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

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Summary Statement: This research shows that the neonicotinoid thiacloprid as active substance and as formulation (Calypso®) negatively affects honey bees by reducing their learning and memory performance, which endangers their survival in natural conditions.

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 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 (PER) 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 3 different sublethal doses. Extinction and generalization tests allowed us to investigate whether bees respond to a learned stimulus, and how selectively. We show that thiacloprid, as active substance and as formulation, poses a substantial risk to honeybees 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) (Bijleveld van Lexmond *et al.*, 2014). 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 nontarget organisms (Bijleveld van Lexmond *et al.*, 2014) even if their effects on honey bees at the colony level have been questioned (Henry *et al.*, 2015; Rundlöf *et al.*, 2015). The effects of Thiacloprid, like other neonicotinoids, acts as an agonist to the insect 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 nicotinic acetylcholine receptors. 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 as well as in learning and memory (Cano Lonzano et al. 1996; Cano Lozano et al. 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 set on the tarsae, antennae, or mouth parts with nectar or sucrose solution, hungry honeybees 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; Müller, 2002; Menzel, 2012).

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 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; Decourtye *et al.*, 2004b; Palmer *et al.* 2013; Stanley *et al.*, 2015; Williamson *et al.*, 2013; Andrione *et al*., 2016). 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 a single active substance, and Calypso®, a formulation containing thiacloprid and other unknown ingredients. 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 restrictions, also in garden shops, because 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 *et al.*, 1998; 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 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 LD50. We find significant negative effects in both forms.

Methods

Sampling

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. 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.

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), either hexanal or 1-Nonanol, 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 (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 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 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 hours after the first conditioning trial. In the first set of experiments (extinction tests), only the CS, hexanal, was tested in 3 extinction trials and was not rewarded with sucrose. In the second set of experiments (generalization tests), in addition to the CS, 1-nonanol, bees were exposed to nonanal and to 2-hexanol 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, contrary to 2-hexanol, 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 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 random effect in the statistical analysis. For example, 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). 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 see if 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 in the course of

the experiment or any bee that extended its proboscis during the 10 sec 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 hours). 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 performed first extinction tests, followed by generalization tests. During an extinction test, the same odor was tested 3 times whereas in a generalization test, the learned odor was tested once and 2 novel odors also one time each.

Appetitive learning: In order to test possible effects on the learning ability and memory formation, bees were treated with the pesticide (or control sucrose solution) 1 hour before the first conditioning trial (= 25 hours before the memory test). These bees correspond to the Treatment group I.

Memory consolidation: To investigate the effects of thiacloprid and Calypso® on the consolidation of the memory, bees were treated 5 hours after conditioning (= 19 hours before the memory test). These bees correspond to the Treatment group II.

Memory retrieval: To test the effect on the retrieval of the memory), bees were treated 23 hours after conditioning (= 1 hour before the memory test). These bees correspond to the 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 can be seen in brackets in the legends of the figures.

Thiacloprid and Calypso® solutions

Stock solution: 10 mg of thiacloprid (98 % purity, Sigma Aldrich) was dissolved in 1 mL acetone (≥99.9%, Sigma-Aldrich) and 39 mL distilled water leading to a concentration of 0.25 g/L. Acetone was chosen as the solvent following the OEPP (European and Mediterranean Plant Protection Organization) guideline (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, 150 ng.µl^{-1} thiacloprid. 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 3 concentrations of thiacloprid (50, 5, and 0.5 ng.µl^{-1}) in a 30 % (w/v) sucrose solution. Using a multipipette, each bee was fed orally with 4 µl 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 ng, 20 ng or 2 ng per bee were given for both thiacloprid as active substance or as 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 with 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 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^{-1}) 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^{-1}) 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 (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 AIC. 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 *Chi-square* 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 the Fischer 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 *Chi-square* 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, χ^2= 12.93, df= 3, P< 0.01) 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® (Table S1, 24.86 %), and strangely enough, 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} of 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 *vs* 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 3 acquisition trials) after feeding the test bees with 69 ng thiacloprid or with 1.2, 12 and 120 ng thiacloprid in the formulation Calypso® 1 hour before 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. 2.a and b). The level of acquisition was significantly lower in bees treated with 69 ng thiacloprid (Fig. 2A, Tukey, P< 0.05) or 120 ng 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 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 dose (12 ng: P< 0.01 and 120 ng: P< 0.0001). The memory tests indicated that thiacloprid at 69 ng per bee did not significantly change the retention scores (percentage of PER during the memory tests) 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 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: χ^2=5.81, *df*= 1, P< 0.05, Calypso®: χ^2=42.35, *df*= 3, P< 0.0001).

To further investigate the effects of thiacloprid and Calypso® on learning performance and 24 hour 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 2 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, *df*= 3, P= 1). 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= 13.07, *df*= 3, P< 0.01) and the highest dose (200 ng) was shown to impair learning the most (Tukey, P< 0.001).

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 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 the memory test 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. 3A2, A, 44 % PER) and the similar odor (Fig. 3A2, B, 42 % 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< 0.001; control *vs* Calypso® 200 ng: P< 0.0001). Fewer bees from these 2 groups responded only to the learned odor (Fig. 3A2 and A3, Fischer exact tests, control *vs* thiacloprid 200 ng: P< 0.01; control *vs*

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. 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= 5.59. *df*= 3, P= 0.13) contrary to bees that ingested Calypso® (χ^2= 10.07, *df*= 3, P< 0.05). The highest dose of Calypso® significantly impaired memory retrieval (P< 0.01), while 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 thiacloprid 5 hours 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, χ^2= 3.05, *df*= 1, P= 0.08) as treated differently later, indicating that the two groups can be compared with respect to memory consolidation. Memory tests 24 hours after acquisition (=19 hours after treatment 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< 0.001) 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 *vs* 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 thiacloprid 19 hours before the memory test had a negative effect on memory retrieval (χ^2= 39.21, *df*= 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. We used for thiacloprid and Calypso® 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 4 groups during learning since treatment with thiacloprid occurred 5 hours after conditioning (Fig. 5A1: χ^2= 0.07, *df*= 3, P= 1). However, a significant effect of treatment

on the acquisition rates was observed in the groups treated with Calypso® (Fig. 5B1: χ^2= 8.78, *df*= 3, P< 0.05). This effect cannot be related to the treatment since it occurred later, and thus has to be considered as a random effect.

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, P< 0.05). The group treated with the highest dose of Calypso® (200 ng) showed also significantly lower retention scores than the control group (Fig. 5B2, P< 0.001). 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< 0.01; 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< 0.001). 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. 5B3, Fischer exact tests, control *vs* 2 ng, P< 0.001; control *vs* 20 ng, P< 0.001).

Taken together (all odors and all doses combined), the memory of bees that ingested thiacloprid 5 hours after acquisition was marginally affected by the treatment (χ^2= 7.39, *df*= 3, P= 0.06). Bees that ingested Calypso® (χ^2= 25.06, *df*= 3, P< 0.0001) were significantly affected by the treatment. Memory consolidation processes were affected for bees treated with 69 ng 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 thiacloprid 1 hour prior to the memory retrieval test.

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 hours later were not significantly different (Fig. 6, χ^2= 0.08, *df*= 1, P= 0.36). Retention 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, Tukey control *vs* 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 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= 0.16). Overall, the treatment with 69 ng thiacloprid 1 hour before the memory test had a negative effect on memory retrieval (χ^2= 13.29, *df*= 1, P< 0.001).

For the generalization test, we used 3 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 hour before retrieval tests.

As expected no effect of treatment was observed during acquisition for all tests as treatment occurred 24 hours later (Fig. 7A1, χ^2= 4.50, *df*= 3, P= 0.21, and Fig. 7B1, χ^2= 3.35, *df*= 3, P= 0.34) Learning rates for the thiacloprid test were again observed lower than for the Calypso® test due to an effect of the experimenter.

The model revealed significant negative effects of the highest doses (Fig. 7A2, thiacloprid 200 ng: P< 0.05 and B2, Calypso® 200 ng: P< 0.01) and middle doses (Fig. 7A2, thiacloprid 20 ng: P< 0.05 and B2, Calypso® 20 ng: P< 0.05) of thiacloprid and Calypso®. Fewer bees from the highest dose of thiacloprid (Fig. 7A3, Fischer exact test, P< 0.0001), the 20 ng dose (P< 0.05), and the highest dose of Calypso® (Fig. 7B3, 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. 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 (Fig. 7A3, B3) except for the two novel odors in the Calypso® test (Fig. 7B2).

Overall (all odors and doses combined), memory retrieval was compromised after treatment with either thiacloprid or Calypso® 1 hour before the generalization tests (thiacloprid: χ^2= 11.92, *df*= 3, P< 0.01, Calypso®: χ^2= 12.17, *df*= 3, P< 0.01). Negative effects were also observed for bees treated with 69 ng 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 Methods S1 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 feeding (Treatment 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 treated with 200 ng thiacloprid or Calypso® 1 hour before the memory test (2 hours before sample collection). Among bees treated with 200 or 20 ng, the higher amount of residues was always found in bees from Treatment group GIII (Fig. 8).

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

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 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 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. A time and dose relationship seem to apply for the high and the middle dose of thiacloprid and Calypso® but not for the low dose. Furthermore, thiacloprid residues in the range of 0.5 to 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, allows 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 (*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 treated 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 alternative hypothesis as reduced US responses were actually observed in bees from Treatment group III, fed 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. 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 hours after acquisition and 19 hours before the memory 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). In the memory consolidation experiment, treatment with 69 ng/bee 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. 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 a treatment 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 from the Treatment group III not showing a PER, possess the memory of the odor, but treatment with thiacloprid 1 hour before the memory test prevent them from successfully retrieving it, suggesting that the 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 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 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 retrieval 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 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 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

No competing interests declared.

Author contributions

L.T, A.A, Ö.K, S.H 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.

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Figures

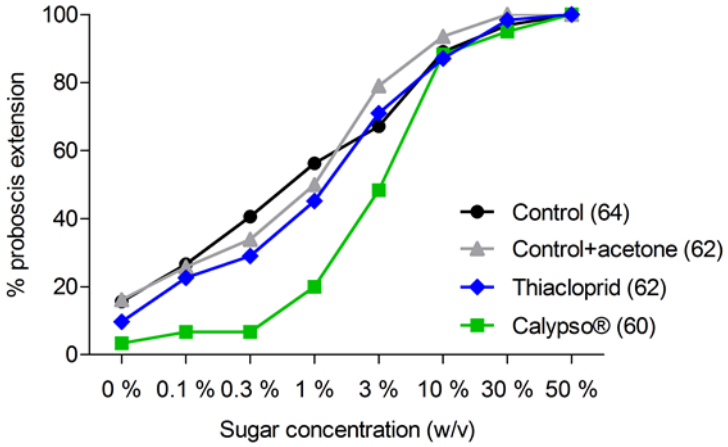


Figure 1. Sucrose responsiveness of control and treated honey bees to different sucrose concentrations. The sucrose solution presented to the bees contained or not (Control), acetone alone (Control+acetone), Thiacloprid as active substance or as formulation (Calypso®) at a concentration of 50 ng.µl^{-1}.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

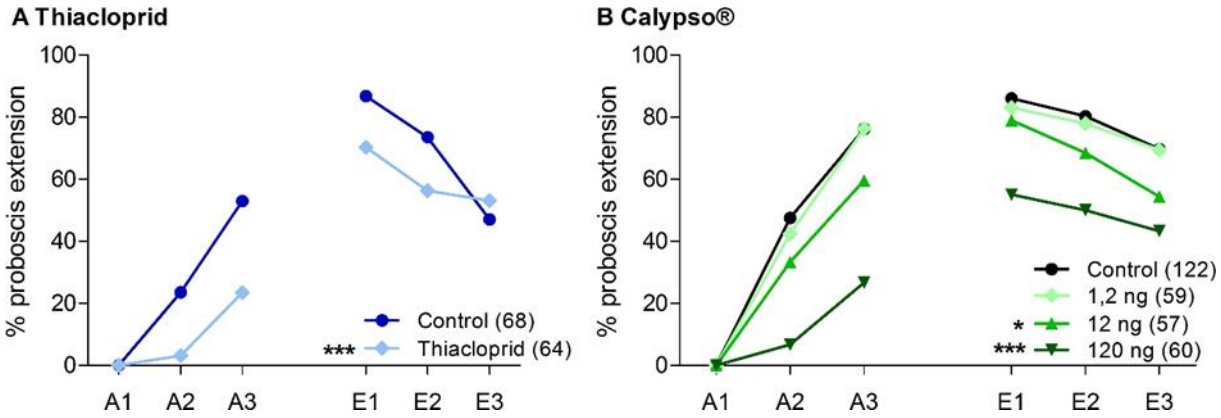


Figure 2. Acquisition functions and retention scores after 24 hours. PER scores were quantified by three acquisition trials (A1, A2, A3) 1 hour after treatment with **A.** 69 ng thiacloprid diluted in sucrose or **B.** 1.2, 12 or 120 ng thiacloprid in Calypso® diluted in sucrose, and three extinction 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 (*, $P < 0.05$ and ***, $P < 0.001$).

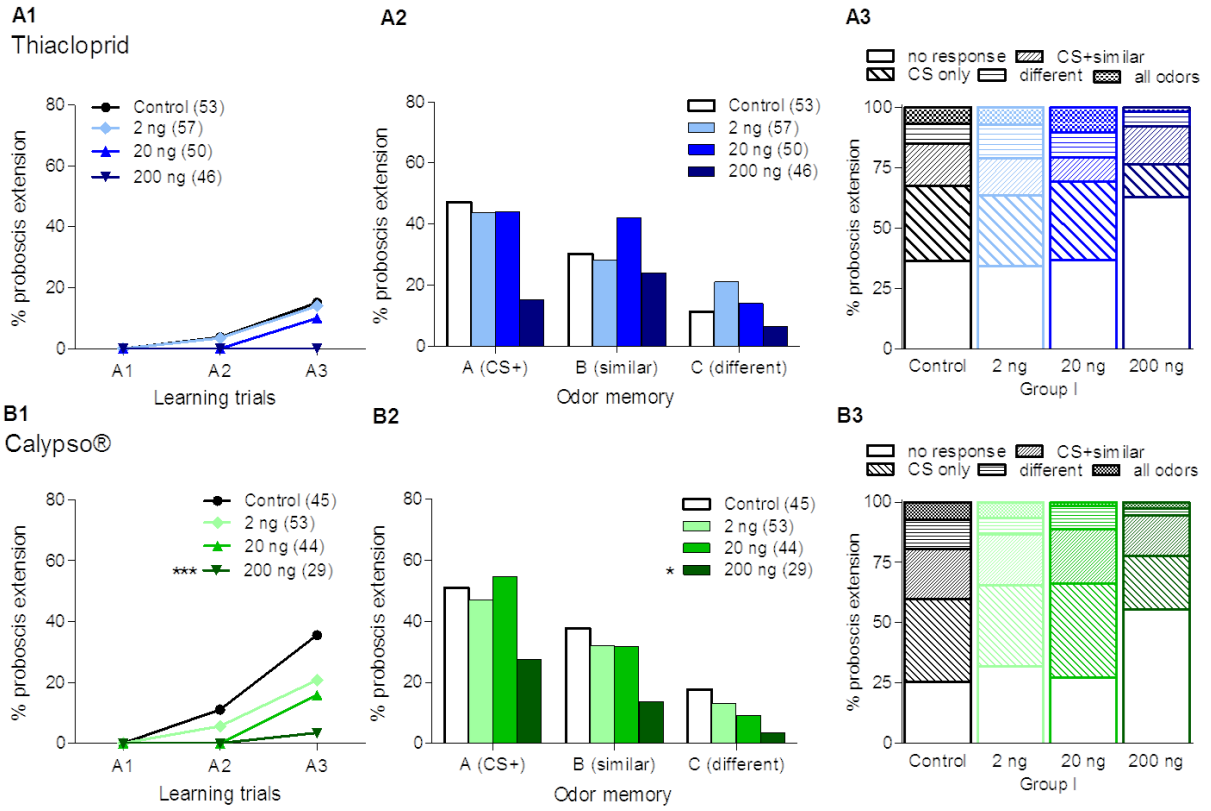


Figure 3. Effect of thiacloprid and Calypso® on acquisition and memory. Retention scores were determined 24 h after acquisition and the treatment occurred 1 hour before acquisition with 2, 20 or 200 ng of either **A.** thiacloprid as active substance diluted in sucrose or **B.** Calypso®, a thiacloprid formulation, also diluted in sucrose. **A1/B1**: Acquisition of CS+ (% PER) during the 3 conditioning trials (A1, A2, A3). **A2/B2**: Retention scores (% PER) during the generalization test, 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 (*, $P < 0.05$ and ***, $P < 0.001$).

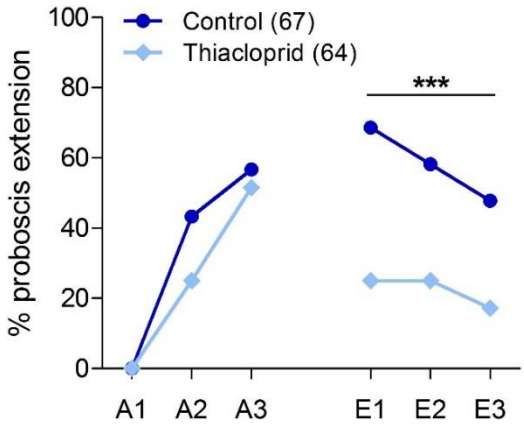


Figure 4. Memory consolidation effect after treatment with thiacloprid. Retention scores were quantified by three extinction trials (E1, E2, E3) testing the probability of PER after odor conditioning 24 hours earlier (acquisition, A1, A2, A3), and 19 hours after treatment (or not, control) with 69 ng 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 (***, $P < 0.001$).

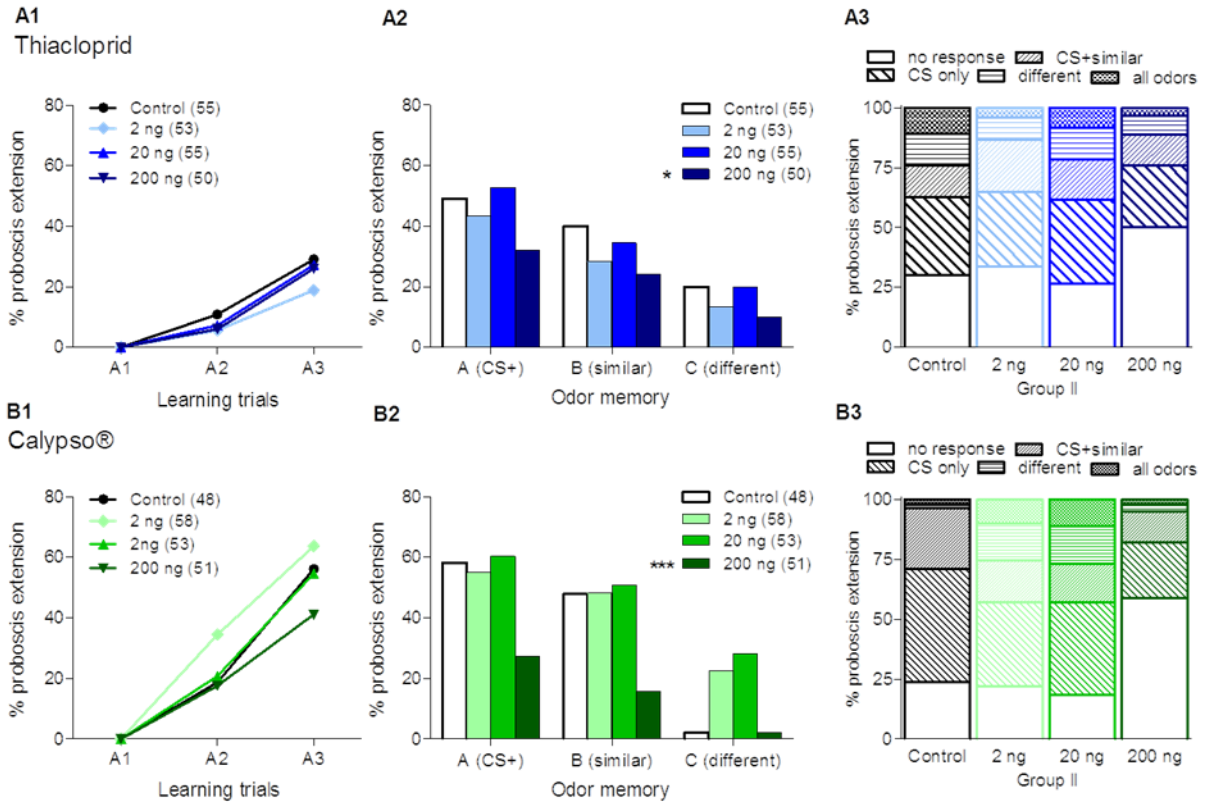


Figure 5. Effect of thiacloprid and Calypso® on memory consolidation. Retention scores were determined 24 h after the last acquisition trial and 19 hours after treatment with 2, 20 or 200 ng of either **A.** thiacloprid as active substance diluted in sucrose or **B.** Calypso®, a thiacloprid formulation, also diluted in sucrose. **A1/B1**: Acquisition of CS+ (% PER) during the 3 conditioning trials (A1, A2, A3). **A2/B2**: Retention scores (% PER) during the generalization test, 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 (*, $P < 0.05$ and ***, $P < 0.001$).

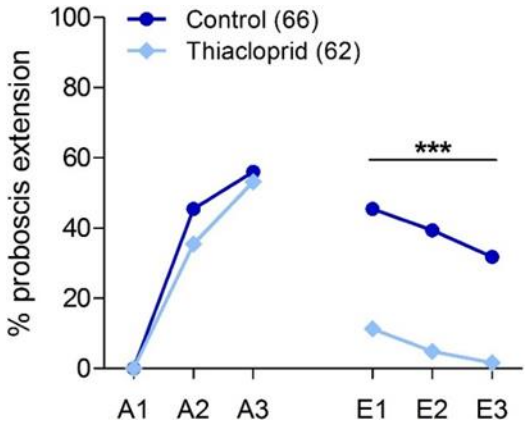


Figure 6. Memory retrieval after treatment with thiacloprid. Retention scores were quantified by three extinction trials (E1, E2, E3) testing the probability of PER after odor conditioning 24 hours earlier (acquisition, A1, A2, A3), and 1 hour after treatment (or not, control) with 69 ng thiacloprid 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 (***, $P < 0.001$).

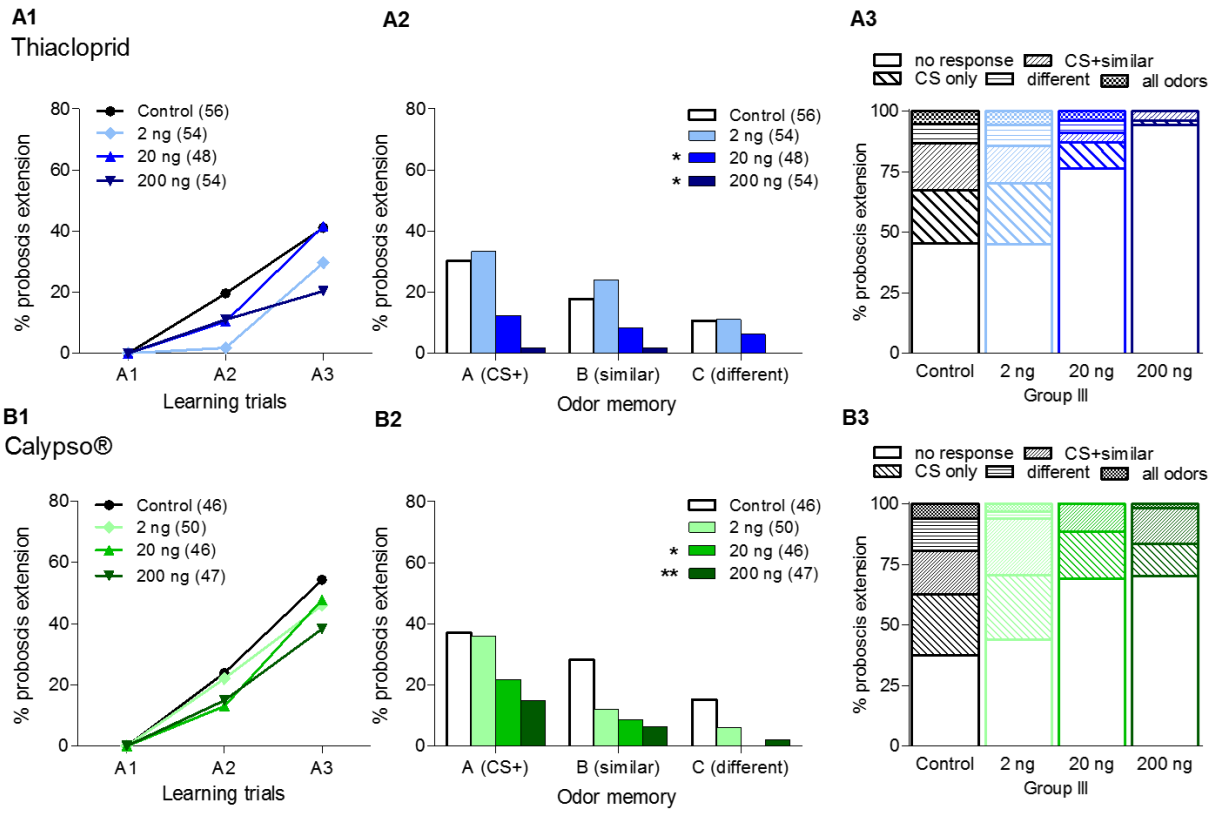


Figure 7. Effect of thiacloprid and Calypso® on memory retrieval. Retention scores were determined 24 h after acquisition and 1 hour after treatment with 2, 20 or 200 ng of either **A.** thiacloprid as active substance diluted in sucrose or **B.** Calypso®, a thiacloprid formulation, also diluted in sucrose. **A1/B1**: Acquisition of CS+ (% PER) during the 3 conditioning trials (A1, A2, A3). **A2/B2**: Retention scores (% PER) during the generalization test, 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 (*, $P < 0.05$; **, $P < 0.01$).

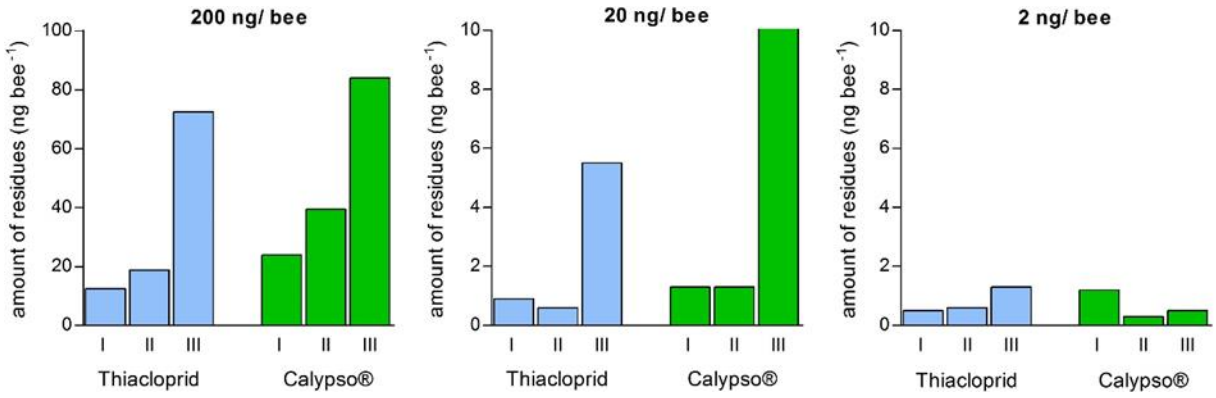


Figure 8. Pesticide residue analyses of honey bees treated with thiacloprid or Calypso® at 200, 20 or 2 ng/bee. Treatment was administered orally 1 hour before learning (I), 5 hours after learning (II) or 1 hour before the memory test (III). The identification and quantification of thiacloprid was performed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

Supporting Information

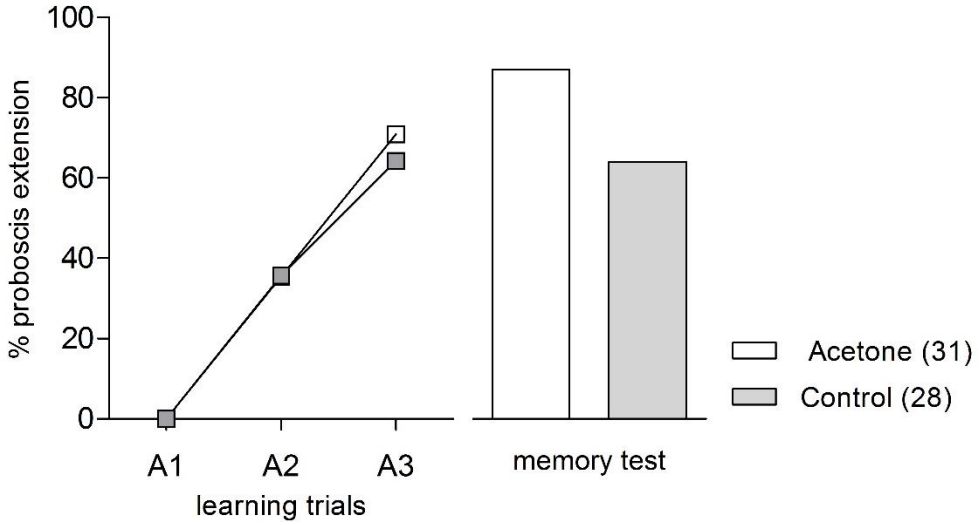


Figure S1. Memory retrieval after treatment with 0.5 % acetone. Retention score was determined 24 h after acquisition and 19 hours after treatment with 0.5 % acetone in sucrose solution. No difference was found between the two groups (Fischer exact test, P = 0.064). The number of individuals in each 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	χ^2= 1.009, *df*= 1, P= 0.32	4.52	χ^2= 1.53, *df*= 1, P= 0.22
thiacloprid 69 ng	1.35		7.31	
control	3.10		0.80	
Calypso® 120 ng	0.00	χ^2= 2.338, *df*= 3, P= 0.51	0.00	χ^2= 1.517, *df*= 3, P= 0.68
Calypso® 12 ng	1.56		0.00	
Calypso® 1.2 ng	3.17		0.00	
control	4.26		16.11	
thiacloprid 200 ng	2.23	χ^2= 4.196, *df*= 3, P= 0.24	8.00	χ^2= 12.93, *df*= 3, **P< 0.01**
thiacloprid 20 ng	3.70		6.04	
thiacloprid 2 ng	1.08		7.65	
control	3.16		5.88	
Calypso® 200 ng	1.70	χ^2= 7.63, *df*= 3, P= 0.054	24.86	χ^2= 35.21, *df*= 3, **P< 0.0001**
Calypso® 20 ng	4.05		10.84	
Calypso® 2 ng	0.00		7.34	

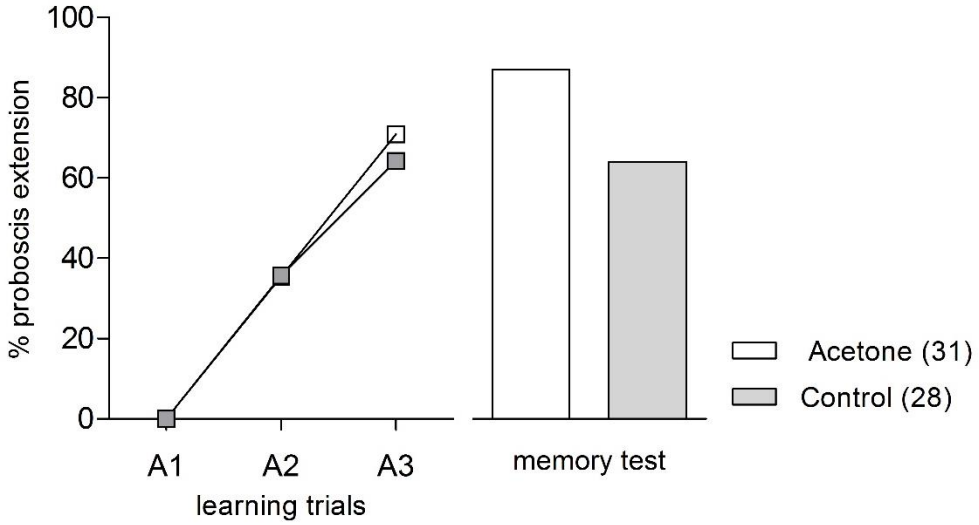
Significant p-values (< 0.05) are showed in bold letters.

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.

Supporting Information



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