible.

Olfactory Conditioning

Shortly before conditioning, the olfactory stimuli was prepared by placing 4 µl of pure odorant, 1-Nonanol (Sigma Aldrich), on a 1.32 cm^2 piece of filter paper inserted in a 20 ml plastic syringe used to deliver odor-filled air to the antennae of the conditioned honey bees.

Olfactory appetitive conditioning was performed according to a standard protocol (Matsumoto *et al.* 2012), using hexanal as the conditioned (reinforced) stimulus (CS) for the first set of experiments and 1-Nonanol for the second set of experiments. We used the following method (Felsenberg *et al.*, 2011) for conditioning. To avoid odor contamination, the conditioning trials and retention tests were performed in front of an exhaust pipe. Before starting with conditioning and retention, the group of harnessed bees was placed for 30 min on the conditioning table in the lab for acclimatization. At the beginning of each trial, each bee was placed at the learning spot for 10 seconds. The CS was presented during 5 seconds to each bee during each trial. The tip of the syringe was placed at the same height as the bee antennae and at a distance of 3 cm. The US (50 % w/v sucrose) was presented 3 seconds after the odor onset and was delivered first to the antennae in order to elicit an extension of the proboscis and then to the proboscis. Each bee was allowed to lick the sucrose solution during 4 seconds. The bee was left on the learning spot for 6 more seconds after the US and was then removed and replaced by the next bee. Each bee received 3 paired CS-US presentations (i.e., conditioning trials) with a 12 minute inter-trial Interval.

A bee was discarded if it did not extend its proboscis when stimulated with sucrose during conditioning. Bees that showed learning extended their proboscis in response to the odor before the sugar reward was delivered (PER) and thus the process of acquisition could be quantified during training. The learning performance is represented as the percentage of bees displaying the conditioned PER at each trial.

Memory test

Memory retention was assessed 24 hours after the first conditioning trial. In the first set of experiments, only the CS, hexanal, was tested in 3 extinction trials. In the second set of experiments, in addition to the CS, 1-nonanol, bees were exposed to 2-hexanol and to nonanal to determine the selectivity of their response to the CS. Nonanal has a high degree of similarity to 1-nonanol and was thus expected to be perceived similarly by the bees. On the contrary, 2-hexanol was expected to be perceived differently (Guerrieri *et al.* 2005) and thus would induce less PER.

For the memory test, each bee is placed on the learning spot for 10 sec. The odor was then presented to each bee with an inter trial interval of 12 min and were not rewarded with sucrose solution (extinction tests). The order of the odor presentation in the second set of experiments was shifted between each trial and this order was taken into account as random effect in the statistical analysis. At the end of the retention tests, each bee was stimulated with a 50 % sucrose solution to see if its unconditioned response to sucrose was still intact. Any bee that failed to display a proboscis extension reflex during the US test was discarded as well as any bee that extended its proboscis during the 10 sec prior to the odor presentation.

Sucrose responsiveness

This test was performed in order to determine whether thiacloprid or Calypso® affected honey bees' motivation for sucrose. Summer honey bees *Apis mellifera carnica* were collected at 2 p.m. from the hives located in the garden of the Institute of Neurobiology of the Free University of Berlin. They were handled the same way as during the preparatory steps for conditioning and tested one hour after catching. The sucrose responsiveness of harnessed bees was assessed by stimulating each bee's antennae with solutions containing 0, 0.1, 0.3, 1, 3, 10 and 30 % (w/v) sucrose (Scheiner *et al.*, 2005; Matsumoto *et al.*, 2012) only, or 50 $ng.\mu l^{-1}$ thiacloprid, 50 $ng.\mu l^{-1}$ Calypso® or 0.5 % acetone. The PER of the bees to the different solutions was carefully observed and noted. Typically, a bee that extends the proboscis to a given concentration of sucrose will respond also when tested for higher concentrations of sucrose. If a bee responded to low concentration(s) of sucrose and then stopped responding to higher concentrations or if a bee did not respond at all to a 50 % (w/v) sucrose stimulation at the end of the test, it was discarded.

Extraction and Quantification of Thiacloprid in Honey Bees

Collected bees were cut into three parts: head, thorax, and abdomen and thirty samples from the same groups of bees were pooled, weighed, and stored in a deep-freezer (-20° C) until the day of the residue analysis. 20 mL of an acetone/water mixture (2:1, v/v) and 20 µL of a surrogate standard solution containing thiacloprid-d4 (1 ng/µl) were added to each sample.

The samples were homogenized with a disperser during three minutes and then centrifuged (10 min at 3000 rpm). 15 mL of the supernatant was removed and after addition of 5 mL sodium chloride-solution (20 %) to this aliquot transferred onto a disposable cartridge filled with diatomaceous earth (ChemElut® cartridges, 20 mL, unbuffered; Agilent, Santa Clara, USA). After a waiting time of 15 minutes the samples were eluted with dichloromethane (2 x 50 mL). The eluates were evaporated to approximately 2 mL by using a rotary evaporator, then transferred to a graduated tube and evaporated to dryness with nitrogen, using a metal block thermostat with a nitrogen blow device. The residual extract was taken up with 50 µL of an internal standard solution containing imidacloprid-d4 (1 ng/µl) and 950 µL of a methanol/water mixture (1:1, v/v), dissolved using an ultrasonic liquid mixer (10 s) and put into a freezer (-18°C) overnight. On the next day, the samples were filtered cold (syringe filter 0.2 µm) and diluted (1:50, v/v) to reduce matrix effects before proceeding with the identification and quantification of thiacloprid using LC-MS/MS. The LC-MS/MS system used was a Nexera X2 HPLC system (Shimadzu Corporation, Kyoto, Japan) coupled to a QTRAP 6500+ mass spectrometer (SCIEX, Framingham, USA) equipped with an electro spray ionization source. For quantification (internal standard method, imidacloprid-d4), the estimation of the limit of detection (LOD) and quantification (LOQ) of the analytes were measured using standards in solvent (concentrations: 0.05, 0.1, 0.5, 1, 5, 10, 25, 50, 100 pg μL^{-1}). LOD and LOQ are given in Tables S2. Since no matrix standards were available, the measurements were carried out with standards in solvent and dilute sample extracts. The value given for each sample represents the average of double-injections. All residues were corrected for recovery using the results for the isotopically labeled surrogate standard thiacloprid-d4 in each single sample (SANTE/ 11945/2015).

Frozen residual thiacloprid-containing sugar solutions were thawed, diluted, and measured to control the active ingredient content with LC-MS/MS. By this the concentrations of the solutions used in the experiments were confirmed. The LOD for thiacloprid in diluted sugar solution was 0.05 pg µL-1 and the LOQ 0.1 pg μL^{-1}.

Statistical Analysis

The responses of each bee were scored as binary responses (PER: 1, no response: 0). We used R (R Core Team, 2012) and lme4 (Bates *et al.*, 2015) to perform a generalized linear mixed model (GLMM) analysis of the relationship between PER and Treatment. As fixed effects, we entered Treatment and Trial number into the model, using the binomial error structure with the logit-link function. Bee identity and Session identity were always used as a random factors.The slopes of the response curves in different treatment groups were compared. Several models (with or without interactions between factors) were tested and the best was selected using AIC (Akaike Information Criterion). All models were validated by

assessing normal Q-Q plots and residual versus fitted data plots. For analyzing the learning curves, the last trial was set to become the intercept of the model. Results are presented as parameter estimate ± standard error and associated P-value. P-values showing the influence of a fixed effect were obtained by analysis of deviance table (Type II Wald *Chi-square* tests) between the full model with the effect against the model without the effect. The results of the memory tests were also analyzed using GLMM, using Treatment and Trial as fixed factors. The random factor Order of Odor Presentation was also included in addition to the usual Bee identity and Session identity factors. For each memory test, the factor Order of Odor was tested and no effect was found. Interaction between the fixed factors was included only if it improved the model. We used the Fischer Exact test to compare proportions of bees showing a PER on the last acquisition trial (A3) and on the first memory test (E1) or CS. Sucrose responsiveness was analyzed with GLMM, using Treatment and Sugar Concentration as fixed effects and Bee identity and Session identity as random factors. We used *Chi-square* tests to compare the mortality and US-tests rates between the doses. Comparisons in which $P<0.05$ were considered significant.

Results

Sucrose responsiveness

To determine whether the effects on learning and memory performance could be due to a change in motivational state, we assessed the effects of thiacloprid as active substance and as formulation (Calypso®) on sucrose responsiveness 1 hour after catching the bees.

Independent groups of bees were presented with different sucrose solutions, containing or not 50 ng/µl of thiacloprid, representing the highest concentration tested during the learning and memory tests. Water and increasing concentrations of sucrose containing or not pesticide were presented to the antennae of each bee. Two controls (Control+acetone and Control) were used in order to test for a possible effect of acetone (0.5 % in sucrose solution) on sucrose responsiveness. No difference was found between the two groups (Control *vs* Control+acetone, Fig. 1, -2.03 ± 3.19, P= 0.52; B: -0.53 ± 2.86, P= 0.85).

All groups showed increasing responses to the ascending sugar concentrations. No statistically significant difference was found between the control groups and the thiacloprid group (Fig. 1, -4.74 ± 3.02, P= 0.12) but a significant difference was found between the slopes of the control group and the Calypso® group (-9.25 ± 4.00, P= 0.02). Tukey post-hoc tests revealed significant differences only for the three lowest sucrose concentrations, between 0.1 and 1 % (w/v) (0.1 %: 10.19 ± 3.58, P= 0.02; 0.3 %: 14.42 ± 3.97, P= 0.0015; 1 %: 12.55 ± 4.27, P= 0.017).

No significant repellent effect of Calypso® was thus revealed for concentrations higher than 1 % (w/v) sucrose.

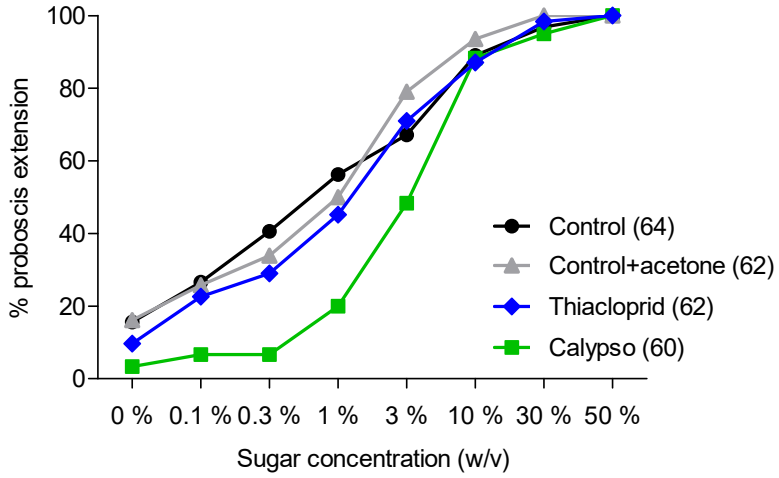

Figure 1. Sucrose responsiveness of control and treated honey bees to different sucrose concentrations. The sucrose solution presented to the bees contained or not (Control), acetone alone (Control+acetone), Thiacloprid as active substance or as formulation (Calypso®) at a concentration of 50 ng.µl^{-1}. The number of individuals in each group is given in brackets in the legend. Significant differences with the control are represented by stars in the legend.

Mortality and US tests

Mortality was evaluated for each dose by the number of bees dying in the time frame between intoxication and the end of the memory tests. The proportion of responses to a 50 % (w/v) sucrose stimulation (US-test) at the end of the memory tests was also assessed. We found no evidence of an increase in mortality as a result of treatment for all doses of thiacloprid and Calypso® used in this study (Table S1). However, we found a significant difference in the proportion of bees responding to the US-test for bees intoxicated with thiacloprid (Table S1, χ^2= 12.93, df= 3, P= 0.0048) or Calypso® (χ^2= 35.21, df= 3, P<0.0001). These differences are due to higher rates of non-responsive bees for the highest dose (200 ng) of Calypso® (24.86 %), and strangely enough, for the control group in the thiacloprid test (16.11 %). The other doses showed no difference with the controls (Table S1).

Appetitive learning

The learning ability was quantified by evaluating the acquisition functions after intoxicating the test bees with 69 ng thiacloprid or with 1.2, 12 and 120 ng thiacloprid in Calypso® 1 hour before onset of conditioning. Three extinction trials were applied in order to test the stability of memory. Control bees learned to associate the conditioned odor with a sucrose reward at a higher level than treated bees (Fig. 2.a and b). The level of acquisition after the third learning trial was significantly lower in bees treated with 69 ng thiacloprid or 120 ng Calypso® (Fig. 2A, thiacloprid: -2.30 ± 0.67, P= 0.02; Fig. 2B, Calypso®: -3.70 ± 0.59, P<

0.0001). The learning rate of bees treated with 12 ng Calypso® was lower than that of controls but not significantly. The learning curves of the control group and the group treated with Calypso® at 1.2 ng did not differ.

Both the control bees and the treated bees from the test with thiacloprid showed a significantly higher PER during the first extinction trial of the memory test 24 hours after acquisition (E1, Fig. 2A) than the last acquisition trial (A3) (Fischer exact test, for all groups: $P < 0.0001$) indicating that memory consolidation took place. The same was observed for the test with Calypso® (Fig. 2B) but the difference was significant only for the highest doses (12 ng: $P= 0.0055$ and 120 ng: $P < 0.0001$). The memory tests indicated that thiacloprid at 69 ng per bee did not significantly change the retention score as compared with the control bees neither during the first (E1) nor the two subsequent extinction trials (E2 and E3) (Fig. 2A). However, bees fed with the highest dose of Calypso® (Fig. 2B) showed a significantly lower retention score during the first extinction test (Fig. 2B, E1) than the controls (120 ng: -3.70 ± 0.59, $P < 0.0001$). No difference was found for the lowest dose (1.2 ng). Overall, the treatment with thiacloprid and Calypso® had a negative effect on learning and memory (thiacloprid: $\chi^2=5.8114$, *df*= 1, P= 0.0159229, Calypso®: $\chi^2=42.352$, *df*= 3, P= $3.378e^{-09}$)

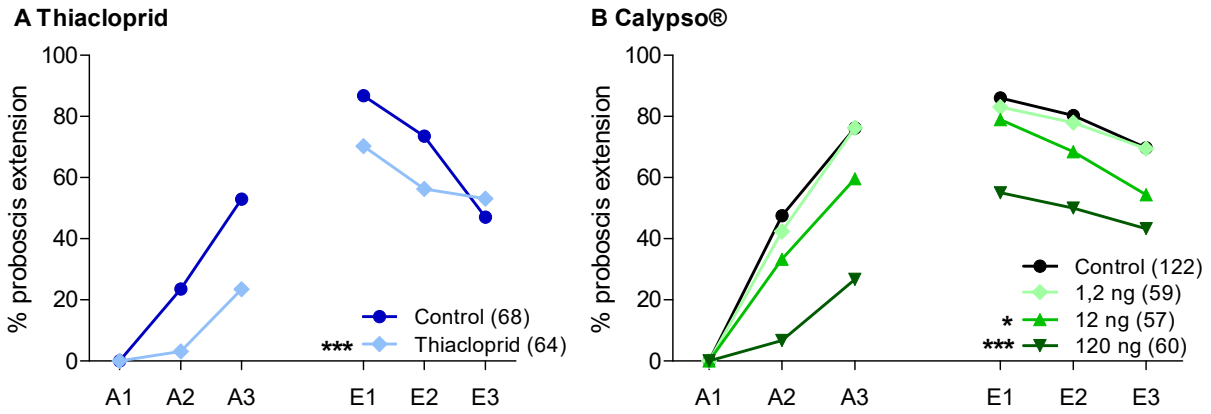

Figure 2. Acquisition functions and retention scores after 24 hours. PER scores were quantified by three acquisition trials (A1, A2, A3) 1 hour after treatment with **A.** 69 ng thiacloprid diluted in sucrose or **B.** 1.2, 12 or 120 ng thiacloprid in Calypso® diluted in sucrose, and three extinction trials (E1, E2, E3) 24 hours after conditioning. Significant differences ($P< 0.05$) with the control are represented by stars in the legend. The number of individuals in each group is given in brackets in the legend.

To further investigate the effects of thiacloprid and Calypso® on learning performance and 24 hour memory retrieval, we replicated this experiment with another range of doses, and also tested the discrimination performance by presenting both the trained odor and two novel odors.

In this particular experiment both control and thiacloprid treated bees learned at very low rates during the three conditioning trials (Fig. 3A1). No significant effects were found between the

acquisition functions of the four groups (χ^2= 0.0019, *df*= 3, P= 0.99998). However, no conditioned response was seen in bees fed with the highest dose of thiacloprid (200 ng) indicating no learning in this group. The control bees from the test with Calypso® (Fig. 3B1) showed better learning rates than the control bees from the test with thiacloprid (Fig. 3A1). Differences in learning performance were detected between bees fed with Calypso® and controls (Fig. 3B1, χ^2= 13.0673, *df*= 3, P= 0.004493) and the highest dose (200 ng) was shown to impair learning the most (-138.3 ± 3.89, P= 0.00038).

Memory tests 24 hours later indicated that control bees in both test series (thiacloprid and Calypso® Fig. 3A2 and B2) responded more to the learned odor 1-nonanol (83 %) than to the rather similar and novel odor nonanal (32 %). The different novel odor 2-hexanol elicited the lowest response (16 %). No significantly different responses were found in the retention trials between the control bees and bees fed with 20 ng or 2 ng thiacloprid (Fig. 3A2) or Calypso® (Fig. 3B2). Bees who received 20 ng of thiacloprid, however, failed to differentiate between the learned odor (Fig. 3B2, A, 40 % PER) and the similar odor (Fig. 3B2, B, 39 % PER). Significantly more bees fed with the highest doses of thiacloprid or Calypso® did not respond to any of the 3 odors (Fig. 3A3 and B3, Fischer exact tests, control *vs* thiacloprid 200 ng: P= 0.0004; control *vs* Calypso® 200 ng: P<0.0001). Less bees from these 2 groups responded only to the learned odor (Fig. 3A2 and A3, Fischer exact tests, control *vs* thiacloprid 200 ng: P= 0.0034; control *vs* Calypso® 200 ng: P= 0.0285). The other groups did not differ from the controls. Bees that ingested 2 ng or 20 ng of thiacloprid showed higher (but not significantly) responses to the dissimilar odor only (Fig. 3A3) than the control bees.

In this latter experiment, the memory of bees that ingested thiacloprid 1 hour before acquisition was not significantly affected by the treatment (χ^2= 5.5862. *df*= 3, P= 0.13357) contrary to bees that ingested Calypso® (χ^2= 10.073, *df*= 3, P= 0.01795). The highest dose of Calypso® significantly impaired memory retrieval (-2.05 ± 0.66, P= 0.002), while the other doses had no significant effects.

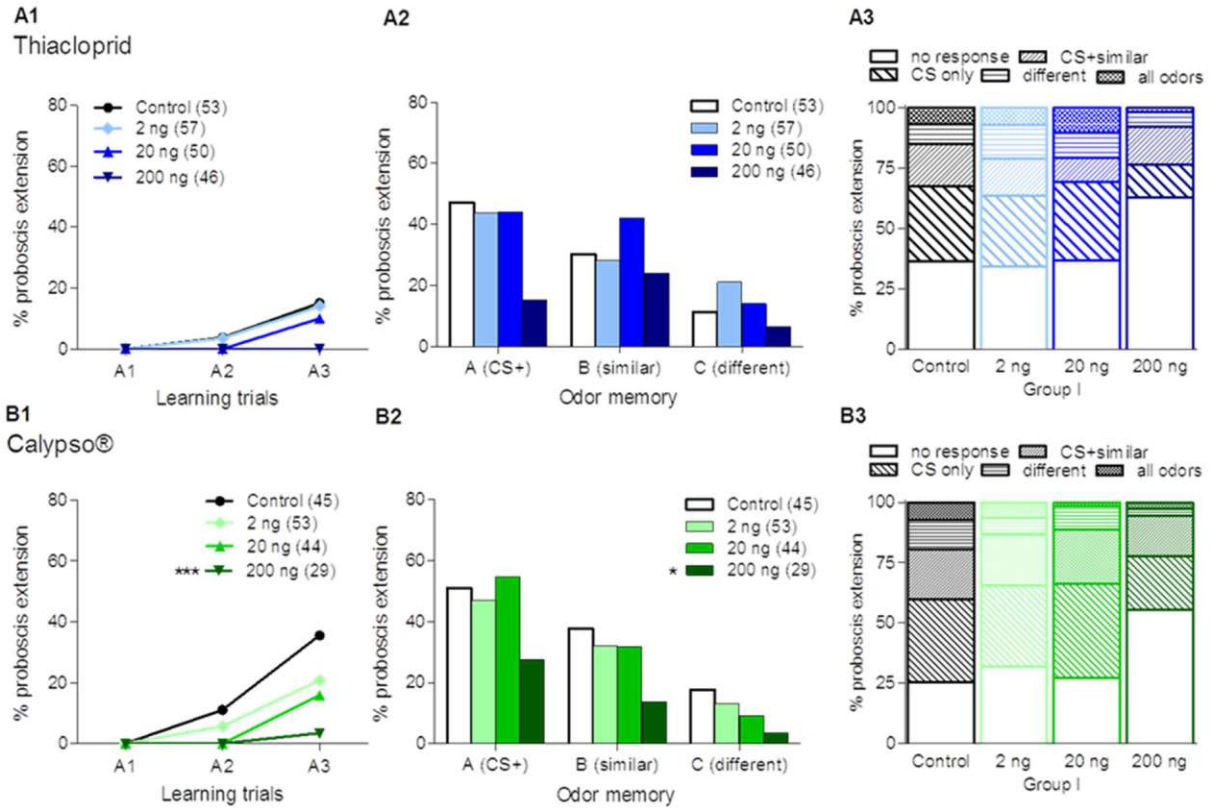

Figure 3. Effect of thiacloprid and Calypso® on acquisition and memory retention. Retention scores were determined 24 h after acquisition and the treatment occurred 1 hour before acquisition with 2, 20 or 200 ng of either **A.** thiacloprid as active substance diluted in sucrose or **B.** Calypso®, a thiacloprid formulation, also diluted in sucrose. **A1/B1**: Acquisition of CS+ (% PER) during the 3 conditioning trials (A1, A2, A3). **A2/B2**: Retention scores (% PER) (A) and generalization (B, C) tests, 24 h after learning. A: CS, conditioned odor (1-nonanol), B: similar odor (nonanal), C: different odor (2-hexanol). **A3/B3**: Distribution of bees according to their individual responses to the odors during the memory tests. Stars in the legend indicate statistically significant differences compared to control ($P<0.05$). The number of individuals in each group is given in brackets in the legend.

Memory Consolidation

In order to investigate the effect of thiacloprid on memory consolidation, treated bees were fed with 69 ng thiacloprid 5 hours after conditioning. Three extinction trials were applied in order to test the stability of memory. No effect on learning was found between the groups (Fig. 4, χ^2= 3.05, *df*= 1, P= 0.08) as treated differently later, indicating that all groups can be compared with respect to memory consolidation.

Memory tests 24 hours after acquisition (=19 hours after intoxication with thiacloprid) showed a significant difference between the control and the treated group. Whereas control bees consolidated their memory overnight, bees treated with thiacloprid showed significantly lower PER for extinction trial 1 (E1) than for A3 (Fischer exact test, P= 0,0001) indicating a loss of the memory consolidation effect. The control group increased its PER to 68.7 % (E1) whereas

the treated group showed only 25 % PER (E1, Fig. 4, control *vs* thiacloprid 19.63 ± 2.59, P< 0.0001) although the last acquisition trial (A3) showed the same PER for both groups. Similar levels of significance were revealed for the two further extinction tests (E2 and E3). Overall, the treatment with 69 ng thiacloprid 19 hours before the memory test had a negative effect on memory retrieval (χ^2= 39.21, *df*= 1, P= 3.8e^{-10}).

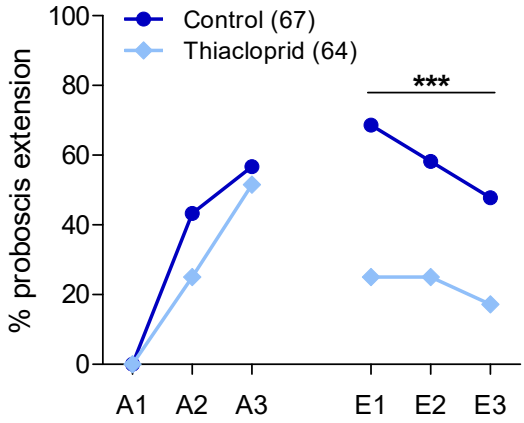

Figure 4. Memory consolidation effect after treatment with thiacloprid. Retention scores were quantified by three extinction trials (E1, E2, E3) testing the probability of PER after odor conditioning 24 hours earlier (acquisition, A1, A2, A3), and 19 hours after treatment (or not, control) with 69 ng of thiacloprid diluted in sucrose. Significant differences (P< 0.05) between the control group and the thiacloprid group are represented by stars in the graph. The number of individuals in each group is given in brackets in the legend.

As for the learning experiment, the memory consolidation experiment was replicated for thiacloprid and Calypso® with a range of doses and with a similar and a different odor in addition to the CS for the memory tests.

No difference was seen between the 4 groups during learning since intoxication with thiacloprid occurred 5 hours after conditioning (Fig. 5A1: χ^2= 0.0688, *df*= 3, P= 0.9953). However, a significant effect of treatment on the acquisition rates was observed in the groups treated with Calypso® (Fig. 5B1: χ^2= 8.7796, *df*= 3, P= 0.03237). This effect cannot be related to the treatment since it occurred later, and thus has to be considered as a random effect. As pointed out above, the rates of acquisition were rather low in these tests leading to stronger random effects and those induced by small differences in hunger dependent motivation.

When tested 24 hours later, bees treated with the highest dose of thiacloprid (200 ng) responded less to the odors than the control bees (Fig. 5A2, -1.48 ± 0.59, P= 0.017). The retention scores to the odors for the 2 other doses were not different from those of the controls. The group treated with the highest dose of Calypso® (200 ng) showed also significantly lower retention scores than the control group (Fig. 5B2, -2.19 ± 0.60, P= 0.00026). Bees fed with the highest doses of thiacloprid or Calypso® did not respond to any of the 3 odors in greater

proportions than control bees (Fig. 5A3, Fischer exact test, control *vs* thiacloprid 200 ng: P= 0.0059; Fig. 5B3, control *vs* Calypso® 200 ng: P<0.0001), and bees treated with 200 ng Calypso® showed significantly lower retention scores for the CS only than the control bees (Fig. 5A3, Fischer exact test, P= 0.0003). Generalization scores were not different between the controls and the Calypso® treated groups with 200 ng when the dissimilar odor was used for the generalization test. However, bees that ingested 20 ng or 2 ng of Calypso® showed higher rates of PER to the dissimilar odor only (C) than the control bees (Fig. 5B3, Fischer exact tests, control *vs* 2 ng, P= 0.0003; control *vs* 20 ng, P= 0.0003).

Taken together, the memory of bees that ingested thiacloprid 5 hours after acquisition was not significantly affected by the treatment (χ^2= 7.3895, *df*= 3, P= 0.06047) contrary to bees that ingested Calypso® (χ^2= 25.057, *df*= 3, P= 1.502e^{-05}). Memory consolidation processes were however affected for bees intoxicated with 69 ng thiacloprid in the preliminary experiment (Fig. 4).

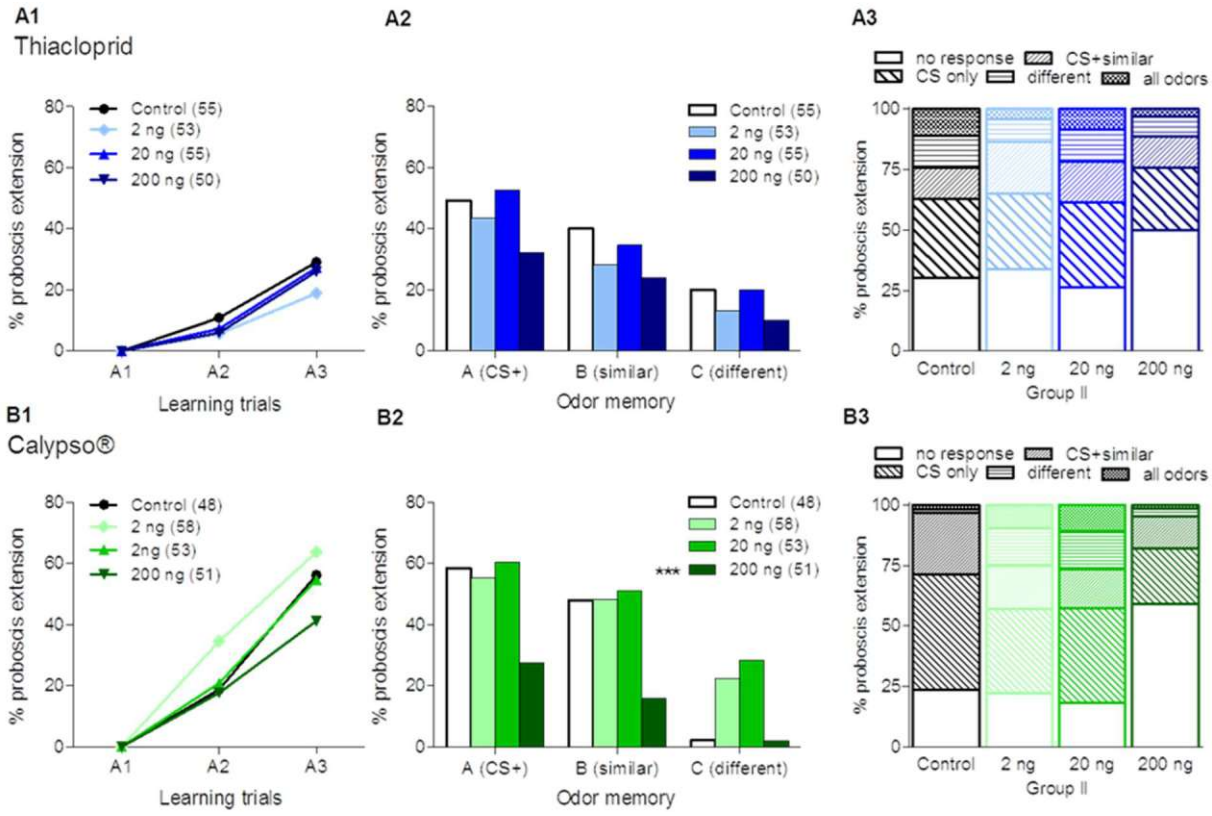

Figure 5. Effect of thiacloprid and Calypso® on memory consolidation. Retention scores were determined 24 h after the last acquisition trial and 19 hours after treatment with 2, 20 or 200 ng of either **A.** thiacloprid as active substance diluted in sucrose or **B.** Calypso®, a thiacloprid formulation, also diluted in sucrose. **A1/B1**: Acquisition of CS+ (% PER) during the 3 conditioning trials (A1, A2, A3). **A2/B2**: Retention scores (% PER) (A) and generalization (B, C) tests, 24 h after learning. A: CS, conditioned odor (1-nonanol), B: similar odor (nonanal), C: different odor (2-hexanol). **A3/B3**: Distribution of bees according to their individual responses to the odors during the memory tests. Stars

in the legend indicate statistically significant differences compared to the control (P<0.05). The number of individuals in each group is given in brackets in the legend.

Memory Retrieval

In order to investigate the effect of thiacloprid on memory retrieval, treated bees were fed with 69 ng thiacloprid 1 hour prior to the memory retrieval test. Three extinction trials were applied in order to test the stability of memory. As expected, the acquisition of the groups treated or not 24 hours later were not significantly different (Fig. 6, $\chi 2$= 0.083, df= 1, P= 0. 36). Retrieval scores revealed great differences between the control and the treated group: 45.5 % of the control and only 11.3 % of the treated bees responded to the CS in the first test (E1, Fig. 6, control vs thiacloprid 4.87 ± 1.02, P< 0.0001). Similar levels of significance were revealed for the two further extinction tests (E2 and E3). On the last extinction trial (E3), none of the treated bees responded to the conditioned odor. Treated bees showed significantly lower retrieval scores during E1 as compared to A3 (Fig. 6, Fischer exact test, P< 0.0001). This was also the case for control bees, but the difference was not significant (P= 0.1571). Overall, the treatment with 69 ng thiacloprid 1 hour before the memory test had a negative effect on memory retrieval ($\chi 2$= 13.29, df= 1, P= 0.0002662).

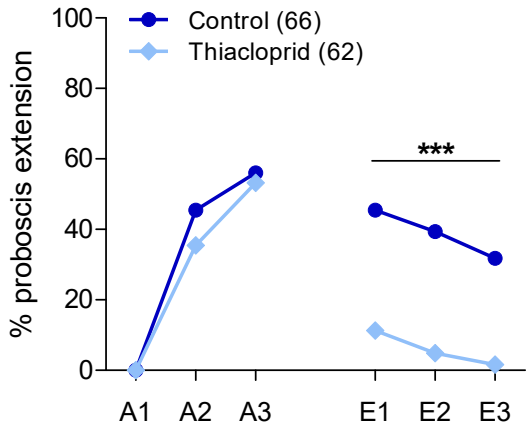

Figure 6. Memory retrieval after treatment with thiacloprid. Retrieval scores were quantified by three extinction trials (E1, E2, E3) testing the probability of PER after odor conditioning 24 hours earlier (acquisition, A1, A2, A3), and 1 hour after treatment (or not, control) with 69 ng thiacloprid diluted in sucrose. Significant differences (P< 0.05) between the control group and the thiacloprid group are represented by stars in the graph. The number of individuals in each group is given in brackets in the legend.

The retrieval tests were replicated for thiacloprid with 3 different doses, and Calypso® was added in these tests also with three different doses. Retention scores were quantified for the CS, and generalization was tested with two new odors (similar and different odors) as in

the experiments reported above on acquisition and memory consolidation. Again treatment was performed 1 hour before retrieval tests.

As expected no effect of treatment was observed during acquisition for all tests as intoxication occurred 24 hours later (Fig. 7A1, χ^2= 4.5033, *df*= 3, P= 0.21200, and Fig. 7B1, χ^2= 3.3456, *df*= 3, P= 0.3413). Learning rates for the thiacloprid test were again observed lower than for the Calypso® test due to an effect of the experimenter.

The retrieval scores revealed significant negative effects of the highest doses (Fig. 7A2, thiacloprid 200 ng: -4.09 ± 1.83, P= 0.025 and B2, Calypso® 200 ng: -2.88 ± 0.90, P= 0.0014) and middle doses (Fig. 7A2, thiacloprid 20 ng: -2.68 ± 1.26, P= 0.036 and B2, Calypso® 20 ng: -2.34 ± 0.91, P= 0.011) of thiacloprid and Calypso®. Less bees from the highest dose of thiacloprid (Fig. 7A3, Fischer exact test, P< 0.0001), the 20 ng dose (P= 0.0327), and the highest dose of Calypso® (Fig. 7B3, P= 0.0279) extended their proboscis to the learned odor only (CS) than the control bees. Significantly more bees fed with 200 ng and 20 ng of thiacloprid or Calypso® did not extend their proboscis to any of the three odors (Fig. 7A3, B3, Fischer exact tests, all groups: P<0.0001). The lowest doses (2 ng) of thiacloprid and Calypso® induced similar memory retrieval rates to the controls except for the similar odor in the Calypso® test to which Calypso® at 2 ng had a similar PER than at 200 and 20 ng.

Overall, memory retrieval was compromised after treatment with either thiacloprid or Calypso® 1 hour before the retrieval tests (thiacloprid: χ^2= 11.9171, *df*= 3, P= 0.007673, Calypso®: χ^2= 12.171, *df*= 3, P= 0.006819). Negative effects were also observed for bees intoxicated with 69 ng thiacloprid in the preliminary experiment (Fig. 6).

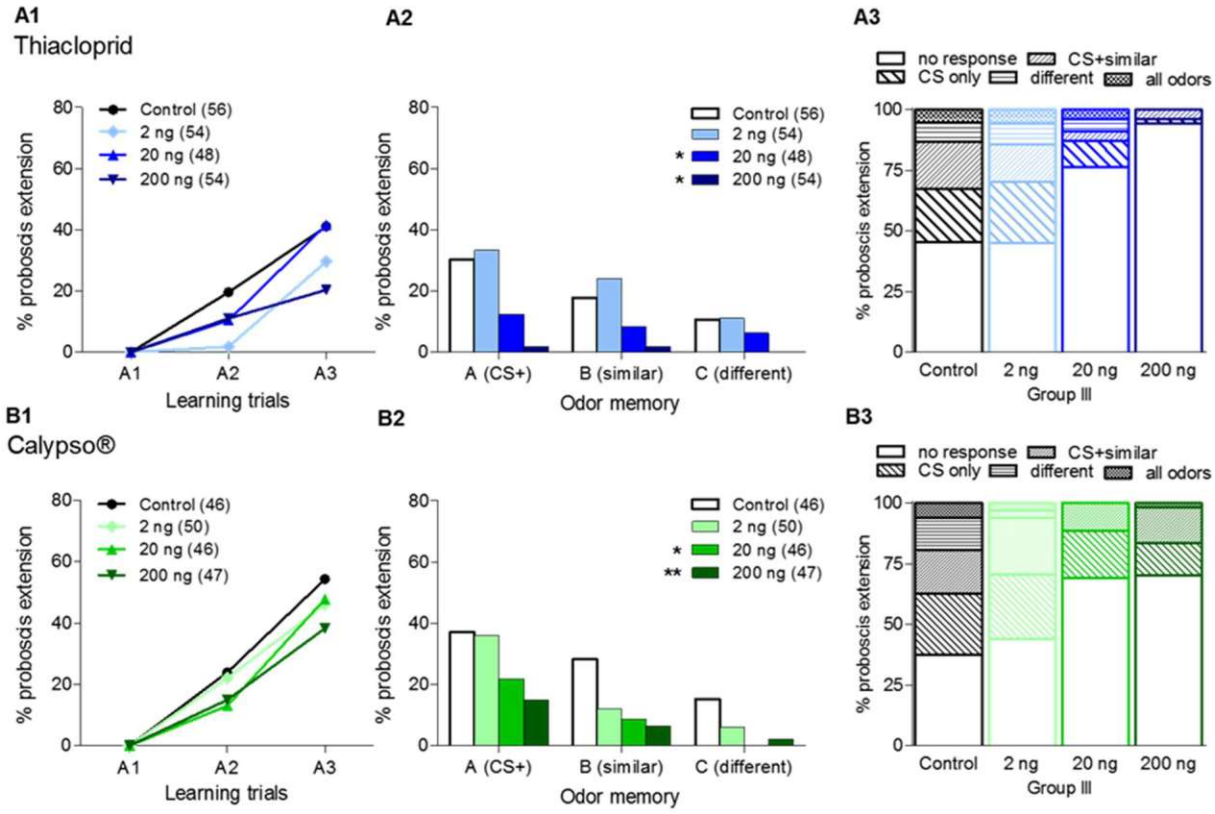

Figure 7. Effect of thiacloprid and Calypso® on memory retrieval. Retention scores were determined 24 h after acquisition and 1 hour after treatment with 2, 20 or 200 ng of either **A.** thiacloprid as active substance diluted in sucrose or **B.** Calypso®, a thiacloprid formulation, also diluted in sucrose. **A1/B1**: Acquisition of CS+ (% PER) during the 3 conditioning trials (A1, A2, A3). **A2/B2**: Retention scores (% PER) (A) and generalization (B, C) tests, 24 h after learning. A: CS, conditioned odor (1-nonanol), B: similar odor (nonanal), C: different odor (2-hexanol). **A3/B3**: Distribution of bees according to their individual responses to the odors during the memory tests. Stars in the legend indicate statistically significant differences compared to the control ($P<0.05$). The number of individuals in each group is given in brackets in the legend.

Residue analysis

The identification and quantification of thiacloprid residues in the body of the test bees was performed using LC-MS/MS (Fig. 8, recovery adjusted to 100 %, see Methods and Table S2). The same bees as the ones used in the tests presented in Fig. 3, 5 and 7 were used for residue analysis. The amount of thiacloprid residues found in the bees from the thiacloprid and the Calypso® groups are correlated with the dose of pesticide fed to the bees (200, 20 or 2 ng/bee) and with the time of intoxication (Intox groups I, II and III). Since all bees were killed directly after the memory test for residue analysis the pesticides were metabolized over different periods of time in the bee body.

The maximum amount of residues was found in bees intoxicated with 200 ng thiacloprid or Calypso® 1 hour before the memory test (2 hours before sample collection). Among bees

intoxicated with 200 or 20 ng, the higher amount of residues was always found in bees from intox group GIII. For Calypso® at 200 ng, the amount of residues found in samples from group III was 2.1 times higher than in group II and 3.5 times higher than in group I. For thiacloprid at 200 ng, residues in group III were 3.9 and 5.8 times higher than in groups II and I respectively (Fig. 8 and Table S2). The residues found for the middle dose of the Calypso® samples were 7.8 times higher in group III than in groups II and I. For thiacloprid at the same dose, the residues in group III were 6 and 9 times higher than in group I and group II respectively. However, no clear difference was seen between the intox groups I and II in bees intoxicated with 20 ng and between any of the 3 intox groups with the lowest dose (2 ng) of thiacloprid or Calypso®.

Notice that the scales in Fig. 8 are not the same for all 3 doses. The amounts of residues found in bees intoxicated with 200 ng were about 10 times higher than the amounts found in bees intoxicated with 20 ng. This corresponds to the order of difference between the 2 applied doses but not for the 20 and 2 ng doses.

It is also interesting that the amounts of thiacloprid residues are always higher for the Calypso® group than for the thiacloprid group (except for the 2 ng dose) despite the fact that bees were intoxicated with the same dose of thiacloprid (the concentration in thiacloprid of the sucrose solutions were verified by LC-MS/MS).

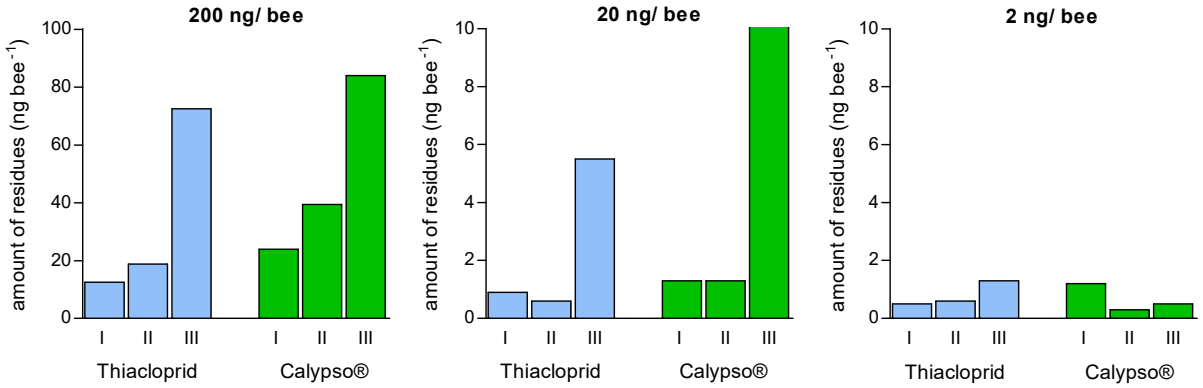

Figure 8. Pesticide residue analyses of honey bees intoxicated with thiacloprid or Calypso® at 200, 20 or 2 ng/bee. Treatment was administrated orally 1 hour before learning (I), 5 hours after learning (II) or 1 hour before the memory test (III). The identification and quantification of thiacloprid was performed using LC-MS/MS.

Table S2 gives details about the amount of residues found in the different body parts of the bees. Except for 2 samples (Calypso® 20 ng II and 2 ng I), the amount of thiacloprid residues were always highest in the bee heads, and they were usually lowest in the thoraces. Bees intoxicated with Calypso® and thiacloprid at 200 ng 1 hour before the memory test showed amounts of residues in the bee heads from 2667.6 to 2957.5 ng/g respectively (Tables

S2). The amounts of thiacloprid (200 ng) found in the thoraces and abdomens were 10 and 6 times respectively lower than in the heads of the thiacloprid treated group 1 hour before the memory test (III). Usually, the closer in time the bees were intoxicated before the memory test, the higher were the amounts of residues. The residues in the heads of bees intoxicated 1 hour before the memory test (2957.5 ng/g) with 200 ng thiacloprid were 6 times and 12 times higher than the residues in the heads of bees intoxicated with the same dose respectively at 19 hours (493.9 ng/g) and 24 hours (241.9 ng/g) before the memory test. The same kind of dose-dependent scheme applied for Calypso® at the middle dose (20 ng). For the lowest dose (2 ng) the amounts of residues were between 1.6 and 46.1 ng/g depending on the time of intoxication and the body part. The time and dose relationship does not seem to apply for this low dose. Furthermore, thiacloprid residues in the range of 0.5 to 13.7 ng/g were also found in the control samples (Table S2). This point will be discussed later.

Discussion

Memory is defined as the ability of an animal to save individually acquired information and retrieve it in the future when needed. In the context of associative learning this means that the CS will elicit the learned response under the control of acquired information. The neural processes involved are highly sensitive to alterations of molecular, cellular, and neural properties of the networks forming and retrieving the respective memory. Here we focused on the neonicotinoid thiacloprid whose adverse effects on memory retrieval during navigation was documented by Fischer *et al.* (2014) and Tison *et al.* (2016). The use of a powerful laboratory training paradigm, the PER conditioning, allows us to show that thiacloprid, fed to the bees as active substance or as formulation (Calypso®) negatively affects appetitive olfactory associative learning, consolidation, and retrieval of memory in honey bees (*Apis mellifera*).

No increased mortality was revealed between control and treated bees in any of the groups studied in the learning and memory tests. This confirms that all chosen doses are sublethal because they do not induce direct mortality of the test animals. However, tests of the unconditioned responses to sucrose revealed that animals intoxicated with the highest doses of Calypso (200 ng/bee) responded less to the US than the control bees, possibly reducing the appetitive strength of the rewarding stimulus. The strength of the appetitive sucrose stimulus could lead to reduced learning (Scheiner *et al.*, 2005; Tan *et al.*, 2014) during acquisition tests. This does not apply to the memory tests since bees were treated after acquisition. An aversive taste of the substance can be excluded as no difference in the sucrose responsiveness was revealed for 30 and 50 % (w/v) sucrose solutions contaminated or not with 200 ng Calypso® (Fig. 1). An acute alteration of the motor function (Williamson *et al.*, 2013; Williamson *et al.*, 2014) would be the most probable hypothesis as reduced US responses were actually observed in bees from Intox group III, intoxicated with 200 ng Calypso® 1 hour before the memory test.

We found that thiacloprid and Calypso® reduce acquisition at the highest dose used in the respective experiment (69 ng/bee thiacloprid and 120 ng/bee Calypso® in Fig. 2, and 200 ng/bee Calypso® in Fig. 3). The lack of a significant effect of thiacloprid in the latter experiment (Fig. 3) may be due to the low learning rate of all bees in this experiment. Most importantly, the retention scores of the treated bees in both experiments were also significantly lower in these respective groups indicating a learning effect rather than a motor effect. An aversive taste or odor of thiacloprid or Calypso® at these doses (200 ng) or lower doses can be excluded since no such effect was found in Fig. 1 or in a previous study with PER tests and free-flying bees (Tison *et al.*, 2016). The reduced appetitive strength of the rewarding stimuli

containing thiacloprid cannot be disentangled from direct effects on the associative process. Taken together, the inhibitory effects on appetitive learning are unlikely to result from direct impairment of neural circuits involved in aversive taste or motor performance. In any case these doses compromise associative learning and as a consequence lead to reduced memory (Fig. 2 and 3).

Decourtye *et al.* (2004b) assume that the consolidation process which ensures the transfer from short-term memory to medium-term memory within 10–15 min after the conditioning trial (Menzel, 1999; Erber *et al.*, 1980) was compromised by imidacloprid in their experiment. We chose to study the effects of thiacloprid on the transfer from middle-term memory to early long-term memory. The intrinsic neurons of the mushroom body, the Kenyon cells play a particularly important role here (Szyszka and Menzel, 2005). Kenyon cells express the main target of thiacloprid (and neonicotinoids in general), the nAChR, at their input sites (Bicker and Kreissl, 1994; Goldberg *et al.*, 1999; Déglise *et al.*, 2002). As a partial agonist of nAChR, thiacloprid could first increase and then decrease cholinergic signaling by competing with the transmitter acetylcholine (ACh) and then by blocking the receptor binding sites (Déglise *et al.*, 2002). It is likely that the input site of the mushroom body is the prominent target of its action on memory consolidation (and memory retrieval, see below). Thiacloprid or Calypso® taken up 5 hours after acquisition and 19 hours before memory retention tests lead to dose dependent loss of retention (Fig. 4 and 5). We selected an interval of 5 hours after acquisition because middle-term memory is converted to early long-term memory during the following period of time (Menzel, 1999; Müller, 2002). Treatment with 69 ng/bee thiacloprid led to significantly reduced retention scores in the experiment of Fig. 4, but only hints of an effect at even higher doses in the experiment of Fig. 5A. Learning performance was particularly low in the latter experiment and normal in the experiments of Fig. 4. We, therefore, consider the results in Fig. 5 less reliable because in the case of Calypso® (200 ng) treatment, learning performance was close to normal, and lead to a significant reduction of retention (Fig. 5B). However, another possibility cannot be ruled out since consolidation processes do not follow only in sequences but also partly in parallel. This applies particularly for the transition to early long-term memory which can be reached either directly from short-term memory or via middle-term memory (Menzel, 1999; Müller, 2002). If the parallel processes are differently affected by thiacloprid and Calypso® (containing also other components than thiacloprid), and different bees may by some unknown reason differ with respect to the sensitivity of these processes, this could explain why some treated bees could still be able to retrieve the memory to the CS. We also asked in these experiments whether the memory content is changed or weakened by the uptake of thiacloprid or Calypso®. To this end we determined the retention scores not only for the trained odor (CS) but also for two other odors, a similar and a different one (Fig. 5B, C).

We found that Calypso® treatment led to a changed generalization gradient for treatment with 2 or 20 ng/bee. The different test odor is not responded to in the control group because these bees discriminate well between the learned odor and the different test odor whereas the 200ng/bee Calypso® treated bees did not respond to the different odor because they did not remember the learned odor.

Memory retrieval to the CS was reduced when the bees were stimulated with the CS 1 hour after treatment with thiacloprid at 20, 69 or 200 ng/bee and with Calypso® at 20 and 200 ng/bee. Retrieval from navigational memory was found to be reduced after acute and chronic treatment with thiacloprid for long-term memory and not for recently stored memory (Fischer *et al.*, 2014; Tison *et al.*, 2016) corroborating our findings here. Taken together, thiacloprid and its formulation Calypso® clearly interfere with processes involved in memory formation and memory retrieval. In the case of a disturbance of the consolidation phase by an intoxication with thiacloprid, tested bees do not remember the odor when stimulated with the CS since they do not possess the memory of it. However, honey bees which do not remember the odor in the memory retrieval test, possess the memory of the odor, but intoxication with thiacloprid 1 hour before the test prevent them from retrieving it, because access to the stored memory is blocked. Our data support the view that the normal function of the nicotinic transmission at the input site of the mushroom bodies is essential for the transition from middle-term memory to early long-term memory and for the read-out from memory. This interpretation is supported by the findings of Himmelreich and Grünewald (2012) and Gauthier and Grünewald (2012) with the exception that the latter authors did not find an effect on memory retrieval when they manipulated the cholinergic transmission.
The multiple tests applied in the retrieval experiments (Fig. 2, 4, 6) allow to address the question, whether extinction learning is compromised after treatment because repeated exposure to the CS without reward leads to the acquisition of a new condition, namely that the CS has changed its value and is now not rewarded anymore (Eisenhardt, 2012; Bitterman *et al.*, 1983). No effect of thiacloprid or Calypso® was found, indicating that this form of learning is not compromised.

The control and treated groups from an experiment as well as the different doses were always tested blindly and in parallel. A difference in the proportion of control bees learning the CS was noticeable between the first set of experiments (Fig. 2, 4 and 6) and the second (Fig. 3, 5 and 7). The latter learned the CS in lower proportions. This can be the result of different factors like the year of the test (2015 oder 2016), the odor used for conditioning (hexanal or 1-nonanol respectively), the bee colonies, the weather conditions and the experimenters performing the tests. The experimenters performing the tests with Calypso® or thiacloprid in

Fig. 3, 5 and 7 clearly had differences in the perception of the PER during acquisition as showed by the different learning rates but rather similar retention scores.

The amounts of residues quantified in the thiacloprid and Calypso® samples by LC-MS/MS indicate effects of both the dose and the time of exposure thus documenting metabolization of thiacloprid in the body of honey bees (Fig. 8 and Table S2). Shorter time between the oral intoxication and the memory tests as well as higher doses were correlated with higher amounts of thiacloprid found in bees. The bee heads were the organs containing the maximum amounts of residues, suggesting a persistence of the substance in the tissues targeted by thiacloprid (i.e. nAChR receptors in the bee brain).

We showed that Calypso® had a repellent effect for sucrose concentrations < 1 % (w/v) (at 50 $ng.\mu l^{-1}$) and stronger detrimental effects on learning and memory than the active substance thiacloprid alone at the same doses. This suggests that additional hazardous components of the formulation might play a role in impairing honey bees' sucrose perception, learning, and memory. We could also see higher amounts of thiacloprid residues in the Calypso® treated bees than in the respective animals treated with similar doses of thiacloprid alone. This could be explained by the fact that agrochemical formulations also contain inerts, which can be found at higher amounts than the active ingredients. Adjuvants added to sprays to improve coverage, penetration, or rain fastness of pesticides are likely to penetrate the waxy cuticle of bees and thus increase the toxicity of other chemicals (Mullin *et al.*, 2015). Calypso® safety data sheet (Bayer CropScience Safety Data Sheet) cites 1,2-benzisothiazol-3(2H)-one as a hazardous component of the formulation. This substance is active against bacteria and fungi and for in-can preservation of pesticide emulsions (DOW, Product Safety Assessment). Other components of the formulation could also be responsible of enhancing the toxicity of thiacloprid. 'Inerts' in pesticide formulations are usually not disclosed by the companies because hidden under the cloak of 'trade secret' (Mullin *et al.*, 2015; Cox and Surgan, 2016). Future experiments will have to test 'inerts' separately on honey bees and representative native pollinators revealing the additive and potentially potentiating effects on the action of the active substance (Mullin *et al.*, 2015; Mullin *et al.*, 2016).

Interestingly very low amounts of thiacloprid residues were also found in the control samples (Table S2). As control samples were always processed before the treated samples, contamination of the samples during residue analysis is excluded. The most probable explanation is natural contamination of foragers from the apiary. Thiacloprid is one of the pesticides most commonly found in apiaries, detected in 64 % of nectar/honey samples (Sanchez-Bayo and Goka, 2014). Also, in a recent study, 42.9% of soil samples were tested positive for thiacloprid, though this compound had not been applied in the previous three years (Botías *et al.*, 2015). Calypso® is a widely used thiacloprid-based formulation in agricultural

fields but also in gardens. Private gardens and a small agricultural area are present around the institute as well as the botanical garden of Berlin, 500 meters from the institute.

This study identified the threshold dose for sublethal effects of thiacloprid on appetitive learning as 69 ng of thiacloprid ingested per bee. An effect on memory retention was seen when 69 ng of thiacloprid was given to bees 5 hours after learning thus documenting effects on memory consolidation. Memory retrieval tested by treatment one hour before the memory test was compromised at doses as low as 20 ng of thiacloprid or Calypso®. Compared with the LD50 doses of oral toxicity of thiacloprid (17320 ng per bee, OEPP), serious sublethal detrimental phenomena were found at much lower doses, ~ 250 to 800 times lower than the LD50.

The test bees were restrained in tubes during 24 hours, a rather unnatural situation. Under field conditions bees have the opportunity to fly freely during foraging, potentially increasing metabolization of the pesticide (higher uptake during flight). The effects on learning and memory reported here could thus be magnified in the field especially in the case of a chronic exposure, more realistic under natural conditions, since thiacloprid would accumulate in the bee bodies over time (Tison *et al.*, 2016). In our previous study, we revealed negative effects of thiacloprid on navigation and foraging behavior (Tison *et al.*, 2016) and interpreted these effects as retrieval blocks of a long-term memory established during orientation flights (Degen *et al.*, 2015). In the context of the data presented here it is also likely that not only the retrieval of a remote memory is impaired but also learning and memory consolidation. Foraging for food is a demanding task that requires the bees to accurately learn and remember which flowers offer the best rewards (Lihoreau *et al.*, 2011). It has been argued that laboratory learning tests are good predictors of foraging efficiency under natural conditions (Raine and Chittka, 2008). As a consequence honey bees exposed to thiacloprid inside the hive via the stored food or outside when foraging on contaminated flowers, are expected to see their learning and memory performances impaired, leading to negative effects on a whole range of different neural and behavioral processes necessary for the survival of the colony (Desneux *et al.*, 2007; Eiri and Nieh, 2012; Fischer *et al.*, 2014; Henry *et al.*, 2012; Schneider *et al.*, 2012; Tison *et al.*, 2016; Yang *et al.*, 2008). This implies that commonly used neonicotinoids are strong candidates for the observed decline in efficiency of pollinators´ populations and that pesticide formulations seem to pose an additional risk to pollinators. Evidence that sublethal doses of thiacloprid are having such negative effects at much lower levels than its LD50 raises important and challenging questions for agricultural management.

List of symbols and abbreviations

ACh acetylcholine

AChE acetylcholinesterase

CS conditioned stimulus

LD50 Lethal Dose 50

nAChR nicotinic acetycholine receptor

PER proboscis extension response

US unconditioned stimulus

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Supplementary Information content

Information about Mortality and response to the US test of bees intoxicated with thiacloprid or Calypso (Table S1), Pesticide residues analysis of honey bees exposed to thiacloprid, as active substance and formulation (Table S2).

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Supporting Information

Table S1. Mortality and response to the US test of bees intoxicated with thiacloprid or Calypso

treatment	% of bees dead after intox	significance (Chi square)	% of bees not responsive to US test (alive bees only)	significance (Chi square)
control	0.45	χ^2= 1.009, *df*= 1,	4.52	χ^2= 1.53, *df*= 1,
thiacloprid 69 ng	1.35	P= 0.3151	7,31	P= 0.2161
control	3.10		0.80	
Calypso® 120 ng	0.00	χ^2= 2.338, *df*= 3,	0.00	χ^2= 1.517, *df*= 3,
Calypso® 12 ng	1.56	P= 0.5053	0.00	P= 0.6784
Calypso® 1.2 ng	3.17		0,00	
control	4.26		16.11	
thiacloprid 200 ng	2.23	χ^2= 4.196. *df*= 3,	8.00	χ^2= 12.93. *df*= 3,
thiacloprid 20 ng	3.70	P= 0.2410	6.04	**P= 0.0048**
thiacloprid 2 ng	1.08		7.65	
control	3.16		5.88	
Calypso® 200 ng	1.70	χ^2= 7.63. *df*= 3,	24.86	χ^2= 35.21. *df*= 3,
Calypso® 20 ng	4.05	P= 0.054	10.84	**P<0.0001**
Calypso® 2 ng	0.00		7.34	

Significant p-values (< 0.05) are showed in bold letters.

Table S2. Pesticide residues analysis of honey bees exposed to thiacloprid active substance and formulation

sample		sample weight (mg) *	thiacloprid residues (ng/g) corrected by thiacloprid-d4 recoveries			(ng/bee)		thiacloprid-d4 recoveries (%)		
		n= 30	**head**	**thorax**	**abdomen**	**whole body**	**whole body**	**head**	**thorax**	**abdomen**
200 ng/bee										
thiacloprid	**I**	3181.4	241.9	12.9	164.5	**419.4**	**12.5**	89	74	75
	II	3131.5	493.9	110.5	174.1	**778.5**	**18.8**	78	69	66
	III	3352.3	2957.5	302.8	477.8	**3738.1**	**72.5**	73	85	69
Calypso®	**I**	3407.1	309.8	92.5	270.4	**672.6**	**23.9**	77	69	64
	II	3284.2	520.0	105.0	510.0	**1135.0**	**39.4**	83	68	52
	III	3148.0	2667.6	362.5	751.5	**3781.6**	**84.0**	83	67	72
20 ng/bee										
thiacloprid	**I**	3086.3	16.7	5.0	9.1	**30.8**	**0.9**	70	57	58
	II	3222.1	19.1	3.1	5.0	**27.2**	**0.6**	62	51	64
	III	3256.0	159.6	25.8	49.8	**235.1**	**5.5**	70	55	54
Calypso®	**I**	3208.0	16.9	9.3	14.1	**40.3**	**1.3**	74	76	64
	II	2999.0	12.8	6.2	17.8	**36.8**	**1.3**	73	57	77
	III	3259.0	388.7	55.1	67.2	**511.1**	**10.2**	78	67	63
2 ng/bee										
thiacloprid	**I**	3231.6	11.1	3.7	4.1	**18.9**	**0.5**	76	63	63
	II	3199.1	9.5	2.8	6.3	**18.7**	**0.6**	64	63	62
	III	2908.6	46.1	9.5	12.3	**67.9**	**1.3**	68	63	53
Calypso®	**I**	3110.1	16.4	20.5	3.9	**40.8**	**1.2**	92	72	72
	II	3223.0	7.5	2.7	1.6	**11.8**	**0.3**	82	37	81
	III	3149.3	15.9	3.9	4.2	**23.9**	**0.5**	76	75	66
control	**I**	2961.6	13.7	3.2	2.4	**19.4**	**0.4**	77	69	69
	II	3071.4	6.3	3.1	8.1	**17.5**	**0.6**	71	70	55
	III	3159.5	7.3	3.1	0.5	**10.9**	**0.2**	68	59	86
LOD §			0.2	0.05	0.04					
LOQ §			0.4	0.1	0.07					

* The sample weight is the sum of the weights of the separated analyzed honey bee body parts.

§ LOD. limit of detection (3 times background noise); LOQ. limit of quantification (10 times background noise). The calculation is based on an average weight of 30 bee body parts each.

Chapter 3

Detrimental effects of clothianidin revealed in field and laboratory studies

Léa Tison, Vanda Púčiková, Alexander Rößner, Susan Gerschewski, and Randolf Menzel

Free University Berlin, Institute for Biology-Neurobiology, D-14195 Berlin, Germany

Corresponding author: E-mail: lea.tison@gmail.com.

Author contributions

L.T and V.P performed the field experiments and analyzed the pesticide residues. L.T, A.R and S.G performed the PER experiments. L.T and R.M designed the experiments. L.T analyzed the data and wrote the manuscript.

Abstract

Ongoing losses of pollinators are of significant international concern because of the essential role they have in our ecosystem, agriculture, and economy. Both chemical and non-chemical stressors have been implicated as possible contributors to their decline but the increasing use of neonicotinoid insecticides has recently emerged as particularly concerning. Here we exposed honey bees orally to sublethal doses of the neonicotinoid clothianidin in the field and in the lab to assess its effects on the foraging behavior, homing success, and learning and memory performances. We found that the foraging span, foraging activity, and recruitment rates at the contaminated feeder decreased significantly due to the chronic exposure to clothianidin at field-realistic concentrations. After analyzing the residues found in honey bee body parts, we found that clothianidin residues were accumulating in their abdomens over time. No difference was found in the homing success nor the flight duration of control and treated bees released at an unexpected location in the field study. A negative effect of the temperature and an influence of the location of the experiment or feeder was however revealed. In the laboratory, Proboscis Extension Response (PER) conditioning revealed a negative impairment of the consolidation and retrieval of the memory of honey bees exposed orally to sublethal doses of clothianidin. We conclude from these results an adverse effect of both acute and chronic exposure to sublethal doses of clothianidin on the foraging behavior and memory performances of honey bees.

Introduction

Pollinating insects contribute significantly to agricultural productivity and the importance of their conservation is no longer up for debate (Klein *et al.*, 2007; Potts *et al.*, 2016). The prevalent use of pesticides in crop protection and especially the extensive use of neonicotinoids as prophylaxis measure in agriculture is suspected of posing a threat to pollinating insects (Brittain and Potts, 2011; van der Sluijs *et al.*, 2013). Indeed, sublethal doses of neonicotinoids were already shown to compromise a large range of behaviors in honey bees (Desneux *et al.*, 2007; Eiri and Nieh, 2012; Fischer *et al.*, 2014; Henry *et al.*, 2012; Schneider *et al.*, 2012; Tison *et al.*, 2016; Yang *et al.*, 2008).

Clothianidin is a neonicotinoid insecticide acting against sucking and chewing pest insects. It is mostly used for coating seeds, but also as foliar and soil application in a variety of crops. Clothianidin is also a component in several other commercial insecticides and the metabolite of another widely used neonicotinoid, thiamethoxam (Nauen *et al.*, 2003). It is an agonist of the nicotine acetylcholine receptors (nAChR) and by binding to the receptor of the neurotransmitter acetylcholine on the post-synaptic membrane, it interferes with the normal transduction of the neural stimulus (Tomiwaza and Casida, 2005). The nAChRs are present in the bee brain, in many areas associated with mechanosensory antennal information, learning and memory formation (Gauthier, 2010). Palmer *et al.* (2013) showed that the neonicotinoids clothianidin and imidacloprid at concentrations < 10nM had a depolarizing effect on the nAChRs in the Kenyon cells. An inhibition of the formation of action potential leads ultimately to the loss of functional capacity of these cells, principal neural components of the mushroom bodies (Szyszka *et al.*, 2005). The consequence would be an impairment of the cognitive abilities that are dependent on this higher order brain structure. Mushroom bodies are essential for associative learning, memory formation, integration of multisensory information, and spatial orientation (Heisenberg, 2003; Menzel, 2012; Palmer *et al.*, 2013).

The EFSA (European Food Safety Authority) identified a risk of clothianidin to bees exposed to contaminated dusts and residues in nectar and pollen from rape (EFSA, 2013). Because of the systemic properties of neonicotinoids, insects can be exposed chronically and acutely in the field. Whereas residues in pollen and nectar result most of the time in chronic exposure of forager bees, residues in water puddles, guttation drops, or in dust drift can lead to acute exposure of foragers or honey bee colonies. The intake from nectar and pollen residues from oilseed rape, at the lowest and highest maximal application rate was estimated by the EFSA (2013) to be respectively 4.27 ng and 13.65 ng per forager bee in one day, both estimations being above the endpoint of acute oral toxicity for clothianidin ($LD50_{48h}$ = 3.7 ng/bee). Our lab and field experiments were conducted with field-realistic concentrations or doses, similar or lower to the EFSA (2013) estimations and to the doses used in several other

studies (Schneider *et al.*, 2012; Fischer *et al.*, 2014; Piiroinen and Goulson, 2016). Reported values of the maximum amounts of clothianidin residues found in the nectar of treated crops vary from 1 to 14 ppb with the average values ranging from 0.3 to 5.4 ppb (Sanchez-Bayo and Goka, 2014; Bonmatin *et al.*, 2015; Botías *et al.*, 2015; Rundlöf *et al.*, 2015).

In 2008, the registration of clothianidin for use on seed corn was revoked in Germany after an incident that resulted in the death of millions of nearby honey bees (Pistorius *et al.*, 2009). The substance is currently subject to a moratorium in the EU, with 2 other neonicotinoids: imidacloprid and thiamethoxam. In recent research, the effects of clothianidin produced variable results depending on the type of experiment (lab or field), the species studied (honey bees, bumble bees, solitary bees), the doses, and the clear competing interests behind some studies.

In this study, we exposed forager honey bees chronically in the field to 4.5 and 9 ppb clothianidin in sucrose solution in order to investigate its effects on foraging behavior and homing success. We have shown in an earlier study that thiacloprid, another neonicotinoid, had dramatic consequences on these behaviors in the field (Tison *et al.*, 2016). Other laboratory and field studies have shown negative effects of clothianidin in honey bees and other bee species (Jin *et al.*, 2015; Brandt *et al.*, 2016; Schneider *et al.*, 2012; Fischer *et al.*, 2014; Piiroinen and Goulson, 2016). However, the effects of clothianidin on individual bees and on colonies have been revealed highly variable and a source of debate (Cutler and Scott Dupree, 2007; Cutler *et al.*, 2014; Franklin *et al.*, 2004; Rundlöf *et al.,* 2015; Rolke *et al.*, 2016; Schmuck and Lewis, 2016). Honey bees forage as single animals, therefore, testing single individuals for their foraging abilities and homing success represents well the conditions faced by bees in nature.

To study the effects of an acute intoxication with clothianidin on different phases of learning and memory, we used the Proboscis Extension Response (PER) conditioning paradigm and 3 different sublethal doses (0.1, 0.3 and 0.8 ng/bee). In the olfactory conditioning, the odor represents the conditioned stimulus (CS) and sucrose the unconditioned stimulus (US). During conditioning, the initially neutral CS becomes associated with the US and subsequently elicits a response, previously elicited only by the US (Bitterman *et al.*, 1983). This olfactory conditioning paradigm has been successfully used over the last 60 years in order to study the processes of learning and memory in honey bees (Decourtye *et al.*, 2005; Williamson and Wright, 2013). Clothianidin was shown to affect learning ability in honey bees (Palmer *et al.*, 2013; Piiroinen and Goulson, 2016). Here we are also testing the effects of clothianidin on memory consolidation and retrieval. Olfactory memory plays an important role in many aspects of honey bee behavior, including recognition of nestmates, foraging, food preferences, social communication, and navigation (Menzel and Müller, 1996). Any disruption in olfactory learning and memory may result in a negative impact on bees' foraging performance (Farooqui, 2013).

Until now, no study has investigated the effects of a neonicotinoid insecticide on such a large range of behaviors through exposing animals to acute doses and chronic concentrations, in addition to building a bridge between lab and field experiments. We found that clothianidin at the sublethal concentrations and doses tested impaired the foraging behavior of bees chronically exposed in the field as well as the learning and memory performances of honey bees acutely intoxicated in the laboratory.

Material and methods

Clothianidin solutions

Stock solution: 10 mg clothianidin ((*E*)-1-(2-chloro-1,3-thiazol-5-ylméthyl)-3-méthyl-2-nitroguanidine, Sigma-Aldrich) diluted in 1 mL acetone (≥99.9 %, Sigma-Aldrich) plus 39 mL distilled water leading to a concentration of 0.25 g/L. Acetone was chosen as the solvent following the EPPO guidelines (1992). The control group was fed sucrose solution without acetone as we demonstrated that acetone (0.05 %) had no effect on sucrose perception (see Sucrose responsiveness in Chapter 2) nor on behavior (Fischer *et al.*, 2014). The clothianidin sucrose solutions used in the field (0.005 and 0.01 ng/µl corresponding to 4.5 and 9 ppb respectively) as well as for the taste and choice experiments (0.01 and 0.25 ng/µl corresponding to 9 and 225 ppb respectively) and the conditioning experiments (0.01, 0.04 ng, and 0.1 ng/µl corresponding to 11.25, 36 and 90 ppb respectively) were freshly made every morning from the stock solution. The concentration of the solutions used were confirmed by LC-MS/MS.

Field experimental design

The experimental area is a highly structured agricultural landscape nearby Großseelheim, Germany. Two colonies housed in two observation hives (W.Seip, Bienenzuchtgerätefabrik) were put up on two opposite sides of a cabin (50°48'51.9"N). Each colony of *Apis mellifera carnica* was equipped with one comb (Deutsch Normal Mass) of sealed brood plus newborn bees and one comb of food originating from the same honey bee colony. The queens were generously provided by the Bieneninstitut Kirchhain and the bees from a local beekeeper. Queens were sisters, open mated, aged 1 year old, and derived from selected breeder colonies of the carnica breeding population of the institute.

Training to the feeders

The experimental set-up was the same as in the Tison *et al.* (2016) study except for the harmonic radar. Two feeders (F1 and F2) were separated by an angle of 90° and established 350 meters northeast and 340 southeast from the cabin respectively. The release site was located 780 meters east of the cabin. A group of foragers from each of the two colonies was trained to its respective feeder and marked individually with number tags on the thorax. A full protocol was kept about the number and identity of bees visiting the control and contaminated feeders every day. The origin of each newly marked bee was also controlled at the respective hive entrance.

Each feeder was placed in a little wooden box to allow for counting the entrances and exits of foragers with a retro-reflective sensor (Baumer GmbH). In order to regulate the traffic, the concentration of the sucrose solution at each feeder was adjusted during the day following

evaluation by the experimenter of the number of trained foragers visiting the feeder. Dance recruitment was induced 24 times on 24 different days (time: 1400 - 1600 hours) by first halving the sucrose concentration at both feeders for one hour and then increasing it twofold for another hour (von Frisch, 1967).

Both control and treated bees foraged first on uncontaminated sucrose solutions during 7 days. Experiment 1 started and one group of bees (treated group) started to forage on a sucrose solution containing clothianidin (4.5 ppb), and the other group (control group) foraged over 7 days at a feeder containing only sucrose solution. The concentration of clothianidin was then raised at the treated feeder to 9 ppb during 11 days. In experiment 2, the feeders´ locations were exchanged in order to exclude any possible landscape effect related to the feeders' position and the two groups of foragers fed at their respective feeder during 13 days.

Homing experiment

Colonies were settled in the field for at least a week before the homing experiments started. After a certain number of days foraging at the feeders, single bees were caught on departure at their respective feeder after they had freely drunk a 1 M sucrose solution containing 4.5 or 9 ppb clothianidin (treated bees) or not (control). They were kept in the dark for 50 min while they were transported into a ventilated glass vial to the release site. Since the harmonic radar could not be used this year, we could not track the trajectories of the bees but we recorded the homing success and flight duration of the bees manually with a timer. A piece of wire of 10 mm (looking like a real transponder but without a diode) was fixed to the number tag on the thorax of each bee in order to avoid that the bee enter its hive before we could read its number. Bees from both feeders were tested each day and the time (between 1200 and 1800 hours), temperature (16-27°C) and wind (< 15 km/h) were noted. No release was made when the sky was evaluated too cloudy or totally overcast, nor when it was raining, and each bee was released only once. We waited 120 min for each released bee before we stopped looking at the entrance of the hive. Bees that did not return to the hive after being released and not seen at the feeder or at the hive entrance on the same or the following days were considered to have died in the field.

Clothianidin residue analysis

Preparation of the bee samples

Bees were caught at their feeder after foraging for a certain number of days and after they had filled their crop with a 1 M sucrose solution contaminated with 9 ppb clothianidin or not (control). They were then kept in the dark for 50 minutes before being killed by chilling and put into a -20° C deep-freezer. We also collected unmarked forager bees at the entrance of the treated and control hives when flying out on a foraging trip in order to assess the in-hive contamination of foragers not visiting the feeders but exposed indirectly to clothianidin inside

the hive via the stored food. All of the collected bees were cut into 3 parts, head, thorax and abdomen. The legs and wings were cut off. Samples from the same foraging groups were pooled (usually 10 organs in each tube) and weighted. 25 µl of surrogate solution (acetamiprid-d3) and 5 ml of acetone were added to each sample, then homogenized with a disperser during three minutes and centrifuged (10 min at 3000 rpm). After centrifugation, 4 ml of supernatant was carefully removed and left to dry in a metal block thermostat with nitrogen blow device. 950 µl of water methanol (1/1, v/v) and 50 µl of internal standard solution containing clothianidin-d3 were added to the dry extract. Samples were then mixed using an ultrasonic liquid mixer and put into the freezer (-18°C) overnight.

Preparation of the wax and honey samples

To 1 g of treated or control honey, 20 µl of surrogate solution (acetamiprid-d3) and 20 ml of acetone/water mixture (3/1, v/v) were added. A piece of wax comb was also sampled in each hive and weighed. 50 µl of surrogate solution and 30 ml of acetone/water (2/1, v/v) were added. Samples were homogenized with a disperser during three minutes and centrifuged (10 minutes at 3000 U/min). 15 mL of the supernatant was removed and after addition of 5 mL sodium chloride-solution (20 %) to this aliquot samples were transferred onto a disposable cartridge filled with diatomaceous earth (ChemElut® cartridges, 20 mL, unbuffered; Agilent, Santa Clara, USA). After a waiting time of 15 minutes the samples were eluted with dichloromethane (2 x 50 mL). The eluates were evaporated to approximately 2 mL by using a rotary evaporator, then transferred to a graduated tube and evaporated to dryness with nitrogen, using a metal block thermostat with a nitrogen blow device. For honey samples, the residual extract was taken up with 50 µl of internal standard solution containing Clothianidin-d3 and 950 µl of methanol/water mixture (1/1, v/v). For wax samples, 1900 µl or 2375 µl of methanol/water (1/1, v/v) and 100 µl or 125 µl of the internal standard solution were added to the control and treated samples respectively. Samples were then dissolved using ultrasonic liquid mixer and put into the freezer (-18°C) overnight.

Identification and quantification of clothianidin residues

On the next day, samples were filtered cold (syringe filter 0.2 m) before proceeding with the identification and quantification of clothianidin by LC-MS/MS. The LC-MS/MS system used was a UltiMateR 3000 RS HPLC (Dionex Corporation, Sunnyvale, USA) coupled to a mass spectrometer QTRAP® 5500 (AB SCIEX, Framingham, USA) equipped with an electrospray ionization (ESI) source. Clothianidin was identified by its retention time and two Multiple Reaction Monitoring (MRM) transitions. The residues in the samples were measured using matrix standards (concentrations: 0.1, 0.5, 1, 5, 10, 25, 50 pg μL^{-1}). The quantification was

carried out by the internal standard method. The value given for each sample represents the average of double-injections. See Table S2 for recoveries, limit of detection (LOD) and limit of quantification (LOQ). These values were determined during the method validation.
Frozen samples of contaminated sucrose solutions were sampled and analyzed by LC-MS/MS. LOD = 0.05 pg μL^{-1} and LOQ = 0.1 pg μL^{-1}.

Sucrose responsiveness and PER conditioning

Sucrose responsiveness

A laboratory and a semi-field choice test were carried out in order to determine whether clothianidin affected honey bees' motivation for sucrose. For both tests, bees were handled the same way as described in Tison *et al.* (2016).
The sucrose responsiveness of the harnessed bees was assessed 1 hour after bees where caught at their hive entrance by stimulating each bee's antennae with solutions containing 0, 0.1, 0.3, 1, 3, 10 and 30 % (w/v) sucrose (Scheiner *et al.*, 2005; Matsumoto *et al.*, 2012) only, or with 0.01 or 0.25 ng/µl (9 ppb respectively) clothianidin. If a bee responded to low concentration(s) of sucrose and then stopped responding to higher concentrations or if a bee did not respond at all to a 50 % (w/v) sucrose stimulation at the end of the test, it was discarded.
In the semi-field choice experiment, a group of bees was trained to a yellow training and feeding platform with 10 mini-feeders in the middle of blue squares, 30 m from the hive. The test platform was composed of six mini-feeders, control or treated, randomly allocated. During the testing of single bees, three feeders from the test platform contained 8 µL of a 1 M control sucrose solution each, and the other three contained 8 µL of a 1 M sucrose solution with clothianidin (9 ppb) each. The number of feeders drunk and the time a bee took to drink at each of the six feeders was recorded. At the end of the test, the bee was killed, and the same test was repeated with a new naive bee.

Sampling and preparation of bees for olfactory conditioning

Summer honey bees *Apis mellifera carnica* were collected at 2 p.m. with a Plexiglas pyramid on their outbound flight at the hive entrance, in the garden of the Institute of Neurobiology of the Free University of Berlin. The bees were then transferred into ventilated glass vials and cooled on ice until immobile. Then they were harnessed individually in tubes that allowed free movements of the mouthparts and antennae (Matsumoto *et al.*, 2012) and at 4 p.m. they were fed to satiation with a 30 % (w/v) sucrose solution and put in a dark and humid box in a 20 °C room until the next morning.
Each bee was fed orally with 8 µl of feeding solution (sucrose solution only or sucrose solution with clothianidin) using a multipette. Bees were fed either 1 hour before the first conditioning trial (learning experiment) with 0.1 ng, 0.3 ng, or 0.8 ng per bee, 5 hours after conditioning

(consolidation experiment) with 0.3 ng per bee or 23 hours after conditioning (retrieval experiment) with 0.3 ng, or 0.8 ng per bee. The control and treated groups were always tested blindly and simultaneously. Mortality was assessed in each experiment and bees were recorded as dead when no movement of the antennae or the abdomen could be seen.

Olfactory conditioning and memory tests

Shortly before conditioning, the olfactory stimuli was prepared by placing 4 µl of pure odorant, hexanal (Sigma Aldrich), on a 1.32 cm^2 piece of filter paper inserted in a 20 ml plastic syringe used to deliver odor-filled air to the antennae of the conditioned honey bees. Olfactory appetitive conditioning was performed according to a standard protocol (Matsumoto *et al.* 2012), using hexanal as the conditioned (reinforced) stimulus (CS). We used the method described in Felsenberg *et al.* (2011) for conditioning bees. The CS was presented during 5 seconds and the US (50 % w/v sucrose) 3 seconds after the odor onset and during 4 seconds. Each bee received 3 paired CS-US presentations (i.e., conditioning trials) with a 12 minute inter-trial Interval. A bee was discarded if it did not extend its proboscis when stimulated with sucrose during conditioning. Bees that extended their proboscis in response to the odor before the sugar reward was delivered (PER) showed learning (acquisition).

Memory retention was assessed 24 hours after the first conditioning trial in 3 extinction trials for the learning and consolidation experiment and 6 extinction trials (3 hour after the first 3 trials) for the retrieval experiment. The CS, hexanal, was presented to each bee with an inter-trial interval of 12 minutes and was not rewarded with sucrose solution (extinction tests). At the end of the retention tests, each bee was stimulated with a 50 % (w/v) sucrose solution to see if its unconditioned response to sucrose was still intact (US test). Any bee that failed the test was discarded as well as any bee that extended its proboscis during the 10 sec prior to the odor presentation.

Statistical analysis

For the statistical analysis of the data, we used R and Prism 5 and 6. The normality of the data was tested using the D'Agostino-Pearson omnibus test. If the data were normally distributed, we used a paired/unpaired t.test or an analysis of variances with Tukey's post-hoc tests. Otherwise non-parametric tests were performed (Mann-Whitney test). The Fischer's Exact Test was used to compare proportions. The survival analysis was conducted using censored Kaplan Meier Log-Rank in R and the influence of multiple variables was investigated using a Cox-regression model (survival package). For the PER data we used the lme4 package to perform a linear mixed effects analysis of the relationship between PER and Treatment. The responses of each bee were scored as binary responses (PER: 1, no response: 0). As fixed effects, we entered Treatment and Trial number into the model, Bee identity and Session identity were always used as random factors. Several models (with or without interactions

between factors) were tested and the best was selected using AIC. All models were validated by assessing normal Q-Q plots and residual versus fitted data plots. This was followed by Overall Likelihood Ratio Tests and Tukey's post-hoc tests (multicomb package). Sucrose responsiveness was also analyzed with GLMM, using Treatment and Sugar Concentration as fixed effects and Bee identity and Session identity as random factors. We used *Chi-square* tests to compare the mortality and US-tests rates between the doses. Comparisons in which $P < 0.05$ were considered significant. The numbers of bees tested for each experiment and test groups are indicated in the legends of the figures and in the text.

Results

Foraging behavior

A full protocol was kept about the number and identity of bees visiting the control and contaminated feeders so as to calculate the foraging span of trained bees. The pre-experiment is a period during which foragers from the control and the future treated hive were not exposed to clothianidin but both to sucrose solution only at their feeder. Seven days after the start of the pre-experiment (Aug. 31. 07), we started with the exposure to clothianidin at the contaminated feeder (4.5 ppb). Bees from the control hive foraged at a feeder containing only sucrose solution. One week later (Aug. 7), the concentration at the contaminated feeder was raised to 9 ppb and remained unchanged during 4 weeks, until the end of the experiment (Sep. 3). Meanwhile, the control bees continued to be exposed to sucrose solution. The exposure to clothianidin stopped during 1 week (Aug. 14 to Aug. 22) because the location of the control and treated feeders was exchanged and bees were trained to the new location.

As the exposure of the treated group to uncontaminated sucrose solution during the pre-experiment or to 4.5 or 9 ppb followed each other, a certain number of treated foragers were exposed to both control sucrose solution and 4.5 ppb or to both 4.5 and 9 ppb, making the calculation of the foraging-span of forager bees from the pre-experiment and experiment 1 (4.5 ppb) not reliable. Thus, the foraging-span of bees at the control and contaminated feeders was calculated for the 4.5 and 9 ppb concentrations taken together. We found that treated bees foraged 2 days less at the contaminated feeder (Fig. 1, 2-9 days, median= 3 days) than control bees at the control feeder (3-11 days, median= 5 days) over the same period of time (Fig. 1, Mann Whitney, P = 0.0005).

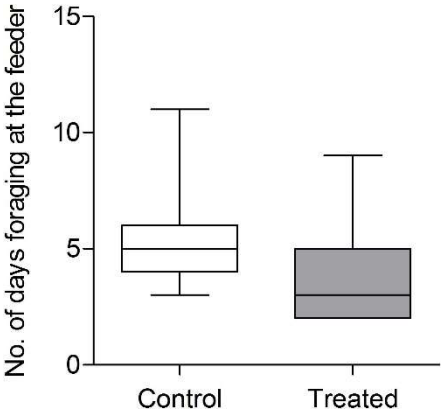

Figure 1. Foraging span of trained bees at the control and treated feeders. The treated feeder contained 4.5 ppb or 9 ppb clothianidin in sucrose solution and the control feeder sucrose solution only. The foraging span was significantly shorter for the treated group of bees (Mann Whitney, P = 0.0005).

We evaluated the amount of sucrose solution collected at both feeders throughout the summer and found no significant difference between the control feeder and the feeder contaminated with 4.5 ppb clothianidin (paired t.test, P = 0.2876). However, when the treated feeder contained 9 ppb clothianidin the difference was significant (paired t.test, P = 0.0061). Taken together the two concentrations and the two experiments, the amount of sucrose collected by bees at the control and treated feeders did not statistically differ (paired t.test, P = 0.61175). During experiment 2, the control bees consumed on average 1.3 times more sugar solution per day than treated bees foraging on 9 ppb (Table S1, paired t.test, P = 0.0099) whereas treated bees exposed to the same concentration but during experiment 1 did not collect less sucrose solution than the control bees (Table S1, paired t.test, P = 0.3338).

The average amount of clothianidin collected per bee and per day is directly linked to the amount of sucrose solution collected at the treated feeder. The estimated exposure was similar between bees exposed to 4.5 ppb clothianidin (12.95 ± 0.45 ng) and bees exposed to 9 ppb during the first experiment (12.90 ± 1.21 ng). Bees exposed to 9 ppb collected on average 20 ml less sucrose per day but were exposed to a higher concentration of clothianidin and the average number of foraging bees was lower (-10 bees). Much fewer bees foraged at the treated feeder contaminated with 9 ppb during experiment 2 (27 ± 2), collecting half as much sucrose solution than bees exposed to the same concentration during experiment 1. The exposure per bee at the treated feeder was however not different and even a bit higher than in the previous experiment (14.32 ± 1.62 ng) due to the lower number of bees foraging.
Interestingly, the average number of bees foraging at the control feeder remained unchanged during experiment 1 and 2 for the 3 groups shown in Table S1 whereas the average number of bees foraging at the treated feeder kept decreasing throughout the summer (50 > 40 > 27) even if higher concentrations of sucrose were used in order to motivate them to visit their feeder (Fig. 2B).
Treated bees performed on average 1.2, 1.5 and 1.7 times less foraging trips per day than control bees in the 3 groups shown in Table S1 (exp. 1, 4.5 ppb and 9 ppb and exp. 2, 9 ppb respectively). From the average number of trips per bee and day and the amount of clothianidin collected per bee and day at the feeder (Table S1), we estimate that on one trip a bee collected on average 0.28 ng clothianidin (about 56 µl of solution of 4.5 ppb solution) and 0.45 ng (about 45 µl of 9 ppb solution) in experiment 1, and 0.53 ng (about 53 µl of 9 ppb solution) in experiment 2.

Reduced sugar consumption is linked to reduced visitation rates of forager bees at the contaminated feeder. Indeed, all experiments and doses taken together, treated bees visited their feeder less frequently than the control bees on the same days (Fig. 2A, paired t.test, P =

0.0010). Similar or higher sucrose concentrations were needed at the contaminated feeder in order to keep the bees visiting the feeder (Fig. 2B, median control and treated = 0.25 M). During the pre-experiment, the number of visits per hour was the same or higher at the future treated feeder. The exposure of one colony with 4.5 ppb clothianidin revealed no significant difference in the feeders' visitation (Fig. 2A, paired t.test C1 *vs* T1, p= 0.6458), however, the exposure to 9 ppb clothianidin at the feeder induced a decrease in the visitation rate. The same average sucrose concentration was used at the control and contaminated feeders and still the treated feeder was on average 33 % less visited than the control feeder (Fig. 2A, paired t.test, P = 0.0002). We did not record the visitation at the feeders between August 15 and August 21 as it was the period during which we switched the positions of the feeders and trained bees to their new feeding location. Only on August 27 and 28, the treated feeder was more visited than the control feeder, but the sucrose concentration in the feeding solution was 0.5 M and in the control feeder only 0.25 M. Every other day during the experiment 2 the treated feeder was visited on average 34 % less than the control feeder (Fig. 2A, paired t.test, P = 0.0189) even if the sucrose concentration at the treated feeder was more than half of the time higher than at the control feeder (median concentration treated: 0.5M; control: 0.25M).

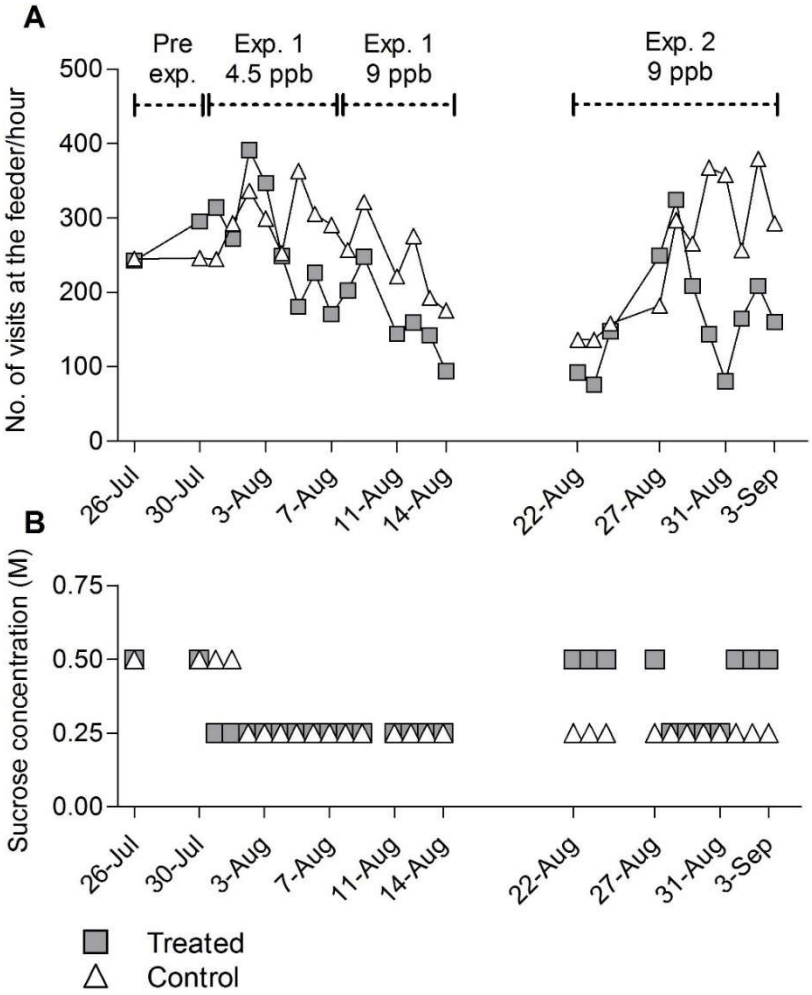

Figure 2. Foraging activity and required sucrose concentrations at the control and treated feeders. A. Number of visits per hour recorded on the same days (*n* = 27 days) during the pre-experiment, experiment 1 (4.5 ppb and 9 ppb) and experiment 2 (9 ppb) at both control (triangles) and treated feeders (squares). The foraging activity of the treated bees is significantly reduced by exposure to clothianidin (paired t.test, P = 0.0010). **B**. Sucrose concentrations used in order to keep a similar number of foragers coming regularly to the control and treated feeders. The same or higher concentrations of sucrose solution were usually used at the treated feeder.

Recruitment of foragers via the waggle dance was induced by raising the sucrose concentration at the feeder (von Frisch, 1967). The sucrose concentration in the feeding solutions during the dance induction in experiment 1 was most of the time the same at both feeders (0.5 M) since the regular sucrose concentration was also similar (Fig. 2B, 0.25 M). However, during experiment 2, the sucrose concentration during dance induction was more than half of the time higher at the treated feeder.

We extracted from the data presented above the regular foraging activity and the foraging activity during dance induction on the same days at both feeders over experiment 1

and 2 for the concentrations 4.5 and 9 ppb. Both control (unfilled marks) and treated bees (filled marks) with 4.5 ppb were able to recruit new untrained foragers in experiment 1 (Fig. 3, Exp.1_4.5 ppb $P < 0.001$). Treated bees exposed to 9 ppb in experiment 1 however did not significantly react to dance induction, contrary to the respective control bees (Fig. 3, Exp.1_9 ppb, $P < 0.05$). The visitation rate at the control feeder was 36 % higher than at the treated feeder during dance induction. In experiment 2, control bees showed increased activity at the feeder during dance induction (38 %) when treated bees with 9 ppb were unable to recruit new untrained foragers to the feeder, but this increase was not statistically significant. The number of visits per hour was 49 % higher at the control feeder than at the treated feeder in experiment 2 (Fig. 3, Exp.2_9 ppb, $P < 0.01$). Overall, the traffic at both feeders decreased during regular foraging and dance induction throughout the summer.

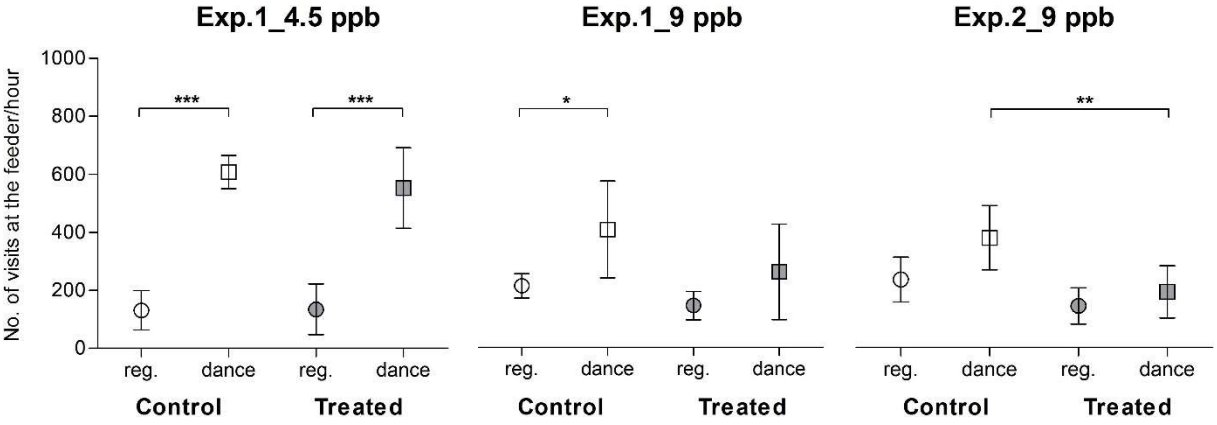

Figure. 3. Number of visits per hour performed by the trained bees from the control and contaminated feeder with clothianidin. Mean (± 95 % confidence limits) number of visits per hour recorded on the same days at both feeders during regular foraging ("reg.", circles) and during dance induction ("dance", squares). Dances were induced at the same time at both feeders on 7 days in exp. 1, 4.5 ppb, 6 days in exp.1, 9 ppb and 11 days in exp. 2, 9 ppb. Both control (unfilled marks) and treated bees (filled marks) with 4.5 ppb were able to recruit new untrained foragers in exp. 1. With 9 ppb in exp. 1, only control bees significantly increased the number of visits per hour at their feeder. In exp. 2 (*n days* = 6, 9 ppb), the number of visits per hour was significantly different at the control and treated feeders. Stars indicate the results of the Tukey post-hoc tests after ANOVA: *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$.

Residue analysis

Bees visiting the control and contaminated feeders were caught at their departure from the feeder, immediately after drinking some sucrose solution containing 9 ppb clothianidin.
In experiment 1 the residues ranged between 2.1 ng/g and 2.9 ng/g and in experiment 2 from 2.4 ng/g to 3.2 ng/g depending on the number of days bees foraged at the feeder before being caught for analysis (Fig. 4). Clothianidin residues were detectable only in the bee abdomens

except in experiment 2, during which clothianidin was detected in the heads of bees which foraged 3-6 days at the contamined feeder (Table S2).
Residues of clothianidin could not be detected in any of the control samples collected at the feeder or at the hive entrance throughout the summer.

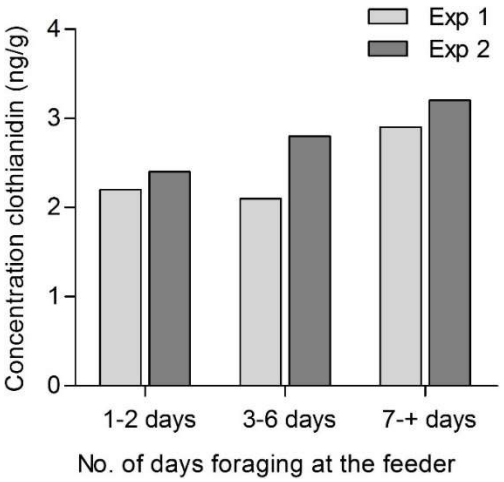

Figure 4. Residues of clothianidin (ng/g) detected in the bees caught at the contaminated feeder with 9 ppb clothianidin. Bees from Exp. 1 were collected on Aug. 16 and bees from Exp. 2 on Sept. 4. Collected bees were grouped according to the number of days they foraged at their feeder before analysis.

In bees caught at the hive entrance, we detected clothianidin residues only in the abdomens of bees collected after 30 days of treatment (Table S2, 2.80 ng/g). Divided by the number of bees in this sample, clothianidin residues amount to 0.23 ng of clothianidin per bee. Clothianidin residues in honey and wax from the control and treated hives were not present in the samples or were under the limit of detection of the LC-MS/MS method used here and thus not presented.

Homing success

Navigation requires the integration of multisensory cues and the retrieval of appropriate memory about the landscape structure. We tested the homing success of bees trained to feeder 1 and 2 during experiments 1 and 2 and thus exposed to 4.5 or 9 ppb.
A survival analysis was conducted on the data and a flight duration of 120 min was settled for bees that did not come back to the hive as it was the minimal time we waited for them at the hive entrance. The flight duration of all other bees was the flight time in minutes from the release site to the hive.
No difference was found in the homing success or flight duration of bees from the pre-experiment (Table 1). No influence of the treatment was revealed on the homing success (Fig.

5, control 87 % return, treated, 87 % return) when the experiment and concentrations were left out from the analysis and only the treatment effect was considered (Fig. 5 and Table 1, 4.5 and 9 ppb together, Kaplan Meier Log Rank test $\chi_1^2 = 1.1$, P = 0.295 and Fischer's Exact test, P = 1). However, we could see a significant difference in the flight duration of control and treated bees (Table 1, Mann Whitney, P = 0.0328). Indeed, control bees flew on average 3 minutes longer than bees exposed to 4.5 and 9 ppb clothianidin.

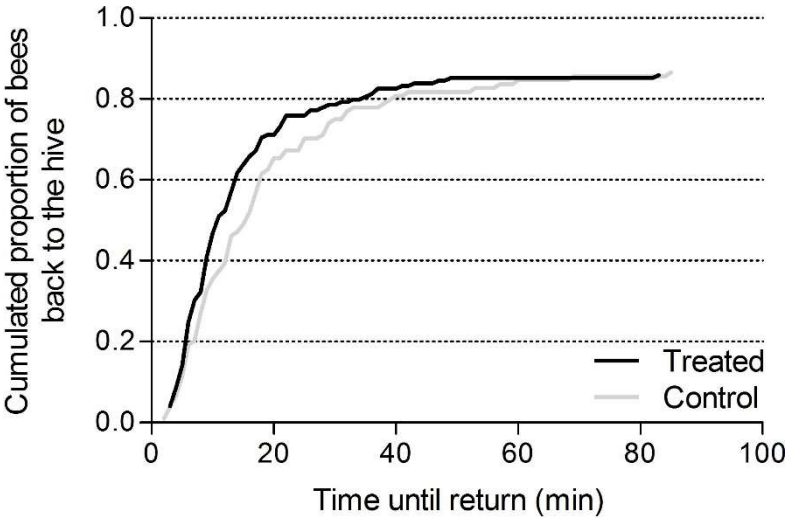

Figure 5. Probability of homing success as a function of time until return. Control and treated (4.5 and 9 ppb) honey bees returned to their hive in similar proportions ($n_{control}$ = 104, 87 % return; $n_{treated}$ = 149, 86 % return; Fisher's exact test, P = 1). The origin of the temporal axis represents the moment of release.

When including the 2 experiments and the two different concentrations of clothianidin (4.5 and 9 ppb) in the analysis, a significant difference in the homing success was revealed (Table 2, Kaplan Meier Log Rank test treatment + concentration + experiment: $\chi_5^2 = 34.9$, P = 1.59e-6). Tukey post-hoc tests revealed no significant difference in the homing success of control and treated bees in experiment 1 or experiment 2 (Table 1, Tukey, P = 0.116 and P = 0.993 respectively). Also, no significant difference in the homing success was seen between bees exposed to 4.5 ppb and their relative controls or bees exposed to 9 ppb and their relative controls (Table 1, Tukey, P = 0.0823 and P = 0.5 respectively). However, we found that the control bees which returned to the hive in experiment 1 flew significantly longer than the treated bees exposed to 4.5 ppb in experiment 1 (Table 1, Mann Whitney, P = 0.0093).

The experiment and thus the time during which the experiment was performed (related to weather conditions and status of the colony) and/or the feeding location had an influence on the homing success and the flight duration. Significant differences were revealed within the control group and within the treated group between bees foraging during experiment 1 or 2 (Tukey, treated, P = 0.00000589; control, P = 0.00481) and between bees exposed to 4.5 or

9 ppb (Tukey, treated, P = 0. 00024; controls, P = 0.0265). Bees exposed to 9 ppb clothianidin at the feeder F1 in experiment 2 had a lower homing success (Table 1, Tukey, P = 0.0282) and flew significantly longer (Mann Whitney, P = 0.0320) than control bees foraging at F1 but in experiment 1. Control bees foraging at F2 during experiment 2 had a lower homing success (Table 1, Tukey, P = 0.00125) and flew significantly longer than treated bees foraging at F2 on 9 ppb during experiment 1 (Mann Whitney, P = 0.0137).

Table 1. Summary of the homing success and flight duration of honey bees released

		Pre-exp.		**Exp. 1**		**Exp. 1**		**Exp. 2**		**Total**	
		No treatment		**control**	**4.5 ppb**	**control**	**9 ppb**	**control**	**9 ppb**	**controls**	**4.5 + 9 ppb**
feeder		F1	F2	F1	F2	F1	F2	F2	F1	F1+F2	F2+F1
n returned / *n* total		24/24	40/42	42/43	47/50	22/26	46/49	26/35	35/50	90/104	128/149
homing success (%) *		100	95	98	94	85	94	74	70	87	86
flight duration	mean	9.63	9.43	18.38	9.94	12.00	14.37	20.54	16.80	16.63	13.41
	± s.e.m.	± 1.53	± 0.84	± 2.47	± 1.04	± 1.80	± 2.04	± 3.36	± 1.98	± 2.08	± 1.01
	median §	8	8.5	13	7	9	10	16	13	13	10
	min.	3	3	2	3	3	3	8	3	2	3
	max.	36	24	69	37	32	83	85	49	85	83

* Kaplan Meier Log Rank test (treatment) Total, χ_1^2 = 1.1, P = 0.295 (Fischer's exact test, P = 1)*;* (treatment + concentration + experiment): χ_5^2 = 34.9, P = 1.59e-6 followed by Tukey post-hoc tests, significant differences: control exp.1 *vs* control exp.2, P = 0.00481; treated exp.1 *vs* treated exp.2, P = 0.00000589; control (4.5 ppb) *vs* control (9 ppb), P = 0.0265 ; treated 4.5 ppb *vs* 9 ppb, P = 0.00024; F1, 9 ppb, control *vs* treated, P = 0.0282; F2, 9 ppb, control *vs* treated, P = 0.00125.

§ Mann Whitney tests, significant differences: control *vs* treated 4.5 ppb, P = 0.0093; Total, P = 0.0328; control exp. 1 (9 ppb) *vs* control exp. 2 (9 ppb), P = 0.0108; F2, 9 ppb, control *vs* treated, P = 0.0320; F2, 9 ppb, control *vs* treated, P = 0.0137.

The influence of multiple variables on the homing success was tested in a cox-regression model (Table 2). The results of the later shows that the variable "treatment" and "concentration" (4.5 or 9 ppb) had no significant negative effect on honey bee survival. However, the period during which the experiment was performed ("experiment") had a significant influence on honey bee survival after reduction of the model based on the AIC (Table 2, model 2). The number of days a bee foraged at its feeder before being released ("time foraging") had a significant influence on the survival in model 2 on the contrary to "time exposure", corresponding to the number of days from the first day of the experiment until a bee was released. At their release, 31 % of the control and 26 % of the treated bees waited for a short time at the release site before starting to fly. This waiting time ("time before flying") was

not different between the control and the treated bees (median waiting time 0 min for both groups) and had no influence on the homing success (Table 2).

Also, the temperature at the release time had a significant effect on honey bee homing abilities in both models. The temperature was lower during the second part of the experiments (average temperature exp. 1 = 21.7 °C, exp. 2 = 18.4 °C) which could have influenced the homing success.

Table 2. Summary of the Cox regression model

Variables	Model 1				Model 2			
	regr. coef	exp (coef)*	Z	P	regr. coef	exp (coef)*	Z	P
treatment	0.101	1.106	0.615	0.539				
concentration	-0.067	0.935	-0.244	0.807				
experiment	-0.837	0.433	-1.763	0.078	-0.478	0.620	-2.625	**0.009**
time foraging ǂ	-0.077	0.926	-1.912	0.056	-0.094	0.910	-2.692	**0.007**
time exposure §	0.029	1.030	0.784	0.433				
temperature	0.085	1.089	2.178	**0.029**	0.056	1.061	2.365	**0.018**
time before flying $	-0.046	0.955	-1.173	0.241	-0.056	0.946	-1.448	0.147
	Rsquare: 0.165 (max possible= 1), Likelihood Ratio Test: 45.62 on 7 df, P = 1.036e-7				*Rsquare: 0.162 (max possible= 1), Likelihood Ratio Test: 44.64 on 4 df, P = 4.727e-9*			

A backward selection on the AIC was performed on model 1 in order to obtain model 2

Values in bold indicate significant differences

*exp (coef) = Hazard ratio

ǂ **time foraging** is the time in days during which a bee is foraging at its feeder before being released

§ **time exposure** is the time in days from the first day of the experiment until the day the bee is released

$ **time before flying** is the short time bees waited at the release site before starting to fly

Based on the crop-emptying measurements by Fournier *et al.* (2014) we calculated that the foragers could have assimilated in 50 min up to about 8 µl of the sucrose solution collected at the treated feeder, corresponding to 0.04 ng (4.5 ppb exposure) and 0.08 ng (9 ppb exposure) clothianidin respectively. This amount is what bees would take up just before flying, in addition to the residues already assimilated over n days foraging at the feeder. This value is a higher estimate because the amount of assimilated sucrose during the 50 minute waiting time may well be much lower depending on the activity of the waiting bee (Rothe, 1989). In any case the partial acute treatment component involved in the homing success experiments adds to the chronic effect.

Sucrose responsiveness and choice experiment

One explanation for lower foraging activity found in treated bees could be an aversive taste of the substance in contaminated sucrose solution. We applied different test conditions to study this question.

First, we used the Proboscis Extension Response (PER) paradigm to test the sucrose responsiveness of hungry foragers to water and 7 different sucrose concentrations (0.1 %, 0.3 %, 1 %, 3 %, 10 %, 30 % and 50 % w/v) containing clothianidin or not, at the same concentration than the one used in the field (9 ppb). No difference was found in the PER of bees stimulated either with the control sucrose solutions or the contaminated sucrose solutions (Fig. 6, logistic regression with random effects “Bee” and “Date”, Sugar concentration x Treatment: $\chi 52 = 6.7745$, $P = 0.238$). As 100 % of bees showed a PER for the 30 % and 50 % sucrose concentrations, both concentrations were excluded from the analysis (the GLMM was run on 5 degrees of freedom). The Tukey’s post-hoc tests between the control and treated groups for each of the different sucrose concentrations revealed no significant difference.

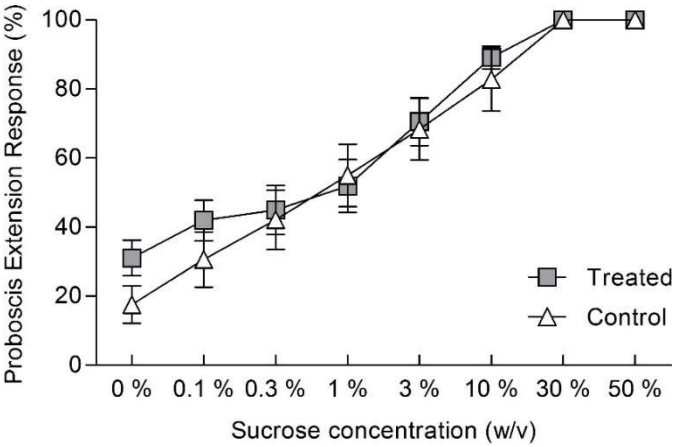

Figure 6. Proboscis extension response (PER) to different sucrose concentrations with or without 9 ppb clothianidin. $n_{control} = 71$. $n_{reated} = 67$. No difference was found between the two groups (logistic regression with random effects; sugar concentration × treatment: $\chi_5^2 = 6.7745$, $P = 0.238$).

In the free flight experiment, 61 bees were trained to fly from the hive to a platform on which 6 mini-feeders were randomly distributed. Three of these feeders contained 8 µl of 1 M sucrose solution, the other three contained the same amount of 1 M sucrose solution plus clothianidin (9 ppb). No significant difference was found in the visitation rate of the control and contaminated feeders (97 bees out of 183 drank at control feeders, and 110 bees out of 183 drank at the contaminated feeders, Fischer’s exact test, $P = 0.2057$). The average (± s.e.m.) drinking time per bee and feeder was 7.62 ± 0.31 sec at the control feeders, and 7.41 ± 0.25 at the contaminated feeders and was not significantly different (Mann Whitney, $P = 0.7287$).

No significant difference was found in the number of bees that aborted the test after drinking at one of the six feeders (8 out of 26 bees after drinking a control feeder, and 14 out of 35 after drinking a treated feeder, Fisher's exact test, P = 0.8008). These results rule out the possibility that clothianidin could have had a repellent taste for honey bees in our field experiment.

Olfactory conditioning

Mortality and US tests

Mortality was evaluated by the number of bees dying in the time frame between intoxication and the end of the memory tests. The proportion of responses to a 50 % (w/v) sucrose stimulation (US-test) at the end of the memory tests was also assessed. Overall, we found no evidence of an increase in mortality as a result of treatment for all doses and timings of intoxication with clothianidin in this study (Fischer's exact test, P = 0.1811). The only significant difference found when taking the experiments separately (Table S3) was for the memory retrieval test (Table S3, χ^2= 18.4, *df*= 3, P= 0.0004) during which the bees treated with 0.8 ng clothianidin died in greater proportions than the controls (12.5 % and 0 % deaths respectively). Also no significant difference was found in the proportion of bees responding to the US-test for bees intoxicated with clothianidin or not (Fischer's exact test, P = 0.8911). However, more bees from the retrieval experiment failed the US test than bees from the learning or consolidation experiment (Table S3). Bees in the retrieval experiment were tested two times for their memory at intervals of 3 hours (6 extinction trials instead of 3) before their US was tested. Bees were probably too weak by the time the US test was performed at the end of the retrieval experiment, explaining the higher mortality rates and lower responses to the US.

Appetitive learning

The learning ability was quantified by evaluating the acquisition functions after intoxicating the test bees with 0.1, 0.3 or 0.8 ng clothianidin 1 hour before onset of conditioning. Then, three extinction trials were performed in order to test the stability of the memory.

Control and treated bees learned to associate the CS with a US (acquisition curves) at a similar level for each of the doses tested. No effect on learning and memory was revealed for the two lowest doses tested (Fig. 7A, 0.1 ng/bee, Analysis of Deviance Table, $\chi2$ = 1.4065, *df* = 1, P = 0.2356; and Fig. 7B, 0.3 ng/bee, $\chi2$ = 0.0799, *df* = 1, P = 0.7774813) even if a tendency of a loss of memory retrieval can be seen for the treated bees (E1, Fig. 7A and B). Contrary to treated bees intoxicated with 0.1 ng/bee, control bees answered in slightly higher proportion to the first extinction trial (E1). Fig. 7B shows lower acquisition rates for both control bees and bees intoxicated with 0.3 ng than with the two other doses tested. The control and treated bees from this test (Fig. 7B, 0.3 ng/bee) showed a memory consolidation effect since they answered to the CS (E1) in higher proportion than they learned it (A3). This consolidation effect was

significant for the control bees (control A3 *vs* E1 -3.28, ± 0.58, P < 0.0001) but not for the treated bees with 0.3 ng clothianidin (treated A3 *vs* E1, -1.38 ± 0.44, P = 0.0610).

On the contrary to the two lowest doses tested, the highest dose of clothianidin (0.8 ng/bee) negatively impaired the memory of honey bees ($\chi 2$ = 9.1735, *df* = 1, P= 0.002455). No difference was revealed in the acquisition functions but each of the 3 extinction trials were significantly different for the control and treated bees (Fig. 7C, control *vs* treated, E1: 3.21, ± 0.90, P = 0.0126; E2: 2.69, ± 0.74, P = 0.0105; E3: 2.08, ± 0.65, P = 0.0467). Control bees showed a consolidation effect overnight (not significant) contrary to treated bees which showed the same proportion of PER for A3 and E1.

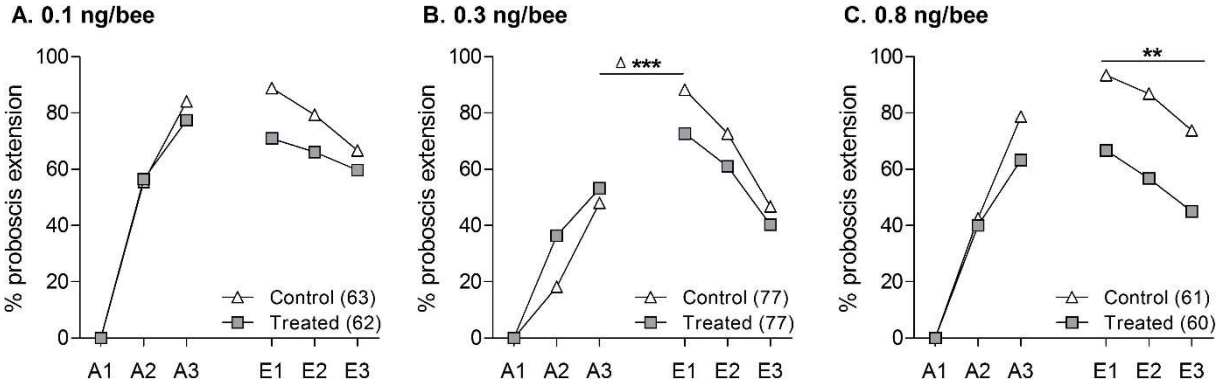

Figure 7. Acquisition functions and retention scores after treatment with clothianidin. PER scores were quantified by three acquisition trials (A1, A2, A3) 1 hour after treatment with **A.** 0.1 ng, **B**. 0.3 ng or **C.** 0.8 ng diluted in sucrose. Memory extinction trials (E1, E2, E3) were performed 24 hours after acquisition. Significant differences (P< 0.05) with the control are represented by stars in the legend. The number of individuals in each group is given in brackets in the legend.

Memory Consolidation

In order to investigate the effect of clothianidin on memory consolidation, treated bees were fed with 0.3 ng clothianidin 5 hours after conditioning. Three extinction trials were applied in order to test the stability of memory. No effect on learning was found between the two groups (Fig. 8, Analysis of Deviance Table, $\chi 2$ = 0.3014, *df* = 1, P = 0.583002) as treated differently later, indicating that all groups can be compared with respect to memory consolidation.

Five hours after acquisition one group of bees was intoxicated with 0.3 ng clothianidin while the control group received sucrose solution only, and 19 hours after intoxication the memory of both groups was tested. The clothianidin treatment had a negative effect on memory retrieval (Fig. 8, $\chi 2$ = 59.8673, df = 1, P = 1.015e-14). Whereas control bees consolidated their memory overnight, bees treated with clothianidin showed significantly lower PER for extinction trial 1 (E1) than for A3 (Fischer's exact test, P = 0,0068) indicating a loss of the memory consolidation effect. The control group increased its PER to 68.7 % (E1) whereas the treated group showed

only 34.9 % PER (Fig. 8, E1 control *vs* treated, 38.43 ± 4.96, P< 0.0001). Similar levels of significance were revealed for the two further extinction tests (E2 and E3).

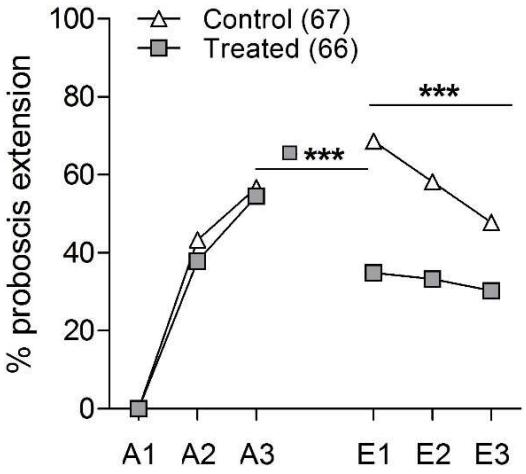

Figure 8. Memory consolidation effect after treatment with clothianidin. Retention scores were quantified by three extinction trials (E1, E2, E3) 24 hours after learning (acquisition, A1, A2, A3), and 19 hours after treatment or not (control) with 0.3 ng of clothianidin diluted in sucrose. Significant differences (P< 0.05) between the control group and the treated group are represented by stars in the graph. The number of individuals in each group is given in brackets in the legend.

Memory Retrieval

In order to investigate the effect of clothianidin on memory retrieval, treated bees were fed with 0.3 ng clothianidin 1 hour prior to the memory retrieval test. Six extinction trials were applied in order to test the stability of memory. Three extinction trials were performed 24 hours after conditioning and 3 further extinction trials 3 hours after the first set of extinction trials.

As expected, the acquisition of the groups treated or not 24 hours later were not significantly different for either of the two doses tested (Fig. 9A, 0.3 ng, $\chi 2 = 0.0014$, *df* = 1, P = 0.970380; Fig. 8B, 0.8 ng, $\chi 2 = 0.2372$, *df* = 1, P = 0.6262201).

For the 0.3 ng dose, the first three extinction tests revealed significant differences between the control and the treated group (Fig. 9A, $\chi 2 = 5.9973$, *df* = 1, P = 0.014328) with 47.6 % of the control and only 22 % of the treated bees responding to the CS in the first test (E1). Whereas the control group showed a strong extinction of the memory along the three first extinction trials, the treated group showed no clear memory extinction pattern. Also, treated bees showed significantly lower retrieval scores during E1 as compared to A3 (Fig. 9A, Fischer's exact test, P < 0.0001). This was also the case for control bees, but the difference was not significant.

The difference between the control and treated group regarding memory retrieval was even more obvious when the treated group was intoxicated with 0.8 ng clothianidin (Fig. 9B, $\chi 2 = 80.4767$, *df* = 1, P < 2e-16). Indeed, 60.6 % of the control bees responded to the CS against only 20.3 % of the treated bees (Fig. 9B, E1, control vs treated, 20.06 ± 2.24, P < 0.0001). On

the contrary to the control group, similar PER rates were revealed for the two further extinction tests (E2 and E3) of the treated group. Also, treated bees showed significantly lower retrieval scores for E1 as for A3 (Fig. 9B, Fischer's exact test, P< 0.0001) whereas control bees showed higher (not significant) retention scores.

The second set of extinction tests revealed no significant difference in the memory retrieval of the control and treated groups with 0.3 ng (Fig. 9A, $\chi 2 = 3.059$, *df* = 1, P = 0.080290) or 0.8 ng (Fig. 9B, $\chi 2 = 1.5572$, *df* = 1, P = 0.2120810). In both cases, the treated groups seemed to regain some memory of the CS during the second set of extinction trials whereas the control group showed comparable (Fig. 9A) or lower memory retrieval rates (Fig. 9B). The recovering of the memory was particularly remarkable in the case of the treatment with 0.8 ng clothianidin as 39.1 % of the treated bees remembered the CS at E4 against only 18.8 % at E3 (Fischer's exact test, P = 0.0029).

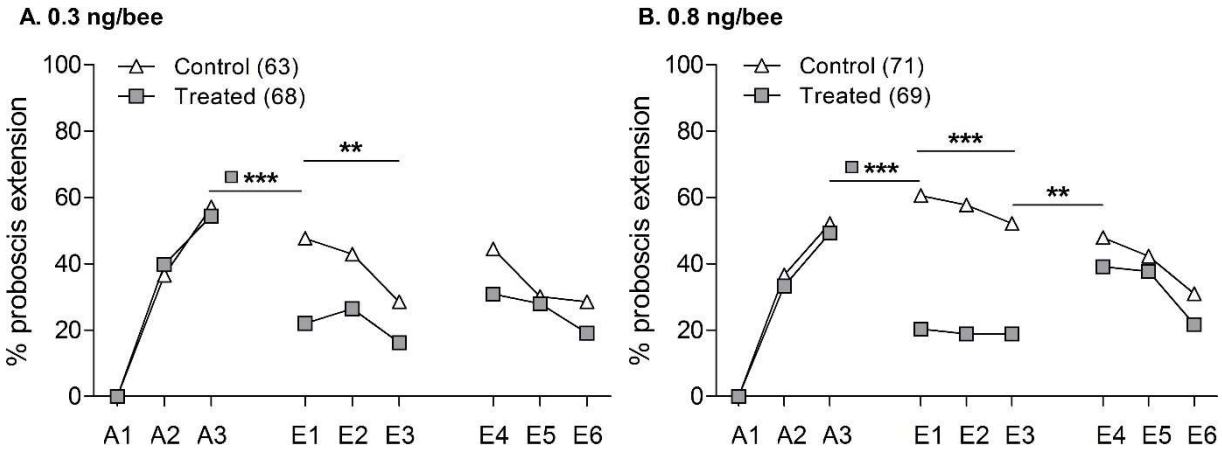

Figure 9. Memory retrieval after treatment with clothianidin. Retrieval scores were quantified by three extinction trials (E1, E2, E3) testing the probability of PER after odor conditioning 24 hours earlier (acquisition, A1, A2, A3), and 1 hour after treatment (or not, control) with **A.** 0.3 ng or **B**. 0.8 ng clothianidin diluted in sucrose. Significant differences (P< 0.05) between the control group and the treated group are represented by stars in the graph. The number of individuals in each group is given in brackets in the legend.

Discussion

Both field and laboratory studies are necessary in biology as the understanding of specific effects on behavior observed in field conditions can often only be studied in controlled laboratory conditions. This is the first study to investigate the effects of a neonicotinoid insecticide on such a large range of behaviors, using both lab and field experiments, acute doses, and chronic concentrations. We found that clothianidin impaired the normal foraging behavior and recruitment rates of honey bees chronically exposed to low concentrations in the field. We also found that memory performances of honey bees acutely intoxicated in the laboratory were impaired by sublethal doses of clothianidin.

On the contrary to the results of our previous field study with thiacloprid (Tison *et al.*, 2016), no impairment of honey bees homing success was revealed in this study (Fig. 5). Schneider *et al.* (2012) found that 100 % of the control and 94.4% of the treated bees returned to the hive during a three-hour observation period immediately after an acute treatment with 0.5 ng clothianidin. Based on the crop-emptying measurements by Fournier *et al.* (2014) the foragers in our experiment could have assimilated in 50 min from 0.04 ng to 0.08 ng clothianidin depending on the concentration at the feeder. This very low “acute” dose (adding up to the chronic exposure) might simply not be sufficient to impair the homing success and navigation of bees. On the contrary to thiacloprid which seem to accumulate also in the bee heads (Tison *et al.*, 2016 and Chapter 2), exposure to clothianidin led to an accumulation of substance (Fig. 4) but only in the abdomens of the foragers (Table S2). The fact that clothianidin was mostly found in the abdomens and not much in the heads where the nAChR are located could explain why the homing success of these bees was not impaired. If the substance is absent or less present in the heads it has less change to interfere with honey bee’s cognitive function of the tested bees.

The results of the survival analysis showed that the variable “treatment” and “concentration” (4.5 or 9 ppb) had no significant negative effect on honey bee survival (Table 2). The number of days a bee foraged at its feeder before being released (“time foraging”) had however a significant effect on the homing success, indicating that the duration of the exposure to clothianidin mattered. We could also show that the experiment and thus the time when the experiment was performed (related to weather conditions and status of the colony) and/or the feeding location had an influence on the homing success and the flight duration. Because these bees foraged at different feeding locations, the effect indicates a site-specific component. Also, the temperature at the release time had a significant effect on honey bee homing abilities. The temperature was lower during the second half of the summer which could have influenced the homing success of bees. Henry *et al.* (2014) showed such an influence of

the weather and temperature in their study with thiamethoxam. However, in a previous study in the same experimental area we revealed no influence of the temperature, probably because the conditions were less variable and the temperature higher throughout the summer (Tison *et al.*, 2016).

We showed that consumption of clothianidin-contaminated nectar by foraging bees led to reduced life-span, foraging efficiency and recruitment rates.

Honey bees visiting a feeder containing clothianidin foraged over shorter periods of time (Fig. 1), probably because they died earlier than the control bees. This result is not surprising as other studies already reported the same effects with other neonicotinoids (Oliveira *et al.*, 2014; Tison *et al.*, 2016). Also, morphological and histochemical alterations were observed in the brain structures and midgut from exposed bees, contributing to the reduction of their lifespan (Oliveira *et al.*, 2014). Furthermore, the overexpression of the vitellogenin transcript in the brains of exposed honey bees could explain the alteration in foraging activity and accelerated aging (Christen *et al.*, 2016).

We showed that bees foraging chronically at a feeder contaminated with 4.5 ppb or 9 ppb clothianidin collected on average respectively about 13 ng or 14 ng clothianidin per bee and per day (Table S1). These estimations based on our measurements correspond to the EFSA (2013) estimations of how much residues a honey bee can collect when foraging on oilseed rape treated with the highest maximal application rate of clothianidin (up to 13.65 ng per forager bee in one day). We found that a forager could collect between 0.28 ng and 0.53 ng of clothianidin on one trip at the feeder depending on the experiment and the concentration of the clothianidin solution at the feeder. Schneider *et al.* (2012) showed that administration of 0.5 ng clothianidin resulted in a significant reduction (31%) of the number of feeder visits per bee compared to the control group. In our experiment we showed that a chronic uptake of similar or lower amounts of clothianidin at the feeder (9 ppb) led to a very similar reduction of the foraging activity at the contaminated feeder (Fig. 2, 33 % in experiment 1 and 34 % in experiment 2). We can imagine that bees in our experiments remained inside the hive until the spiking effect of clothianidin ceased and they were able to fly out to the feeder again (Schneider *et al.*, 2012).

A prolonged stay inside the hive was not used for dance communication because dance activity was highly affected by a chronic uptake of clothianidin (Fig. 3), as was already shown with imidacloprid (Eiri and Nieh, 2012) and thiacloprid (Tison *et al.*, 2016). Even higher concentrations of sucrose at the contaminated feeder could not totally compensate for the reduced dance activity during the dance-induction periods. The results on regular foraging activity and dance performance show that clothianidin most likely alter the motivation to forage rather than the sensory or motor components of foraging. A reduced visitation of flowers by

bees would impair pollination efficiency in the long-term (van der Sluijs *et al.*, 2013) leading to dramatic consequence on honey bees, biodiversity and agriculture.

De Brito Sanchez *et al.* (2011) found that bees reject highly concentrated bitter and saline solutions when they are free to express avoidance behaviors but not when they are immobilized in the laboratory. The combination of both semi-field and laboratory experiments (sucrose responsiveness, Fig. 6) provides a solid base to argue that clothianidin has no repellent effect. At an artificial feeder, we observed that honey bees were unable to perceive clothianidin in sucrose solutions and would thus be unable to actively prefer or avoid the intake of clothianidin in treated environments, supporting the findings of Kessler *et al.* (2015). In their study, none of the concentrations of clothianidin tested (greater than in our experiment) altered the spiking activity of sucrose sensitive gustatory neurons in the bumble bees' or the honey bees' sensilla (Kessler *et al.*, 2015). It is clear that honey bees foraging on treated crops would thus ingest contaminated nectar, as they are not able to distinguish between treated and untreated sugar solution.

The effects of sublethal doses of clothianidin on the learning and memory of honey bees was investigated in the laboratory. First we showed that the acquisition rates of honey bees intoxicated with 0.1, 0.3 or 0.8 ng cothianidin one hour before learning were not different from those of the controls. However, negative effects on memory retrieval were revealed for these bees and the highest doses tested showed the strongest effects (Fig. 7). Since the learning of the association CS-US was not impaired by intoxication with clothianidin 1 hour before learning, the early long-term memory formation was probably affected by the substance immediately after acquisition. It was assumed that the consolidation process which ensures the transfer from short-term memory to middle-term memory within 10–15 min after the conditioning trial (Menzel, 1999; Erber *et al.*, 1980) is affected by exposure to imidacloprid, another neonicotinoid (Decourtye *et al.*, 2004). Similar impairment might be an explanation to our findings here.

Then we showed that the consolidation and memory retrieval of an olfactory association was compromised by intoxication with 0.3 ng 5 hours after the learning of it (Fig. 8). We also showed that 0.3 and 0.8 ng/bee strongly impairs the retrieval of the memory when the intoxication occurred 1 hour before the memory test but that the partial recovery of the memory was possible after a 3 hour wait time (Fig. 9).

In a previous study with the neonicotinoid thiacloprid (see Chapter 2) we showed that memory consolidation was impaired. Another study indicated that imidacloprid impaired long term memory consolidation (Williamson and Wright, 2013). By intoxicating bees 5 hours after the acquisition trials, we chose to study the transfer from median term memory to early long-term memory (Menzel, 1999; Müller, 2002). Long-term memory is formed after acquisition by

processes involved in memory consolidation. Distributed memory traces are established in the antennal lobes, the mushroom bodies and most likely in other neuropils (Menzel, 2012). Andrione *et al.* (2016) showed that the neonicotinoid imidacloprid impairs olfactory coding in the antennal lobe. Palmer *et al.* (2013) showed that an inhibition of the formation of action potential by exposure to clothianidin leads to the loss of functional capacity of Kenyon cells, intrinsic neurons of the mushroom bodies (Szyszka *et al.*, 2005) which express the nAChR at their input sites (Bicker and Kreissl, 1994; Goldberg *et al.*, 1999; Déglise *et al.*, 2002). The nAChR are also expressed in the peripheral olfactory pathway (Andrione *et al.*, 2016), but since we showed that clothianidin does not seem to interfere with olfactory coding it is most likely that the input site of the mushroom body is the prominent target of its action on memory consolidation and memory retrieval. The mushroom bodies are essential for mediating multisensory integration, learning, memory, spatial orientation, etc. (Heisenberg, 2003; Menzel, 2012; Palmer *et al.*, 2013; Zars *et al.*, 2000) and any impairment of their structure or function is expected to have dramatic consequences. The exact mechanisms of clothianidin action on the memory consolidation processes are unknown. Our data support the view that the normal function of the nicotinic transmission at the input site of the mushroom bodies is essential for the transition from middle term memory to early long-term memory and for the read-out from memory.

The three extinction trials performed during the memory retrieval tests allow to address the question whether extinction learning is compromised after treatment with clothianidin. Indeed, repeated exposure to the CS without rewarding it with sugar leads to the acquisition of a new condition that the CS is not rewarded anymore with sugar (Eisenhardt, 2012; Bitterman *et al.*, 1983). Extinction curves can be seen most of the time for the control and treated groups. Two tests showed however rather flat extinction curves for the treated group: the consolidation experiment when bees were intoxicated with 0.3 ng 19 hours before the memory retention tests (Fig. 8) and the retrieval test (extinctions E1-E3) when intoxication with 0.8 ng occurred 1 hour before the test (Fig. 9B). These two cases indicate an impairment of this particular form of learning (extinction learning). Consequences in the field would be an impairment of foraging behavior by compromising the ability of bees to switch food source when the flowers are no longer profitable.

In the case of the retrieval experiment, the use of two sets of extinction trials allows us to address the important question of whether an acute intoxication can lead to irreversible damage to the memory or whether the memory or some traces of it could be regained after a certain period of time. It seems that bees tested for their memory one hour after being intoxicated and then 3 hours after the first memory test, remembered the CS better in the second test, indicating that recovery of memory is possible when the memory is already formed. It has been shown that spontaneous recovery can occur with the passage of time after

extinction (Bitterman *et al.*, 1983; Sandoz and Pham-Delegue, 2004). In the first set of extinction trials, honey bees which do not remember the odor possess the memory of the odor, but intoxication with clothianidin 1 hour before the test prevent them from retrieving it, because access to the stored memory is blocked. After a recovery time of 3 hours, access to the stored memory seems possible again, at rates almost as good as the control bees. The results of the consolidation experiment show however that the possible recovery of the memory is not a question of time after intoxication but rather linked to the moment when intoxication occurs. The memory remains fragile until the consolidation processes have been completely terminated (Menzel, 1999). An intoxication 19 hours before the memory retrieval tests would leave enough time for bees to recover from an acute intoxication and retrieve their memory but intoxication with clothianidin during memory formation and consolidation seems to cause irreversible damage to the memory.

We revealed negative effects of thiacloprid on navigation and foraging behavior in a previous study (Tison *et al.*, 2016) and interpreted these effects as retrieval blocks of a long-term memory established during orientation flights early in the life of a foraging bee (Degen *et al.*, 2015). This study revealed negative effects on foraging behavior but no effect on the homing success, suggesting that the retrieval of a remote memory used in such test conditions (i.e. to find the way back to the hive) was not impaired by the exposure to clothianidin. This contrasts with the PER data since the retrieval of a remote memory but also the memory consolidation were impaired by an acute exposure with clothianidin. Laboratory experiments were shown to be good predictors of foraging efficiency under natural conditions (Raine and Chittka, 2008). The results of the laboratory experiments are in concordance with the impairment of foraging efficiency observed in the field. The retrieval of memories plays a crucial role in bee foraging behavior (Dukas *et al.*, 2008) and an impairment of these functions could have serious implications for colony fitness in the field since bees are constantly confronted to new learning and memory tasks in natural conditions (i.e. which flowers offer the best rewards (Biernaskie *et al.*, 2009; Lihoreau *et al.*, 2011)).

List of symbols and abbreviations

ACh acetylcholine

AChE acetylcholinesterase

CS conditioned stimulus

nAChR nicotinic acetycholine receptor

PER proboscis extension response

US unconditioned stimulus

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Supplementary Information content

Information about the sucrose consumption at the feeders (Table S1). Pesticide residues analysis of honey bees exposed to clothianidin (Table S2). Mortality and response to the US test of bees intoxicated with clothianidin (Table S3),

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Supporting Information

Table S1: Sucrose consumption at the feeders and estimated amounts of clothianidin collected.

	duration feeder open (min)	total consump -tion /day (ml)	No. of bees at the feeder	estimated sucrose collected /bee /day (ml)	estimated clothianidin collected /bee /day (ng)	estimated No. of trips /bee /day
4.5 ppb, experiment 1						
control	457.71 ± 17.54	113.86 ± 10.02	41 ± 1	2.75 ± 0.24		55.26 ± 3.46
treated	463.14 ± 16.65	127.29 ± 3.73	50 ± 1	2.59 ± 0.09	12.95 ± 0.45	45.60 ± 6.12
9 ppb, experiment 1						
control	401.00 ± 44.89	113.00 ± 14.00	37 ± 3	3.00 ± 0.14		43.93 ± 3.27
treated	421.86 ± 47.25	106.57 ± 15.26	40 ± 4	2.58 ± 0.24	12.90 ± 1.21	28.88 ± 2.78
9 ppb, experiment 2						
control	416.36 ± 9.86	71.05 ± 5.39	38 ± 2	1.91 ± 0.16		46.17 ± 3.82
treated	384.27 ± 15.96	52.73 ± 5.30	27 ± 2	1.43 ± 0.16	14.32 ± 1.62	27.03 ± 2.21

Numbers shown are means ± s.e.m.

Table S2. Pesticide residues analysis of honey bees chronically exposed to clothianidin in the field

	days of exposure *	sample weight (mg) #	clothianidin residues (ng/g)			(ng/bee)	
			head	thorax	abdomen	**whole body**	**whole body**
Feeder Exp. 1	1-2 days	894.5	n.d.	n.d.	2.2	**2.2**	**0.2**
	3-6 days	1249.5	n.d.	n.d.	2.1	**2.1**	**0.2**
	7-+ days	1051.3	n.d.	n.d.	2.9	**2.9**	**0.3**
Feeder Exp. 2	1-2 days	982.7	n.d.	n.d.	2.4	**2.4**	**0.2**
	3-6 days	954.0	2.8	n.d.	n.d.	**2.8**	**0.3**
	7-+ days	1002.3	n.d.	n.d.	3.2	**3.2**	**0.3**
Hive	7 days	764.1	n.d.	n.d.	n.d.	**n.d.**	**n.d.**
	30 days	829.8	n.d.	n.d.	2.8	**2.8**	**0.2**
LOD *§*			*1.2*	*0.4*	*0.5*		
LOQ *§*			*2.4*	*0.8*	*1.2*		
Recovery (% RSD)			*81 % (5.5 %)*	*87.6 % (7 %)*	*104.8 % (10 %)*		

No residues were detected in any of the control samples analyzed

* Days of exposure Feeders: number of days foraging at the feeders before sample collection / Hive: number of days since the beginning of the experiment

The sample weight is the sum of the weights of the separated analyzed honey bee body parts (from 9 to 13 bees/sample)

n.d. not detectable

§ LOD, limit of detection (3 times background noise); LOQ, limit of quantification (10 times background noise). The calculation is based on an average weight of 10 bee body parts each.

Table S3. Mortality and response to the US test of bees intoxicated with clothianidin

type of test	treatment	% of bees dead after intox	significance (Chi square)	% of bees not responsive to US test (alive bees only)	significance (Chi square)
Learning	control	1.56	χ^2= 2.70, *df*= 5, P= 0.7466	0.00*	χ^2= 5.56, *df*= 3, P= 0.1320
	0.1 ng	0.00		0.00*	
	control	2.53		0.00	
	0.3 ng	1.30		4.05	
	control	1.61		1.64	
	0.8 ng	0.00		6.66	
Consolidation	control	1.45	χ^2= 0.00, *df*= 1, P= 0.9835	2.94	χ^2= 0.30, *df*= 1, P= 0.5854
	0.3 ng	1.49		1.52	
Retrieval §	control	2.60	χ^2= 18.4, *df*= 3, P= **0.0004**	22.22	χ^2= 1.91, *df*= 3, P= 0.5914
	0.3 ng	1.22		11.76	
	control	0.00		18.31	
	0.8 ng	12.5		15.94	

* Groups from a same test (treated and relative control) for which the % equals to 0 were not included in the *Chi-square* test.

§ Bees in the retrieval experiment were tested two times for their memory at 3 hours interval (6 extinction trials instead of 3 in the other experiments) before they were tested for their US.

Significant p-values (< 0.05) are showed in bold letters.

Chapter 3 - Perspectives

Video recordings

In both hives, videos were recorded on the lower comb, on the side of the observation hive where bees were dancing the most (dance floor). In each set-up, a camera (Pi Cam NOIR, Raspberry Pi) was filming the entire lower comb through the Plexiglas of the observation hive, in a dark box with one multiple red LED light placed above the camera, in direction to the lower comb. We filmed dancing bees on 24 different days during dance induction (average of 2 h per day). The aim with this data is to automatically quantify the number of waggle runs as well as their direction by using a combination of 2 custom-made softwares. The first one will detect number tags on the thorax of bees and track their trajectory on the comb. The second one is a matlab script written by Dr. Tim Landgraf which should allow the automatic detection of waggle runs and their direction among the trajectories tracked by the first software. The output would be a count of the number of waggle runs as well as the direction of each run, extracted from x and y coordinates.

The comparison of the video recordings with the electric field recordings will allow a precise detection and quantification of dances as well as an evaluation of the quality of the video-tracking and electric field recording methods.

Electric field recordings

The electric fields emitted by dancing bees (Greggers *et al.*, 2013) consist of low-frequency (movements of the abdomen, 16 Hz on average) and high-frequency (buzzing of the wings, 230 Hz) components synchronization, leading to an average of three to seven electric pulses per waggle. Like it was done in the Tison *et al.* (2016) study with thiacloprid, electric-field measurements were performed at the same time on both sides of the lower comb in the control and treated hives in order to study honey bees' dancing performances during dance induction. We recorded electric fields of dancing bees on 24 different days (average of 2 h per day). 4 microphones were positioned in the dance area (14 cm^2 covered) on each side of both treated and control observation hives. The 8 electrodes per hive were powered by a lithium battery and were connected to a stereo amplifier (Audiobox 1818 VSL, Presonus, Baton Rouge, US) with a sample rate of 44.1 kHz. Each amplifier was connected to a laptop, and the software Presonus Studio One (version 2.4) was used for saving the data as WAV files. Both hives were closed separately in a faraday cage and the batteries, amplifiers, and laptops were placed outside of the cage.

The quality of these recordings will allow us to use the cutting-edge program for analyzing electric field recordings developed by Aron Dür at the institute. His program is in the final stage

of development and will allow us to extract and quantify several parameters characteristic of honey bee dance activity inside the hive. The number of waggles, the percentage of time spent fanning and the stop signals can be automatically detected and quantified with this program. Depending on the outcome of the analysis of the videos and electric field recordings, the results about dance communication will be published together with the data presented in the above manuscript or in a separate research article or short communication.

General discussion

Pesticide exposure contributes to the current decline in populations of pollinating insects and other beneficial arthropods (Desneux *et al.,* 2007; Goulson, 2013; Goulson *et al.*, 2015; Potts *et al.*, 2016). Insecticides are normally applied in ways to mitigate their impact on bees, but mitigation strategies are not possible with neonicotinoids as they are applied as prophylaxis treatment and are thus always present in the plant. In the past 20 years, there has been a rapid increase in the use of neonicotinoids (Elbert *et al.*, 2008) with improved selectivity for insects relative to vertebrates (Tomizawa and Casida, 2003, 2005). Due to their systemic properties, neonicotinoids can be found everywhere, not only in pollen and nectar of treated plants but also in the wildflowers of field margins (Botias *et al.*, 2015), in leaf litter, soil and water. There is a risk for non-target pollinators to be adversely affected via consumption of contaminated nectar and pollen or via direct acute contact exposure (Blacquiere *et al.*, 2012; Goulson, 2013; Krupke *et al.*, 2012). Sublethal and chronic exposure is the most likely scenario in the field as neonicotinoids concentrations found in nectar and pollen are usually too low to induce direct lethal effects.

I focused my research on two substances: thiacloprid, a widely used cyano-substituted neonicotinoid thought to be less toxic to honey bees and of which use has increased in the last years, and clothianidin, a nitro-substituted neonicotinoid mostly applied as seed-treatment and currently subject to a moratorium in the EU.

Honey bees (*Apis mellifera carnica*) were chronically exposed to field-realistic concentrations of thiacloprid or clothianidin in the field for several weeks. We found that the foraging behavior and recruitment rates via dance communication were impaired by both thiacloprid and clothianidin. Previous studies also demonstrated a reduced foraging activity of honey bees on sucrose solutions contaminated with thiacloprid (Schmuck *et al.*, 2003), imidacloprid (Eiri and Nieh, 2012; Colin *et al.*, 2004; Yang *et al.*, 2008), or clothianidin (Schneider *et al.*, 2012).

The recruitment of new foragers to the feeder via dance communication was measured with counters at the feeders' entrance and via electric field recordings. The dances were also filmed with video cameras. However, the videos of the exposure to thiacloprid could not be used and analyzed due to low quality and bias in the set-up. The electric field recordings of the field exposure to clothianidin will be soon analyzed with the program currently developed by Aron Dür in the institute. The video recordings also need to be analyzed and we are still trying to figure out the best way how, in collaboration with Dr. Tim Landgraf.

We tried to compensate for the reduced foraging activity by increasing the sucrose concentration at the contaminated feeders, but the reduced foraging and dance activity could never be totally compensated for. For both substances, we showed via laboratory PER tests and semi-field tests that the effects observed in the field were not due to a repellent taste. This was not very surprising as the same kind of result was found via different methods for other neonicotinoids (Kessler *et al.*, 2015).

Impaired foraging can lead to poor nutrition, making bees more susceptible to other disturbances like weather conditions, additional pesticides intoxication, parasites, and pathogens (Vidau *et al.*, 2011; Pettis *et al.* 2012; Di Prisco *et al.*, 2016). In addition, neonicotinoids interfere with brood development and shorten lifespans of adults (Henry *et al.* 2012, 2015; Wu *et al.* 2011; Desneux *et al.* 2007), maybe because of an overexpression of the vitellogenin transcript in the brain (Christen *et al.*, 2016). We have confirmed this in our experiments as bees exposed to thiacloprid or clothianidin foraged on average respectively 0.8 and 1.4 days less at their feeder than non-exposed bees.

Furthermore, the residue levels increased both in the foragers and in the nest mates over time especially for thiacloprid. Indeed, adult bees are not directly killed by foraging on the substance as the concentrations they are exposed to at the feeder are very low. We calculated that a foraging bee collected on average 216 ng of thiacloprid on one trip at the feeder which is 80 times less than the acute oral $LD50^{(48h)}$ of 17320 ng/bee. A bee foraging on clothianidin collected on average between 0.28 ng and 0.53 ng clothianidin (about 50 µl of solution) depending on the concentration used at the feeder (4.5 or 9 ppb). This is between 13 and 7 times less than the acute $LD50^{(48h)}$ of 3.7 ng/bee. Furthermore, only a small fraction of this amount is actually taken-up by bees on their return flight, the rest being stored into the colony (Tison *et al*., 2016). In natural conditions, the amount of substance a bee metabolizes when foraging on contaminated nectar sources is very hard to quantify as it depends on the distance from the food source to the hive, the flight time during foraging, the motivational state (Balderrama *et al.*, 1992) and the reward rate (Balderrama *et al.*, 1992; Fournier *et al.*, 2014). The contaminated nectar and pollen brought back to the colony is then fed to the larvae and consumed by other foragers, also during winter time when the foraging season is over. The chronic esposure to neonicotinoids thus not only lasts during the flowering season but as long as bees remain in their contamined colony.

Relatively to the LD50, we exposed bees to much lower doses of thiacloprid than clothianidin but the effects observed in the field were stronger for thiacloprid. The homing success and navigation performances of bees were impaired for bees exposed to thiacloprid but no difference was found in the homing success of bees exposed to clothianidin, supporting previous studies (Schneider *et al.*, 2012). The effects of clothianidin on the homing success and flight duration appeared dependant on the landscape context (here the feeding location)

and the weather conditions (Henry *et al.*, 2012, 2014) which seemed to be also the case for thiacloprid but only for specific flight patterns.

We revealed dramatic effects of a sub-lethal concentration of thiacloprid on forager survival (24 % risk of homing failure for treated bees against 8 % for control bees) with potential contributions to collapse risk. Higher risks are observed when the homing task is more challenging (Henry *et al.*, 2012). Impact studies in which bees are fed inside of the colony or conducted on honey bee colonies placed in the immediate proximity of treated crops are likely to severely underestimate sublethal pesticide effects as they are not challenging bees to forage at all or far from the colony.

Analysis of the flight tracks recorded with the harmonic radar in the experiment with thiacloprid showed that the active and recently acquired navigation memory which would have brought the animals back to the hive (vector memory) is not compromised. The transition from vector flight to homing flight requires the activation of a different reference system, the activation of a remote memory acquired during the exploratory orientation flights (Degen *et al.*, 2015) and/or the foraging flights before training to the feeder (less relevant). With this catch-and-release experiment, we could document that the ability of a bee to retrieve this remote memory is compromised. Thiacloprid act as a blocker of the nAChRs, highly present receptor in the mushroom body which is a key structure in multimodal integration, learning and memory formation as well as memory retrieval in honey bees (Heisenberg, 2003; Menzel, 2012). Also, the thiacloprid treatment was shown once more to reduce the flight speed of exposed bees (Fischer *et al.*, 2014).

In collaboration with Dr. Tim Landgraf, the flights tracked with the harmonic radar are being further analyzed. Certain criteria are defined and different hypotheses put forward about what we expect from a fying bee in order to locate itself and navigate in such highly structured landscape. We know that bees could not use path integration since they were transported to an unexpected release site. In addition to the use of cues such as the sun compass or the polarized light, bees have to use their memory of the landscape and landmarks present in the area in order to perform successful homing flights. We have noticed that the landscape offered strong cues to the bees such as roads, possibly helping them to orientate or misguiding them. The analysis of the ground structure and particularly the elongated landmarks in relation to the bees' in-flight decisions is of particular interest to us and is currently investigated. We expect promising results from these analyses.

In order to study the influence of thiacloprid, Calypso® and clothianidin on honey bees' learning and memory performances, we applied a laboratory standard procedure, the Proboscis Extension Response (PER) conditioning.

We showed that thiacloprid poses a substantial risk to honey bees by disrupting their learning and memory consolidation functions at doses as low as 69 ng/bee and their memory retrieval functions at doses as low as 20 ng/bee. These doses correspond respectively to 250 times and 860 times less than the acute oral $LD50^{(48H)}$ of thiacloprid. Also, residues of thiacloprid found in the bee bodies and especially in the heads were shown to be correlated to the dose applied and the time between intoxication and collection of samples. We can speculate an accumulation and persistence of the substance in the tissues targeted by thiacloprid (i.e. nAChR receptors in the bee brain), supporting observations from the field study (Tison *et al.*, 2016).

The consolidation and the retrieval of memory was also impaired in the case of bees exposed acutely to sublethal doses of clothianidin, up to 12 times lower than its the $LD50^{(48H)}$. No effect on acquisition functions was however revealed.

Furthermore, we found that the thiacloprid-based formulation Calypso®, widely used on plants and flowers in gardens and horticultural nurseries (also during flowering), significantly impaired the learning and memory performances of honey bees. Their learning function was impaired at the sublethal doses of 120 and 200 ng/bee and their memory consolidation at 200 ng/bee. These doses are contained in about 1 µl of of the ready-to-spray formulation. Doses as low as 69 ng/bee had significant negative effects on memory retrieval and bees intoxicated with 2 or 20 ng/bee Calypso® failed to differentiate between learned and novel odor. It also seemed that the active substance thiacloprid in Calypso® tend to accumulate in the bees body and especially in the heads in higher proportions that the active substance alone.

We suggested an impairment of the mushroom bodies' function at their input site as an explanation to the results observed on memory consolidation and retrieval. The intrinsic neurons of the mushroom body, the Kenyon cells play a particularly important role in the learning and memory processes (Szyszka and Menzel, 2005) and they were shown to express nAChRs (the main target of neonicotinoids) at their input sites (Bicker and Kreissl, 1994; Goldberg *et al.*, 1999; Déglise *et al.*, 2002). Furthermore, some studies already shown that their physiology and function was impaired by exposure to neonicotinoid insecticides (Palmer *et al.*, 2013; Peng and Yang, 2016).

The laboratory tests performed specify and confirm the results obtained in the field studies presented in chapter 1 (Tison *et al.*, 2016) and 3 and in other field studies (Fischer *et al.*, 2014; Schneider *et al.*, 2012). Such tests have been considered as good predictors of the foraging efficiency under natural conditions (Raine and Chittka, 2008) and thus indicate once more, that thiacloprid and clothianidin are not unharmful substances for bees, even at very low sublethal doses. Honey bees exposed to thiacloprid or clothianidin inside the hive via the stored food or outside when foraging on contaminated flowers, are expected to see their learning and memory

performances impaired. These functions are however highly important for a large range of neural and behavioral processes such as efficient foraging, navigation, and communication.
Furthermore, neonicotinoids formulations used on ornamentals such as Calypso® lead to residues 12-16 times greater than found on crop plants (Hopwood *et al.* 2012) and they can be found in bee friendly plants produced by horticultural nurseries (Goulson 2013; Hopwood *et al.* 2013). Foliar sprays of such neonicotinoid formulation during flowering could have disastrous outcomes on the health of exposed pollinators.
For Mullin *et al.* (2015) "the formulation and not just the dose makes the poison". Future studies should concentrate their efforts on investigating the effects of neonicotinoids not only as active substances but also as formulations and as mixtures of pesticides. The potentially synergistic and long-term impacts of pesticide mixtures on insect individuals, colonies, and populations remain largely unresolved (van der Sluijs *et al.*, 2015; Pisa *et al.*, 2015). We know that thiacloprid is often used together with other pesticides in mixtures (Mullin *et al.*, 2010), and some synergism and concentration addition effects have already been observed between thiacloprid and imidacloprid in *Daphnia magna* (Pavlaki *et al.*, 2011) and earthworms *Eisenia fetida* (Gomez-Eyles *et al.*, 2009) as well as between thiacloprid and ergosterol biosynthesis inhibiting fungicides in honey bees, for which the synergistic factor can be as high as 560-fold (Iwasa *et al.*, 2004; Schmuck *et al.*, 2003).
Research on the effects of pescides mixtures are urging if we want to understand the consequences of realistic field exposure to pollinators to the coktails of pesticides found in apiaries, treated fields or field margins (Mullin *et al.*, 2010; Botias *et al.*, 2015; David *et al.*, 2016).

Beneficial insects are killed by acute exposure to neonicotinoids sprays and by chronic exposure to poisonous pollen and nectar or poisoned preys such as aphids for ladybugs (Hopwood *et al.* 2013). Biocontrol represents more than $4 billion a year to agriculture, and bees provide up to $19 billon of pollination services. The value of beneficial insects thus exceeds by far the $2.6 billion of neonicotinoid sales (Losey and Vaughan, 2006; Jeschke *et al.* 2011). In addition to the risk established for honey bees, large negative impacts of neonicotinoids have been revealed on bumble bee colony growth and reproduction (Whitehorn *et al.,* 2012, Goulson 2015; Rundlöf *et al.,* 2015) but also on solitary bees such as *Osmia* (Rundlöf *et al.,* 2015). The risk of neonicotinoids to bumble bees or solitary bees is expected to be about 2 to 3 times as high as for honey bees due to the different sensitivity among the species (Sanchez-Bayo and Goka, 2014). Whether this is due to greater exposure or greater sensitivity is difficult to say.
There is growing evidence that butterflies are also negatively affected by neonicotinoids (Guilburn *et al.*, 2015). Soil applications kill ground dwelling beneficials such as ground beetles

and earthworms as well. Neonicotinoids are also killing birds (Goulson, 2014) and a major route of exposure is through seed treatments. Indeed, some birds can be killed by eating just one poisoned seed (Mineau and Palmer 2013). Of the commercial neonicotinoids, acetamiprid, imidacloprid, and thiacloprid are considered most toxic to birds, and thiacloprid to fish (Tomizawa and Casida, 2005). Although seed treatments prevent pesticide drift and the environmental problems of sprays, the vast acreage of exposure is becoming a problem (Goulson 2013). Furthermore, the overuse and persistent exposure to neonicotinoids leads to resistance of insect pests (Jeschke *et al.*, 2011; Mineau and Palmer, 2013).

The indiscriminate use of persistent, broad-spectrum insecticides, and the abandonment of traditional practices such as crop rotations, led to huge pest outbreaks in the 60s. The pest insects had all become resistant, while their natural enemies had largely been eradicated. As a result, an approach called integrated pest management (IPM) had been developed in order to minimise pesticide use. The idea is simple: only apply pesticide when pest pressure reaches an economic threshold, encourage natural enemies and use crop rotations and other cultural controls to diminish or suppress pests. The use the insecticides is used as a last resort and substances that persist in the environment are preferably not used.
Neonicotinoids allow growers to ignore good farming methods and IPM approaches in favor of systemic protection. Seed treatments are applied before pests are seen, and may actually not be needed. This approach can lead to unnecessary expense and no improvement in yield in years of low pest pressure (Gray, 2011; Seagraves and Lundgren 2012).
Agronomic studies about the costs and benefits associated with the use of neonicotinoids are urgently needed in order to help authorities to make an informed decision. The protection of pollinators through reduced use or removal of very toxic chemicals from markets should be balanced against the need to ensure agricultural yields and food security. Research is needed to provide viable alternatives to conventional highchemical- input systems. Education and training of farmers and the public are also necessary to ensure the safe use of pesticides.
It has been suggested that if neonicotinoids are forbidden, farmers would go back on using old generations of pesticides which are much worse for the environment. But this seems unlikely, since the use of most of such chemicals if not all, have been withdrawn. Another argument is that we would not have enough food to feed the global population as yields would be probably diminish. There is no evidence that yields would be drastically reduced if IPM methods are applied. Moreover, we already grow largely enough food to feed the global population now and in the future, we just waste enormous amounts of it.

A step has been taken to reduce the exposure to neonicotinoids in Europe but the moratorium was only for 3 substances (clothianidin, imidacloprid and thiamethoxam) and so many derogations exist that these substances are actually still used quite a lot. The use of

other neonicotinoids like thiacloprid has increased in the last years since the use of other substances has been suspended. Moreover, neonicotinoids are likely to be found at high levels in soils for many years due to their persistence in the environment so bees will still be exposed for quite a long time.

The loss of bees has attracted attention because our food supply depends on them. If we want to ensure healthy populations of honey bees, bumble bees, and other wild pollinating insects we need investment in research and support for sustainable farming systems with reduced inputs; and it is the role of governments to take actions. We need to find ways to produce food in a sustainable way which incorporates the needs of biodiversity. The conservation of the biological diversity of pollinators should be motivated not only by immediate benefits from ecosystem services (Gill *et al.*, 2016) but also by the arguments for conservation (Kleijn *et al.*, 2015).

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