

RESEARCH ARTICLE

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

Léa Tison*, Sophie Holtz, Amy Adeoye, Önder Kalkan, Nina S. Irmisch, Nadja Lehmann and Randolph Menzel

ABSTRACT

Learning and memory play a central role in the behavior and communication of foraging bees. We have previously shown that chronic uptake of the neonicotinoid thiacloprid affects the behavior of honey bees 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 conditioning) in order to assess which processes, acquisition, memory consolidation and/or memory retrieval were compromised after bees were fed either with thiacloprid or the formulation of thiacloprid named Calypso® at different sublethal doses. Extinction and generalization tests allowed us to investigate 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. These data support and specify the data collected in the field.

KEY WORDS: *Apis mellifera*, Pesticide, Neonicotinoid, PER, Proboscis extension conditioning

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) (Bijleveld van Lexmond et al., 2015). Although the putative causes of the recent decline in pollinators are still under investigation, the extensive use of pesticides against pest insects for crop protection has contributed to the loss of many pollinators (Brittain and 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. Neonicotinoids are systemic insecticides, providing protection to the plant against insect pests and arthropods feeding upon it (Tomizawa and Casida, 2005). There has been increasing evidence that these systemic insecticides also pose serious risk of impacts on some non-target organisms (Bijleveld van Lexmond et al., 2015), even if their effects on honey bees at the colony level have been questioned (Henry et al., 2015; Rundlöf et al., 2015). Thiacloprid, like other neonicotinoids, acts as an agonist to the insect's nicotinic acetylcholine receptor (nAChR).

Acetylcholinesterase can break down acetylcholine but not neonicotinoids, leading to paralysis and eventually death due to the constant activation of the nAChRs. 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 receptor and the α -BGT-insensitive receptor (Gauthier et al., 2006). These receptors are involved in tactile and olfactory sensations as well as in learning and memory (Cano Lonzano et al., 1996, 2001; Dacher et al., 2005; Barbara et al., 2008), which are all essential functions for foraging behavior.

Sugars are important appetitive stimuli for honey bees, controlling feeding behavior, foraging and recruitment during social communication. In addition, sucrose serves as a reinforcing stimulus for instrumental and operant associative learning (Hammer and Menzel, 1995). When stimulating the gustatory receptors located on the tarsi, antennae or mouthparts 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 olfactory 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 both 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, 2012; Müller, 2002).

Olfactory memory plays an important role in many aspects of honey bee behavior, including recognition of nest mates, 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 their foraging performance (Farooqui, 2013). The PER assay can be used for estimating the sublethal effects of pesticides in bees and it has been used in a number of studies already (Decourtye et al., 2005; Williamson and Wright, 2013). Negative effects of imidacloprid, clothianidin and thiamethoxam were observed on odor coding and olfactory learning and memory of honey bees and bumble bees (Decourtye et al., 2004a,b; Palmer et al., 2013; Stanley et al., 2015; Williamson et al., 2013; Andrienne et al., 2016). These three substances are the most studied neonicotinoids and actions have already been taken in Europe to suspend them (EFSA, 2012a,b). 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 a single active substance, and Calypso®, a formulation containing thiacloprid and other unknown ingredients.

Department of Biology, Chemistry and Pharmacy, Institute of Biology-Neurobiology, Free University, 14195 Berlin, Germany.

*Author for correspondence (lea.tison@gmail.com)

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Calypso® is a ‘ready to use’ spray formulation used against sucking and chewing insect pests on a large number of plants, flowers, fruit trees and vegetables, also during the flowering period. It is sold without restriction in garden centres because it is declared safe to bees. Co-formulants and supplemental adjuvants in pesticide formulations often enhance the pesticidal efficacy as well as inadvertently the non-target effects of the active ingredient after application (Holloway et al., 1994; Holloway, 1998; Sorgan 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 as a single active substance or as an ingredient in a formulated, commercially available pesticide (Calypso®) on learning, memory formation and memory retrieval. We tested different concentrations, all of which were significantly lower than the median lethal dose (LD₅₀). We found significant negative effects in both forms.

MATERIALS AND METHODS

Sampling

Summer honey bees *Apis mellifera carnica* Pollman 1879 were collected on their outbound flight, using a Plexiglas pyramid at the hive entrance, at 14:00 h in the garden of the Institute of Neurobiology of the Free University of Berlin, Germany. The bees were then transferred to ventilated glass vials and cooled on ice until immobile. They were harnessed individually in tubes that allowed free movements of the mouthparts and antennae (Matsumoto et al., 2012). At 16:00 h 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.

Sucrose responsiveness

In order to determine whether thiacloprid or Calypso® affects honey bees’ motivation for sucrose, the sucrose responsiveness of harnessed bees was assessed by stimulating each bee’s antennae with different concentrations of sucrose solutions: 0, 0.1, 0.3, 1, 3, 10 and 30% (w/v) (Scheiner et al., 2005; Matsumoto et al., 2012), containing [or not (control)] 50 ng µl⁻¹ thiacloprid, 50 ng µl⁻¹ Calypso® or 0.5% acetone (this is the maximum concentration of acetone in sucrose solution to which bees were exposed in the memory experiments). 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.

Olfactory conditioning

Shortly before conditioning, the olfactory stimuli were prepared by placing 4 µl of pure odorant (Sigma-Aldrich, St Louis, MO, USA), either hexanal or 1-nonanol, on a 1.32 cm² piece of filter paper inserted into 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 (extinction tests) and 1-nonanol for the second set of experiments (generalization tests). We used a similar set-up as described in Felsenberg et al. (2011) for conditioning. The CS was presented for 5 s, and the US (50% w/v sucrose) was presented for 4 s, 3 s after the CS. Each bee received three paired CS–US presentations (i.e. conditioning trials) with a 12 min 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.

Memory retrieval was assessed 24 h after the first conditioning trial. In the first set of experiments (extinction tests), only the CS (hexanal) was tested in three extinction trials and the bees were not rewarded with sucrose. In the second set of experiments (generalization tests), in addition to the CS (1-nonanol), bees were exposed to nonanol and to 2-hexanol to determine the selectivity of their response to the CS. Nonanol has a high degree of similarity to 1-nonanol and was thus expected to be perceived similarly by the bees, contrary to 2-hexanol, which was expected to be perceived differently (Guerrieri et al., 2005). In the generalization tests, odors were presented to each bee (one time each) with an inter-trial interval of 12 min and the bees were not rewarded with sucrose solution. The order of odor presentation in the generalization tests was shifted between each trial and this order was taken into account as a random effect in the statistical analysis, e.g. Trial 1: odors A, B, C were presented in this order (order 1); Trial 2: B, C, A (order 2); Trial 3: C, A, B (order 3); and Trial 4: back to order A, B, C (order 1). The order did not differ between different bees within a trial. The order of the odor presentation was tested in the model and had no effect. At the end of the memory tests, each bee was stimulated with a 50% (w/v) sucrose solution to determine whether its unconditioned response to sucrose was still intact (US test). Any bee that failed to display a PER during the US test was discarded, as well as any bee that died during the course of the experiment or any bee that extended its proboscis during the 10 s prior to odor presentation. The percentage of bees excluded in each test group can be seen in the supporting information (Table S1).

Experimental design

The time interval between conditioning (acquisition) and the memory test was always the same (24 h). The time of feeding and the doses used were the variable parameters. In order to test the effects of thiacloprid and Calypso® on different phases of learning and memory, we used different tests (extinction or generalization) and time intervals between the acute oral treatment with the pesticide and the memory test (Treatment Group). For each Treatment Group, we first performed the extinction tests, followed by the generalization tests. During an extinction test, the same odor was tested three times whereas in a generalization test, the learned odor was tested once and two novel odors were each tested once.

Appetitive learning

In order to test the possible effects on learning ability and memory formation, bees were treated with the pesticide (or control sucrose solution) 1 h before the first conditioning trial (i.e. 25 h before the memory test). These bees correspond to Treatment Group I.

Memory consolidation

To investigate the effects of thiacloprid and Calypso® on the consolidation of the memory, bees were treated 5 h after conditioning (i.e. 19 h before the memory test). These bees correspond to Treatment Group II.

Memory retrieval

To test the effect on memory retrieval, bees were treated 23 h after conditioning (i.e. 1 h before the memory test). These bees correspond to Treatment Group III.

Every day, about 30 bees per substance (thiacloprid or Calypso®) representing the different treatment groups and doses were tested blindly and simultaneously. Honey bee mortality was assessed throughout the experiment. The number of bees used in total for each dose and within each treatment group was usually between 50 and 60 bees. The exact number of bees used is given in parentheses in the figures.

Thiacloprid and Calypso® solutions

Stock solution: 10 mg of thiacloprid (98% purity, Sigma-Aldrich) was dissolved in 1 ml of acetone ($\geq 99.9\%$, Sigma-Aldrich) and 39 ml of distilled water leading to a concentration of 0.25 g l^{-1} . Acetone was chosen as the solvent following the European and Mediterranean Plant Protection Organization guidelines (OEPP, 1992). The control group was fed sucrose solution without acetone as we demonstrated that acetone had no effect on sucrose perception (Fig. 1) nor on memory retrieval (Fig. S1). Calypso® stock solution was directly taken from the Calypso® ‘ready to spray’ formulated pesticide bottle (Schädlingsfrei Calypso® Perfekt AF, Bayer AG, Leverkusen, NRW, Germany; $150 \text{ ng } \mu\text{l}^{-1}$ thiacloprid). The Calypso® safety sheet (for 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 are unknown.

Thiacloprid and Calypso® stock solutions were then diluted in order to obtain three concentrations of thiacloprid (50, 5 and $0.5 \text{ ng } \mu\text{l}^{-1}$) in a 30% (w/v) sucrose solution. Using a multipipette, each bee was fed orally with $4 \text{ } \mu\text{l}$ of control or contaminated feeding solution. The total amount of pesticide fed to the bees in the preliminary experiments (extinction tests) was 69 ng of thiacloprid as a single active substance. Because the results were promising, we then used the concentrations 120, 12 and 1.2 ng of thiacloprid as an ingredient in the formulation Calypso®. In the second set of experiments (generalization tests), 200, 20 or 2 ng bee^{-1} were given for both thiacloprid as active substance or as an ingredient of the formulation Calypso®.

Extraction and quantification of thiacloprid in honey bees by LC–MS/MS

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

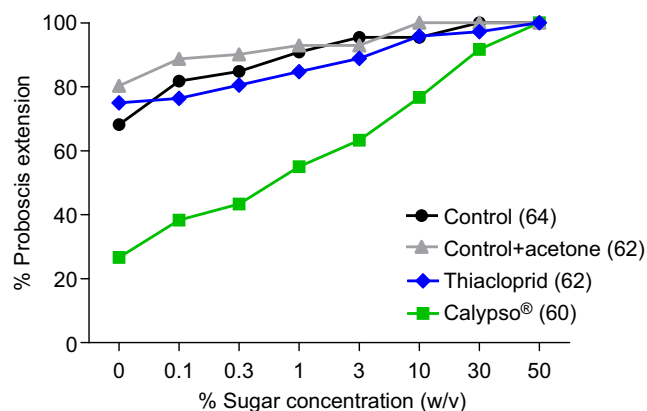


Fig. 1. Sucrose responsiveness of control and treated honey bees to different sucrose concentrations. The sucrose solution presented to the bees contained (or not, control), acetone alone (control+acetone), thiacloprid as active substance or as a formulation (Calypso®) at a concentration of $50 \text{ ng } \mu\text{l}^{-1}$. The number of individuals in each group is given in parentheses.

with liquid chromatography–tandem mass spectrometry (LC–MS/MS) for thiacloprid content. Thiacloprid and Calypso® stock solutions and feeding sucrose solutions were also analyzed for thiacloprid content.

Collected bees were cut into three parts: head, thorax and abdomen, and 30 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. Twenty ml of an acetone/water mixture (2:1, v:v) and $20 \text{ } \mu\text{l}$ of a surrogate standard solution containing thiacloprid-d4 ($1 \text{ ng } \mu\text{l}^{-1}$) were added to each sample. The samples were homogenized with a MICCRA KT disperser (MICCRA GmbH, Müllheim, BW, Germany) for 3 min and then centrifuged (10 min at 3000 rpm). Fifteen ml of the supernatant was removed and, after adding 5 ml of sodium chloride solution (20%) to this aliquot, transferred onto a disposable cartridge filled with diatomaceous earth (ChemElut® cartridges, 20 ml, unbuffered; Agilent, Santa Clara, CA, USA). After a waiting time of 15 min, the samples were eluted with dichloromethane ($2 \times 50 \text{ ml}$). The eluates were evaporated to $\sim 2 \text{ ml}$ 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 $50 \text{ } \mu\text{l}$ of an internal standard solution containing imidacloprid-d4 ($1 \text{ ng } \mu\text{l}^{-1}$) and $950 \text{ } \mu\text{l}$ 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.

The next day, the samples were filtered cold (syringe filter $0.2 \text{ } \mu\text{m}$) 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, MA, USA) equipped with an electrospray 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, $100 \text{ pg } \mu\text{l}^{-1}$). LOD and LOQ are given in Table S2.

As 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 doing this the concentrations of the solutions used in the experiments were confirmed. The LOD for thiacloprid in diluted sugar solution was $0.05 \text{ pg } \mu\text{l}^{-1}$ and the LOQ was $0.1 \text{ pg } \mu\text{l}^{-1}$.

Statistical analysis

The responses of each bee were scored as binary responses (PER, 1; no response, 0). We used R (<http://www.r-project.org>) and lme4 (Bates et al., 2015) to perform a generalized linear mixed model (GLMM) analysis of the relationship between PER and Treatment (the variable treatment represents the substance tested at different concentrations) (fixed effects: Treatment and Trial number; random factors: Bee identity, Session identity and Order of Odor Presentation for the memory tests). Several models (with or without interactions between factors) were tested and the best was selected using the Akaike information criterion. All models were validated by assessing normal Q–Q plots and residual versus fitted

data plots. P -values showing the influence of a fixed effect were obtained by analysis of deviance table (Type II Wald χ^2 tests) between the full model with the effect against the model without the effect. After the ANOVA, the *lsmeans* function was used and followed by Tukey *post hoc* tests. The P -value was adjusted to the number of estimates compared. We used Fisher's exact test to compare proportions. Sucrose responsiveness was also analyzed with GLMM (fixed effects: Treatment and Sugar Concentration; random factors: Bee identity and Session identity). We used χ^2 tests to compare the mortality and US tests rates between the doses.

RESULTS

Mortality and US tests

Mortality was evaluated for each dose by the number of bees dying in the time frame between treatment 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 treated with thiacloprid (Table S1, $\chi^2=12.93$, d.f.=3, $P<0.01$) or Calypso[®] ($\chi^2=35.21$, d.f.=3, $P<0.0001$). These differences are due to higher rates of non-responsive bees for the highest dose (200 ng) of Calypso[®] (Table S1, 24.86%) and, surprisingly, for the control group in the thiacloprid test (16.11%).

Sucrose responsiveness

Independent groups of bees were presented with increasing concentrations of sucrose solutions, containing (or not) 50 ng μl^{-1} thiacloprid, representing the highest concentration tested during the learning and memory tests. No effect of acetone (0.5% in sucrose solution) on sucrose responsiveness was revealed as no difference was found between the two groups (control versus control+acetone, Fig. 1, $P=0.52$). All groups showed increasing responses to the ascending sugar concentrations. No statistically significant difference was revealed by the model between the control groups and the thiacloprid group (Fig. 1, $P=0.12$) but a significant difference was found between the control group and the Calypso[®] group ($P<0.05$). Tukey *post hoc* tests revealed significant differences only for the three lowest sucrose concentrations, between 0.1% and 1% (w/v) (0.1%, $P<0.05$; 0.3%, $P<0.01$; 1%, $P<0.05$).

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

Appetitive learning

The learning ability was quantified by evaluating the acquisition functions (represented by three acquisition trials) after feeding the test bees with 69 ng of thiacloprid or with 1.2 ng, 12 ng and 120 ng

of thiacloprid in the formulation Calypso[®] 1 h before the onset of conditioning. An extinction test was performed in order to test the stability of memory by applying three extinction trials.

Control bees learned to associate the CS with a US at a higher level than treated bees (Fig. 2A,B). The level of acquisition was significantly lower in bees treated with 69 ng of thiacloprid (Fig. 2A, Tukey, $P<0.05$) or 120 ng of Calypso[®] (Fig. 2B, Tukey, $P<0.0001$).

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 h after acquisition (E1, Fig. 2A) than the last acquisition trial (A3) (Fisher's 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, $P<0.01$; 120 ng, $P<0.0001$). The memory tests indicated that thiacloprid at 69 ng bee^{-1} did not significantly change the retention scores (percentage of PER during the memory tests) as compared with the control bees either during the first (E1) or 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 E1 (Fig. 2B, E1) than the controls (Tukey, $P<0.0001$). No difference was found for the lowest dose (1.2 ng). Overall (all odors and doses combined), the treatment with thiacloprid and Calypso[®] had a negative effect on learning and memory (thiacloprid, $\chi^2=5.81$, d.f.=1, $P<0.05$; Calypso[®], $\chi^2=42.35$, d.f.=3, $P<0.0001$).

To further investigate the effects of thiacloprid and Calypso[®] on learning performance and 24 h memory retrieval, we performed a generalization test with another range of doses. The generalization test allows us to test 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. 3Ai). No significant effects were found between the acquisition functions of the four groups ($\chi^2=0$, d.f.=3, $P=1$). However, no conditioned response was observed 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. 3Bi) showed better learning rates than the control bees from the test with thiacloprid (Fig. 3Ai). Differences in learning performance were detected between bees fed with Calypso[®] and controls (Fig. 3Bi, $\chi^2=13.07$, d.f.=3, $P<0.01$), and the highest dose (200 ng) was shown to impair learning the most (Tukey, $P<0.001$).

Memory tests 24 h later indicated that control bees in both test series (thiacloprid and Calypso[®], Fig. 3Aii,Bii) responded more to the learned odor, 1-nonanol, than to the rather similar and novel odor, nonanal. The different novel odor, 2-hexanol, elicited the lowest response. No significantly different responses were found in

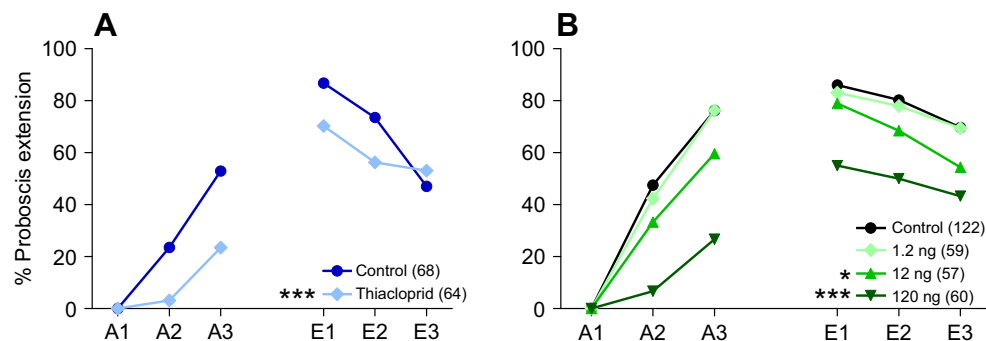


Fig. 2. Acquisition functions and retention scores after 24 h. PER scores were quantified by three acquisition trials (A1, A2, A3) 1 h after treatment with (A) 69 ng of thiacloprid diluted in sucrose or (B) 1.2, 12 or 120 ng of thiacloprid in Calypso[®] diluted in sucrose, and three extinction trials (E1, E2, E3) 24 h after conditioning. Significant differences ($P<0.05$) with the control are represented by asterisks in the keys. The number of individuals in each group is given in parentheses (* $P<0.05$; *** $P<0.001$).

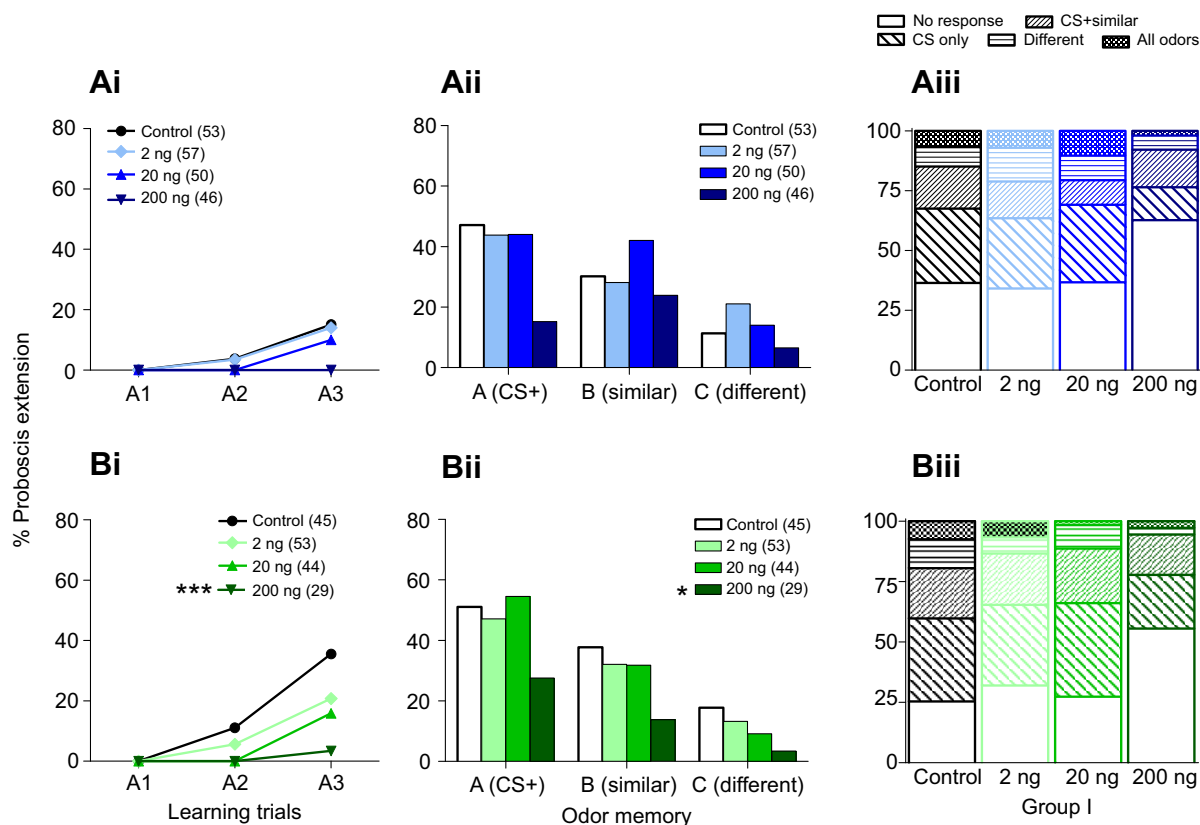


Fig. 3. Effect of thiacloprid and Calypso® on acquisition and memory. Retention scores were determined 24 h after acquisition and the treatment occurred 1 h before acquisition with 2, 20 or 200 ng of either (A) thiacloprid as active substance diluted in sucrose or (B) Calypso®, a thiacloprid formulation, also diluted in sucrose. (Ai,Bi) Acquisition of CS+ (% PER) during the three conditioning trials (A1, A2, A3). (Aii,Bii) Retention scores (% PER) during the generalization test, 24 h after learning. A (CS+), conditioned odor (1-nonanol); B (similar), similar odor (nonanol); C (different), different odor (2-hexanol). (Aiii,Biii) Distribution of bees according to their individual responses to the odors during the memory tests. Asterisks in the keys indicate statistically significant differences compared with control ($P < 0.05$). The number of individuals in each group is given in parentheses (* $P < 0.05$; *** $P < 0.001$).

the memory test between the control bees and bees fed with 20 ng or 2 ng of thiacloprid (Fig. 3Aii) or Calypso® (Fig. 3Bii). Bees who received 20 ng of thiacloprid, however, failed to differentiate between the learned odor [Fig. 3Aii, i.e. A (CS+), 44% PER] and the similar odor [Fig. 3Aii, i.e. B (similar), 42% PER]. Significantly more bees fed with the highest doses of thiacloprid or Calypso® did not respond to any of the three odors (Fig. 3Aiii,Biii, Fisher's exact tests, control versus thiacloprid 200 ng: $P < 0.001$; control versus Calypso® 200 ng: $P < 0.0001$). Fewer bees from these two groups responded only to the learned odor (Fig. 3Aii,Aiii, Fisher's exact tests, control versus thiacloprid 200 ng: $P < 0.01$; control versus Calypso® 200 ng: $P < 0.05$). Bees that ingested 2 ng or 20 ng of thiacloprid showed higher (but not significantly) responses to the different odor only (Fig. 3Aiii) than the control bees.

In this latter experiment, the memory of bees that ingested thiacloprid 1 h before acquisition was not significantly affected by the treatment ($\chi^2 = 5.59$, d.f.=3, $P = 0.13$) contrary to bees that ingested Calypso® ($\chi^2 = 10.07$, d.f.=3, $P < 0.05$). The highest dose of Calypso® significantly impaired memory retrieval ($P < 0.01$), whereas the other doses had no significant effects.

Memory consolidation

In order to investigate the effects of thiacloprid on memory consolidation, the test bees were fed with 69 ng of thiacloprid 5 h after conditioning.

First, an extinction test was performed in order to test the stability of the memory. No effect on learning was found between the control

and the treated group (Fig. 4, $\chi^2 = 3.05$, d.f.=1, $P = 0.08$) because treatment occurred after conditioning, indicating that the two groups can be compared with respect to memory consolidation.

Memory tests 24 h after acquisition (=19 h after treatment with thiacloprid) showed a significant difference between the control and the treated group. Whereas control bees consolidated their memory

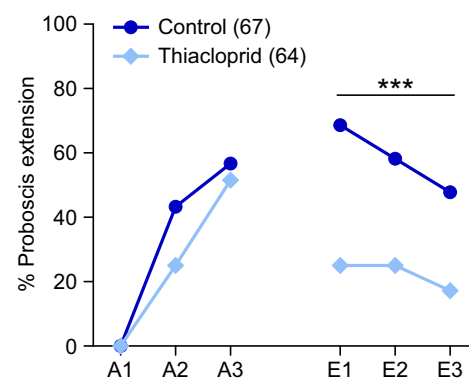


Fig. 4. Memory consolidation effect after treatment with thiacloprid.

Retention scores were quantified by three extinction trials (E1, E2, E3) testing the probability of PER after odor conditioning 24 h earlier (acquisition, A1, A2, A3), and 19 h 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 asterisks. The number of individuals in each group is given in parentheses (*** $P < 0.001$).

overnight, bees treated with thiacloprid showed significantly lower PER for extinction trial 1 (E1) than for A3 (Fisher's exact test, $P<0.001$), 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, Tukey, control versus thiacloprid, $P<0.0001$) 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 (Fig. 4, E2 and E3). Overall, the treatment with 69 ng of thiacloprid 19 h before the memory test had a negative effect on memory retrieval ($\chi^2=39.21$, d.f.=1, $P<0.0001$).

We then performed a generalization test with a similar and a different odor in addition to the CS for the memory tests. For thiacloprid and Calypso®, we used another range of doses in order to test lower, similar or higher doses than those tested in the extinction tests.

No difference was seen between the four groups during learning because treatment with thiacloprid occurred 5 h after conditioning (Fig. 5Ai: $\chi^2=0.07$, d.f.=3, $P=1$). However, a significant effect of treatment on the acquisition rates was observed in the groups treated with Calypso® (Fig. 5Bi: $\chi^2=8.78$, d.f.=3, $P<0.05$). This effect cannot be related to the treatment because it occurred later, and thus has to be considered as a random effect.

When tested 24 h later, bees treated with the highest dose of thiacloprid (200 ng) responded less to the odors than the control bees (Fig. 5Aii, $P<0.05$). The group treated with the highest dose of

Calypso® (200 ng) also showed significantly lower retention scores than the control group (Fig. 5Bii, $P<0.001$). Bees fed with the highest doses of thiacloprid or Calypso® did not respond to any of the three odors in greater proportions than control bees (Fig. 5Aiii, Fisher's exact test, control versus thiacloprid 200 ng, $P<0.01$; Fig. 5Biii, control versus Calypso® 200 ng, $P<0.0001$), and bees treated with 200 ng of Calypso® showed significantly lower retention scores for the CS only than the control bees (Fig. 5Aiii, Fisher's exact test, $P<0.001$). Retention scores differed between the controls and the Calypso®-treated groups with 20 ng or 2 ng; indeed, they showed higher rates of PER to the different odor only (C) than the control bees (Fig. 5Biii, Fisher's exact tests, control versus 2 ng, $P<0.001$; control versus 20 ng, $P<0.001$).

Taken together (all odors and all doses combined), the memory of bees that ingested thiacloprid 5 h after acquisition was marginally affected by the treatment ($\chi^2=7.39$, d.f.=3, $P=0.06$). Bees that ingested Calypso® ($\chi^2=25.06$, d.f.=3, $P<0.0001$) were significantly affected by the treatment. Memory consolidation processes were affected for bees treated with 69 ng of thiacloprid in the preliminary memory extinction tests (Fig. 4).

Memory retrieval

In order to investigate the effect of thiacloprid on memory retrieval, the test bees were fed with 69 ng of thiacloprid 1 h prior to the memory retrieval test.

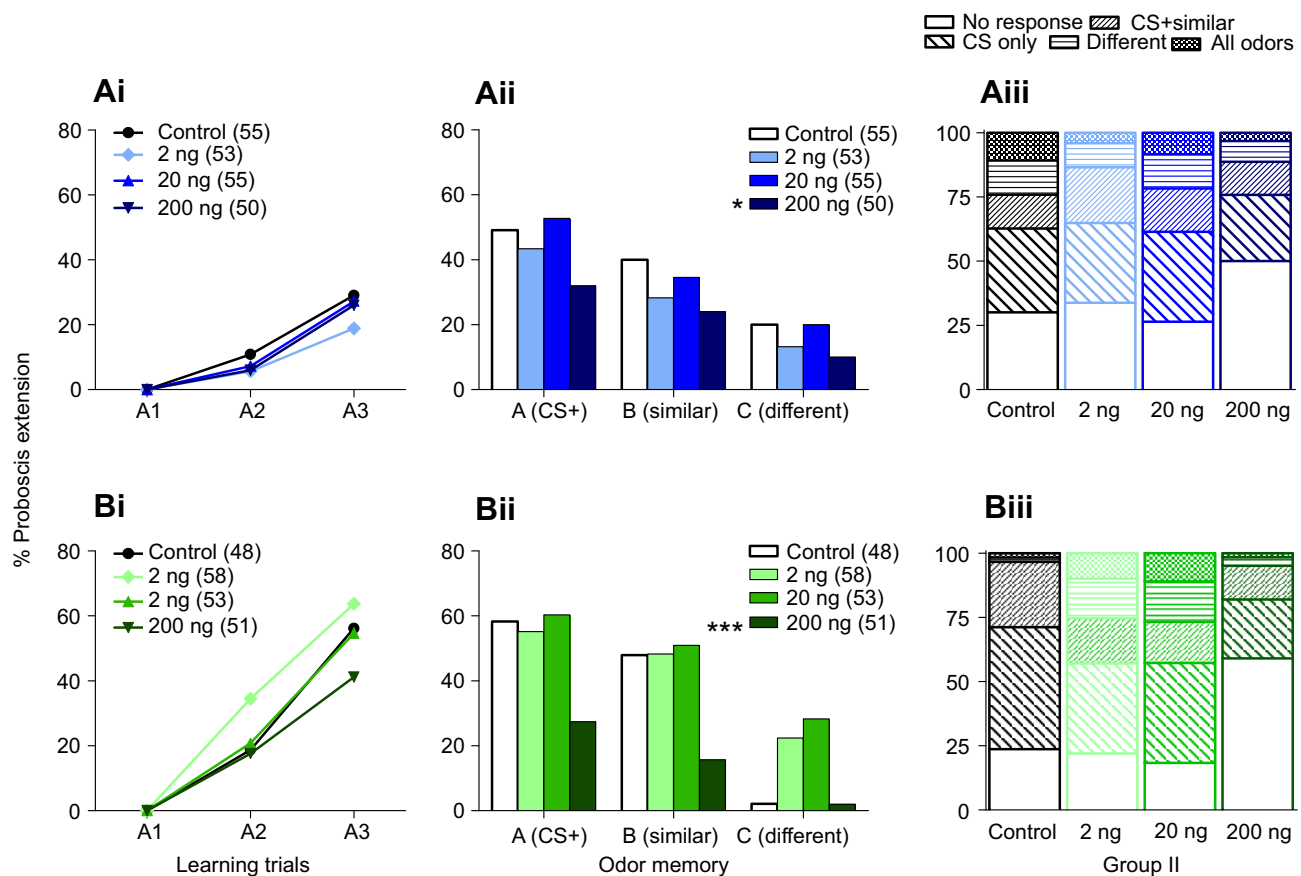


Fig. 5. Effect of thiacloprid and Calypso® on memory consolidation. Retention scores were determined 24 h after the last acquisition trial and 19 h after treatment with 2, 20 or 200 ng of either (A) thiacloprid as active substance diluted in sucrose or (B) Calypso®, a thiacloprid formulation, also diluted in sucrose. (Ai,Bi) Acquisition of CS+ (% PER) during the three conditioning trials (A1, A2, A3). (Aii,Bii) Retention scores (% PER) during the generalization test, 24 h after learning. A (CS+), conditioned odor (1-nonanol); B (similar), similar odor (nonanal); C (different), different odor (2-hexanol). (Aiii,Biii) Distribution of bees according to their individual responses to the odors during the memory tests. Asterisks in the keys indicate statistically significant differences compared with the control ($P<0.05$). The number of individuals in each group is given in parentheses (* $P<0.05$; *** $P<0.001$).

We first performed an extinction test in order to test the stability of memory. As expected, the acquisition of the groups treated (or not) 24 h later were not significantly different (Fig. 6, $\chi^2=0.08$, d.f.=1, $P=0.36$). Retention scores revealed significant 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, Tukey, control versus thiacloprid, $P<0.0001$). 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 retention scores during E1 as compared with A3 (Fig. 6, Fisher's exact test, $P<0.0001$). This was also the case for control bees, but the difference was not significant ($P=0.16$). Overall, the treatment with 69 ng of thiacloprid 1 h before the memory test had a negative effect on memory retrieval ($\chi^2=13.29$, d.f.=1, $P<0.001$).

For the generalization test, we used three different doses of thiacloprid and Calypso® and the retention scores were quantified for the CS, and two new odors (similar and different odors). Again, treatment was performed 1 h before retrieval tests.

As expected, no effect of treatment was observed during acquisition for all tests as treatment occurred 24 h later (Fig. 7Ai, $\chi^2=4.50$, d.f.=3, $P=0.21$; Fig. 7Bi, $\chi^2=3.35$, d.f.=3, $P=0.34$). Learning rates for the thiacloprid test were again observed to be lower than for the Calypso® test due to an effect of the experimenter.

The model revealed significant negative effects of the highest doses (Fig. 7Aii, thiacloprid 200 ng, $P<0.05$; Fig. 7Bii, Calypso® 200 ng, $P<0.01$) and middle doses (Fig. 7Aii, thiacloprid 20 ng, $P<0.05$; Fig. 7Bii, Calypso® 20 ng, $P<0.05$) of thiacloprid and Calypso®. Fewer bees from the highest dose of thiacloprid (Fig. 7Aiii, Fisher's exact test, $P<0.0001$), the 20 ng dose ($P<0.05$) and the highest dose of Calypso® (Fig. 7Biii, $P<0.05$) 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. 7Aiii, Biii, Fisher's exact tests, all groups: $P<0.0001$). The lowest doses (2 ng) of thiacloprid and Calypso® induced similar memory retrieval rates to the controls (Fig. 7Aiii, Biii) except for the two novel odors in the Calypso® test (Fig. 7Bii).

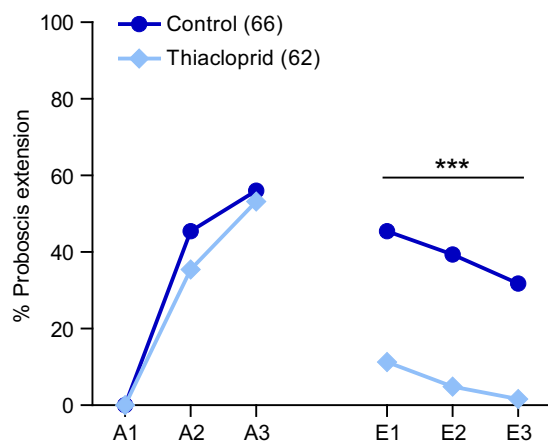


Fig. 6. Memory retrieval after treatment with thiacloprid. Retention scores were quantified by three extinction trials (E1, E2, E3) testing the probability of PER after odor conditioning 24 h earlier (acquisition, A1, A2, A3), and 1 h after treatment (or not, control) with 69 ng of thiacloprid diluted in sucrose. Significant differences ($P<0.05$) between the control group and the thiacloprid group are represented by asterisks. The number of individuals in each group is given in parentheses (*** $P<0.001$).

Overall (all odors and doses combined), memory retrieval was compromised after treatment with either thiacloprid or Calypso® 1 h before the generalization tests (thiacloprid, $\chi^2=11.92$, d.f.=3, $P<0.01$; Calypso®, $\chi^2=12.17$, d.f.=3, $P<0.01$). Negative effects were also observed for bees treated with 69 ng of thiacloprid in the preliminary experiment (Fig. 6).

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 Table S2). The same bees as the ones used in the tests presented in Figs 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⁻¹) and with the time of feeding (Treatment Groups I, II and III). As 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 treated with 200 ng of thiacloprid or Calypso® 1 h before the memory test (2 h before sample collection). Among the bees treated with 200 ng or 20 ng, the higher amount of residues was always found in bees from Treatment Group III (Fig. 8).

Notice that the scales in Fig. 8 are not the same for all three doses. The amounts of residues found in bees treated with 200 ng were ~10 times higher than the amounts found in bees treated with 20 ng, corresponding to the order of difference between the two applied doses.

The amounts of thiacloprid residues were always higher for the Calypso® group than for the thiacloprid group (except for the 2 ng dose) despite the fact that bees were treated with the same dose of thiacloprid (the concentration in thiacloprid of the sucrose solutions were verified by LC–MS/MS).

Table S2 gives details about the amount of residues found in the different body parts of the bees. Except for two samples (Calypso® 20 ng Treatment Group II and 2 ng Treatment Group I), the amount of thiacloprid residues was always highest in the bee heads, and they were usually lowest in the thoraces. A time and dose relationship seems to apply for the high and the middle doses of thiacloprid and Calypso® but not for the low dose. Furthermore, thiacloprid residues in the range 0.5–13.7 ng g⁻¹ 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 the molecular and cellular 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, allowed us to show that thiacloprid, fed to the bees as single active substance or as ingredient of the commercially available pesticide formulation (Calypso®), negatively affects appetitive olfactory associative learning, consolidation and retrieval of memory in honey bees (*A. 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

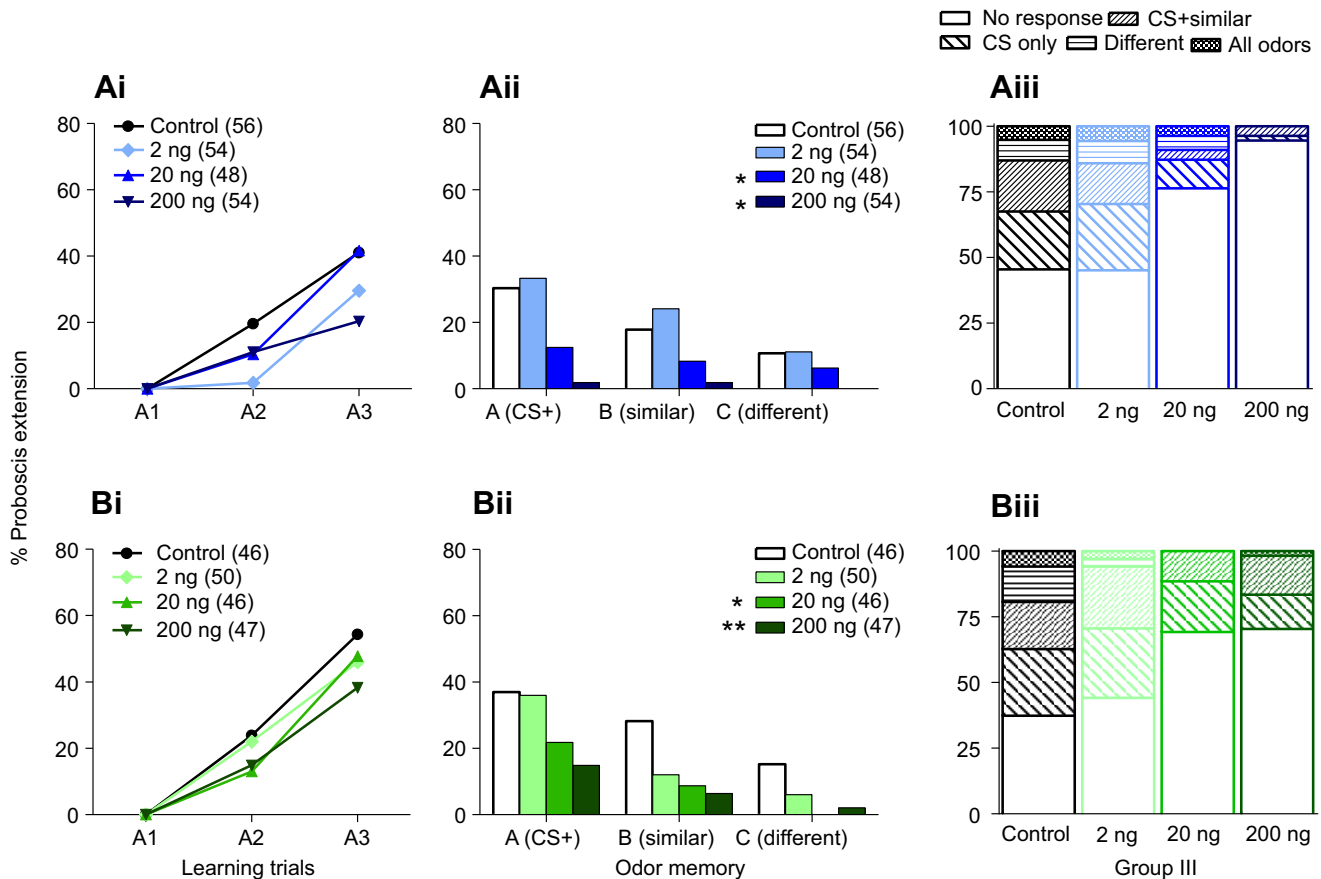


Fig. 7. Effect of thiacloprid and Calypso® on memory retrieval. Retention scores were determined 24 h after acquisition and 1 h after treatment with 2, 20 or 200 ng of either (A) thiacloprid as active substance diluted in sucrose or (B) Calypso®, a thiacloprid formulation, also diluted in sucrose. (Ai,Bi) Acquisition of CS+ (% PER) during the 3 conditioning trials (A1, A2, A3). (Aii,Bii) Retention scores (% PER) during the generalization test, 24 h after learning. A (CS+), conditioned odor (1-nonanol); B (similar), similar odor (nonanol); C (different), different odor (2-hexanol). (Aiii,Biii) Distribution of bees according to their individual responses to the odors during the memory tests. Asterisks in the keys indicate statistically significant differences compared with the control ($P < 0.05$). The number of individuals in each group is given in parentheses (* $P < 0.05$; ** $P < 0.01$).

unconditioned responses to sucrose revealed that animals treated with the highest doses of Calypso® (200 ng bee⁻¹) 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 because 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 of Calypso® (Fig. 1). An acute alteration of the motor function (Williamson et al., 2013, 2014) would be the most probable alternative hypothesis as reduced US responses were actually observed in bees from Treatment Group III, fed with 200 ng of Calypso® 1 h before the memory test.

We found that thiacloprid and Calypso® reduced acquisition at the highest dose used in the respective experiment (69 ng bee⁻¹ thiacloprid and 120 ng bee⁻¹ Calypso® in Fig. 2, and 200 ng bee⁻¹

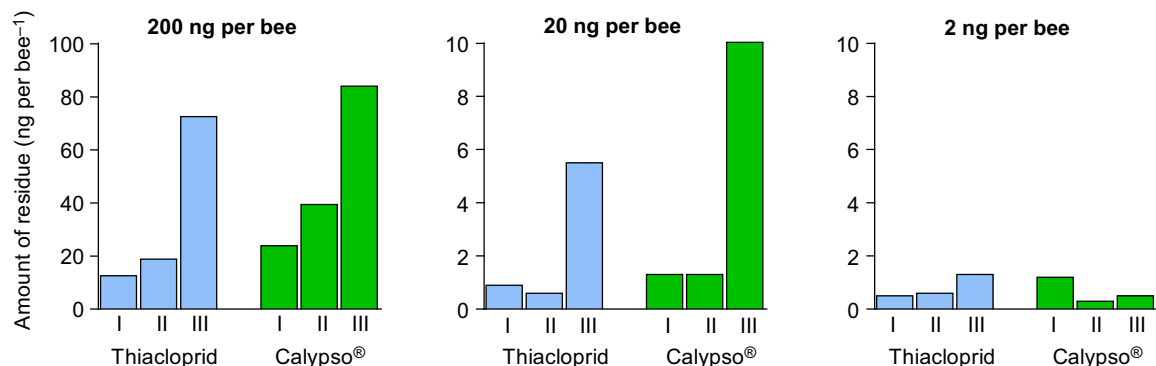


Fig. 8. Pesticide residue analyses of honey bees treated with thiacloprid or Calypso® at 200, 20 or 2 ng per bee. Treatment was administered orally 1 h before learning (I), 5 h after learning (II) or 1 h before the memory test (III). The identification and quantification of thiacloprid was performed using LC–MS/MS.

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 because 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 (Figs 2 and 3).

Decourtye et al. (2004b) assume that the consolidation process, which ensures the transfer from short-term memory to mid-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 mid-term memory to early long-term memory. 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). Thiacloprid or Calypso® taken up 5 h after acquisition and 19 h before the memory tests led to dose-dependent loss of retention (Figs 4 and 5). We selected an interval of 5 h after acquisition because mid-term memory is converted to early long-term memory during the following period of time (Menzel, 1999; Müller, 2002). In the memory consolidation experiment, treatment with 69 ng per bee of thiacloprid led to significantly reduced retention scores in the extinction test (Fig. 4), but only hints of an effect at even higher doses were observed in the generalization test (Fig. 5Ai–iii). 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 led to a significant reduction of retention (Fig. 5Bi–iii). However, another possibility cannot be ruled out because consolidation processes do not only follow 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 mid-term memory (Menzel, 1999; Müller, 2002). If the parallel processes are differently affected by thiacloprid and Calypso® (which contains other components in addition to 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 were 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. 5). We found that Calypso® treatment led to a changed generalization gradient for treatment with 2 or 20 ng per bee. The different test odor was not responded to in the control group because these bees discriminated well between the learned odor and the different test odor whereas the bees treated with

Calypso® at 200 ng per bee 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 h after treatment with thiacloprid at 20, 69 or 200 ng per bee and with Calypso® at 20 and 200 ng per 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 a treatment with thiacloprid, tested bees did not remember the odor when stimulated with the CS because they did not possess the memory of it. However, honey bees from Treatment Group III not showing a PER, possessed the memory of the odor, but treatment with thiacloprid 1 h before the memory test prevented them from successfully retrieving it, suggesting that the access to the stored memory was 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 mid-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 (Figs 2, 4 and 6) allowed us to address the question of 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 (Figs 2, 4 and 6) and the second (Figs 3, 5 and 7). The latter learned the CS in lower proportions. This can be the result of different factors such as the year of the test (2015 or 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 Figs 3, 5 and 7 clearly had differences in the perception of the PER during acquisition as shown 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 the metabolism of thiacloprid in the body of honey bees (Fig. 8 and Table S2). Shorter time between oral treatment and 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 μl^{-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 revealed 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). The Calypso[®] safety data sheet [Bayer CropScience safety data sheet according to regulation (EC) no. 1907/2006 CALYPSO 4/11 version 6/GB revision date: 22.09.2015 102000007569 print date: 21.03.2016; <https://cropscience.bayer.co.uk/api/DocumentDownload/Download/?id=2242>] 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 they are hidden under the cloak of 'trade secret' (Mullin et al., 2015; Cox and Sorgan, 2006). 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, 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 tested positive for thiacloprid, although 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 m 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 retrieval was seen when 69 ng of thiacloprid was given to bees 5 h after learning thus documenting the effects on memory consolidation. Memory retrieval tested by treatment 1 h 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 (17,320 ng per bee, OEPP), serious sublethal detrimental phenomena were found at much lower doses, ~250–800 times lower than the LD₅₀.

The test bees were restrained in tubes for 24 h, which is a rather unnatural situation. Under field conditions, bees have the opportunity to fly freely during foraging, potentially increasing the metabolism 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, because 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 is the retrieval of a remote memory 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, 2007). 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 experience impaired learning and memory performances, possibly leading to negative effects on a whole range of behaviors necessary for the survival of the individual and consequently 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 the 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.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: L.T., R.M.; Methodology: L.T., S.H.; Validation: L.T., R.M.; Formal analysis: L.T.; Investigation: L.T., S.H., A.A., Ö.K., N.S.I., N.L.; Data curation: L.T.; Writing - original draft: L.T.; Writing - review & editing: L.T., R.M.; Supervision: L.T., R.M.; Project administration: L.T., R.M.; Funding acquisition: R.M.

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Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.154518.supplemental>

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Supporting Information

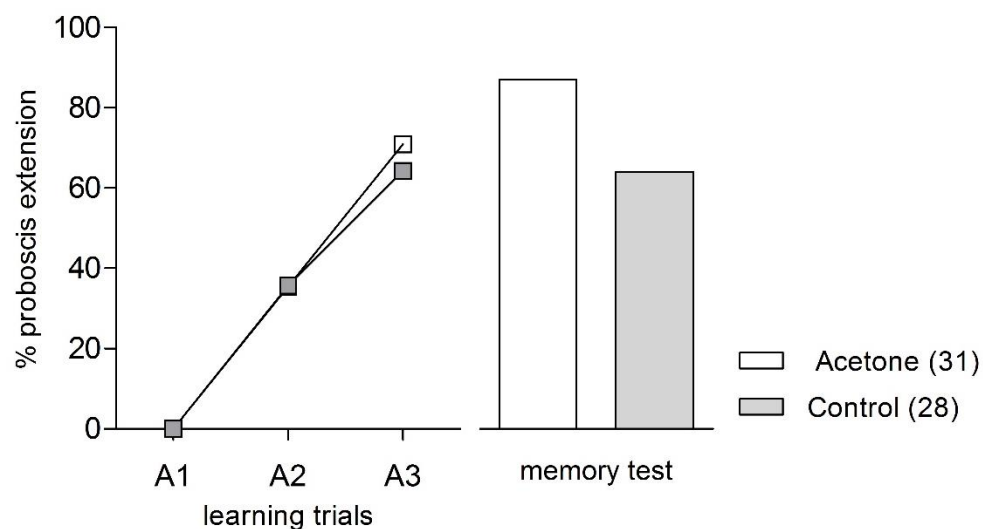


Figure S1. Memory retrieval after treatment with 0.5 % acetone. Retention score was determined 24 h after acquisition and 19 hours after treatment with 0.5 % acetone in sucrose solution. No difference was found between the two groups (Fischer exact test, $P = 0.064$). The number of individuals in each group is given in brackets in the legend.

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	$\chi^2 = 1.009$, $df = 1$, $P = 0.32$	4.52	$\chi^2 = 1.53$, $df = 1$, $P = 0.22$
thiacloprid 69 ng	1.35		7.31	
control	3.10	$\chi^2 = 2.338$, $df = 3$, $P = 0.51$	0.80	$\chi^2 = 1.517$, $df = 3$, $P = 0.68$
Calypso® 120 ng	0.00		0.00	
Calypso® 12 ng	1.56		0.00	
Calypso® 1.2 ng	3.17		0.00	
control	4.26	$\chi^2 = 4.196$, $df = 3$, $P = 0.24$	16.11	$\chi^2 = 12.93$, $df = 3$, $P < 0.01$
thiacloprid 200 ng	2.23		8.00	
thiacloprid 20 ng	3.70		6.04	
thiacloprid 2 ng	1.08		7.65	
control	3.16	$\chi^2 = 7.63$, $df = 3$, $P = 0.054$	5.88	$\chi^2 = 35.21$, $df = 3$, $P < 0.0001$
Calypso® 200 ng	1.70		24.86	
Calypso® 20 ng	4.05		10.84	
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, as active substance and formulation

sample		sample weight (mg) * n= 30	thiacloprid residues (ng/g) corrected by thiacloprid-d4 recoveries (ng/bee)					thiacloprid-d4 recoveries (%)		
			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 honeybee 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.