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# Adverse Effects of the Neonicotinoid Thiacloprid Assessed on an Ant Used as a Biological Model*

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## Abstract

Thiacloprid has been banned in many European countries for its toxicity, but is nevertheless still used outside the EU. Using the ant *Myrmica sabuleti* as a model organism, we sought a concentration of thiacloprid that would be low in environmental impact, but nonetheless harmful to an insect. Assessed on ten biological traits, a sub-lethal 2 µg/L concentration in the sugar water diet of the ants impacted their locomotion and other motor-linked behavioral traits such as orientation, moving on an unfamiliar device, moving on a rough surface, brood caring and progressing along a twist and turns path. Sensory perception appeared unaffected. Behaviors involving cognition or related in some way to it, such as social relationships, ability to leave an enclosure, learning and memory also appeared unaffected, but their outcomes could be entangled with those of locomotor impairment. The effect of thiacloprid on locomotion vanished in about 30 hours. Adverse effects were still present with the 0.1 and 0.01 µg/L concentrations, but at a lower level. Partial physiological adaptation at the individual level occurred for the 2 µg/L concentration, less so for 0.1 µg/L and not for 0.01 µg/L. Thiacloprid should thus be used at the lowest still active concentration producing a significant damaging behavioral effect with no physiological adaptation. This concentration remains to be specifically defined for targeted pest species.

## Keywords

Cognition, Insecticide Toxicity, Motor-Linked Impairments, Neonicotinoid, Physiological Adaptation, Sensory Perception, Thiacloprid

*In loving memory of Marie-Claire Cammaerts (1947-2024), née Tricot, who passed away before her experimental work could be published.

## 1. Introduction

Among the classes of insecticides available on the market, namely organochlorids, organophosphorids, carbamates, pyrethrinoids, formamidines and neonicotinoids, this last is currently the most used [1], representing ca 27% of the world insecticide market [2]. The intense use of insecticides, especially neonicotinoids, has led to an important decline of insects, including those useful in terms of ecosystem services and agricultural production [3] [4], as well as to an adverse impact on aquatic invertebrates, particularly on insects, less so on crustaceans [5] [6]. An open-field assessment of aquatic insects pointed out the relation between this negative impact and the delivered amounts (*i.e.*, concentrations) of a neonicotinoid insecticide, thiacloprid [7].

Thiacloprid and four other neonicotinoid molecules, imidacloprid, clothianidin, thiamethoxam and acetamiprid, were approved in 2011 as active substances for plant protection in the European Union [8]. However, already in 2013, in order to protect honeybees, the use of imidacloprid, clothianidin, and thiamethoxam was restricted to greenhouses or, if outdoors, after flowering or on winter cereals [9]. Further, the European Regulation of 2018 imposed that the seeds treated with these three neonicotinoids have to be used in permanent greenhouses and that the resulting crops have to stay inside during their entire life-cycle [10]. As regards thiacloprid, its use was simply banned in February 2020 [11]. Nevertheless, eleven EU countries asked and were granted emergency authorizations for the use of neonicotinoids during the 2020/21 sugar beet growing season and among these countries, only Poland asked the permission for using thiacloprid [12].

Today, thiacloprid is totally banned for utilization in the EU, and furthermore, in February 2024, France banned the placement on the French market of fruit and vegetables containing thiacloprid residuals over the lowest maximum residue level of 0.01 mg/kg, whatever their geographical origin. This is the lowest concentration level that can be detected using the most modern reliable analytical methods (LOD). The publication of a new European regulation based on this LOD concentration for all edible plants (including cereals) and extended to all EU countries is expected for December 2024 and to be in force by mid-2025 [13].

Nevertheless, banned neonicotinoid substances continue to be produced in Europe for agricultural use and are sold to many foreign countries where they are legally used [14] [15], with regulatory threshold levels varying enormously between them. For instance, the acute threshold of thiacloprid concentration in freshwater varies by a factor of 223 (*i.e.*, 0.0912 µg/L in the EU as compared to 20.35 µg/L in Canada) [2]. Moreover, concerning mammals' health, although thiacloprid is labeled as carcinogen (category 2) and toxic for reproduction (category 1B) and for other organs, a 2019 peer review of its risk assessment for human health considered that insufficient relevant toxicology studies have been made on this substance and its metabolites [16]. However, more recent experimental research on the effect of oral administration of a thiacloprid and clothianidin mixture on rats demonstrated several behavioral impairments, including on short-term memory, as well as histological brain damages [17]. Thiacloprid harmful growth and maturation effects on soil earthworms were also demonstrated in laboratory experiments [18] [19]. This pesticide has a half-life in soil up to 3 (assessed in the field) to 74 days and even much more (in the laboratory) [20] and moreover bio-accumulates in earthworms [19].

Neonicotinoids act on the nervous system as agonists to the nicotinic acetylcholine receptor. Being unable to be broken down by acetylcholinesterase, they trigger a constant activation of this receptor with, as possible ultimate consequences, paralysis and death. These pesticides affect the development of insects from the first larval stage up to the adult as this development is controlled by neurosecretions. Thiacloprid is a N-cyanoamidine neonicotinoid, a compound much less toxic for bees than N-nitroguanidine molecules such as imidacloprid and thiamethoxam, thanks to the functional detoxification enzyme expression of their CYP9Q3 cytochrome P450 gene [21] [22]. Over a 3-year replicated honeybee colony feeding study totaling 30 hives, bees were chronically fed either with sugar syrup or with a 0.2 mg/L or 2 mg/L thiacloprid solution in sugar syrup. In this experiment, no significant differences were found concerning the number of adults and brood cells, weight gain, mortality or the overwintering success between a thiacloprid-free control feeding and a diet with thiacloprid [23]. Nevertheless, other studies showed that thiacloprid has harmful effects. When honeybees were fed with a sublethal dose of thiacloprid during the entire larval stage (*i.e.*, 7 days), the survival rate of the larvae and pupae was reduced, and bees had a delayed development with a lower size and bodyweight [24]. Reduced survival was also observed in honeybee workers fed during 13 days with a thiacloprid 0.6 to 2.0 mg/L concentration in their usual sucrose solution, with the additional observation of a dysbiosis of their gut microbiome, however reversible when returning to a normal diet [25].

The impact of another neonicotinoid on insect development was unambiguously demonstrated by Schläppi *et al.* [26] on young colonies of the Formicine ant *Lasius niger* chronically exposed for 64 weeks to two different sub-lethal concentrations (4.5 μg/L and 30 μg/L) of thiamethoxam in their drinking water. Although this product did not affect queen mortality, the numbers of larvae as well as the reared workers' weight were significantly reduced in the group of colonies that received the high concentration as compared to a control group exposed to pure water. Thus, although thiamethoxam has $4.10^3$ to $4.10^4$ times less the affinity of thiacloprid to the binding site of another neonicotinoid (imidacloprid taken as a reference) at the nicotinic receptors of two investigated aphids and a locust [27], it nevertheless represents a detrimental risk on the ants' development.

Other effects of thiacloprid are more specific to insect behavior. At concentrations used in the environment, it causes in the honeybees' brain a transcriptional alteration of nuclear genes associated with the mitochondrial oxidative phosphorylation, what affects the energy metabolism and may compromise foraging [28]. A sublethal concentration of 6.5 μg/ml thiacloprid provided during 24 hours in the pre-treatment food of honeybees changed their food choice towards polyfloral honey and lowered their mobility if they had eaten polyfloral honey before the experiment [29]. Perhaps more significant, when honeybees were exposed to sublethal doses of thiacloprid for several weeks in the field, their foraging behavior, homing, navigation and social communication were impaired [30]. Sublethal doses of 0.5 to 50 ng/µl thiacloprid in a 30% sucrose solution or a 200 ng/bee 40% commercial thiacloprid formulation (Calypso®) affects honeybees: individually orally fed with 69 ng of such thiacloprid product, the bees associated less a conditioned stimulus with a sugared unconditioned stimulus than control bees. With a higher dose of thiacloprid (200 ng), no appetitive associative learning was observed and memory consolidation and retrieval were significantly impaired. In the field, this certainly affects a whole range of behaviors and may contribute to the decline of honeybee colonies [31].

If the effect of neonicotinoids, including thiacloprid, on the development and survival of insect pests and of some useful insects (e.g., bees) is now well documented, the impact of such chemicals on their physiological and ethological traits (e.g., on orientation, activity, memorization…) would benefit from further research. This is why we intended to examine the adverse effects of thiacloprid on several physiological and ethological traits of an ant species used as a biological model.

For conducting the present work, we used the Myrmicine *Myrmica sabuleti*, Meinert 1861, which biology we know rather well, namely its visual perception, conditioning acquisition, recruitment system [32], and ontogenesis of some of its skills [33]. Several of its workers' cognitive abilities have been investigated. Among others, they can count and add numbers of elements, have a number line, can acquire the notion of zero, and can expect the next element of an increasing or decreasing arithmetic or geometric sequence. A summary of these studies can be found in Cammaerts and Cammaerts [34]-[36].

The *M. sabuleti* workers' behavioral traits that were compared to a diet with or without thiacloprid were their food intake, general activity, locomotion speed on a smooth and on a rough surface, progression through a twists and turns path, orientation ability, exploratory or audacity reaction in presence of a novel situation, escaping ability form an enclosure, social relationship with nestmates, conditioning acquisition, individual adaptation to the effect of the insecticide, as well as their dependence on its consumption. We also studied the loss of the effect of thiacloprid after its consumption was stopped.

Knowing the impacts of low insecticide concentrations may help define the doses and the time periods to be used for minimizing their adverse effects. The aim of the present work is to make such an investigation for three concentrations of thiacloprid on an ant species used as a biological model, and to imagine a less environmental harmful way of using this insecticide.

## 2. Materials

### 2.1. Collection and Maintenance of Ants

The experimental work was conducted on five distinct colonies of *M. sabuleti* collected in May 2021 in the Aise valley (Ardenne, Belgium) and labeled A to E. Each colony contained ca 500 workers, a queen, larvae and eggs, and was maintained in one to three glass tubes half-filled with water, the ants being separated from the water by a cotton plug. The nest tubes of each colony were laid in a tray (34 cm × 23 cm × 4 cm), which borders were slightly talked for preventing ants from escaping. The trays served as foraging areas where pieces of mealworms (*Tenebrio molitor* (Linnaeus, 1758) larvae) were delivered three times per week and as in the wild, this species of ant actively feeds on aphid honeydew, a 15% sugar water solution was permanently provided in a 0.5 × 5 cm cotton-plugged tube. This constituted the normal diet of the colony. The lighting of the laboratory varied between ca 330 lux while working on ants and ca 110 lux while not doing so, the temperature equaled ca 20˚C, the humidity ca 80% and the electromagnetic field ca 2 $\mu Wm^2$, all conditions suitable for the species. "Nestmates" designate workers of the same colony.

### 2.2. Solution of Thiacloprid Given to the Ants

Hundred mg of thiacloprid (purity 99.8%) manufactured in Switzerland (Pestanal TM, CAS number 111988-49-9) were used to make dilutions in water until obtaining a stock-solution of 2 µg per liter kept at −25˚C when not used. In this solution, the usually used amount of sugar intended for feeding the ants was added, what allowed obtaining a 2 µg/L solution of thiacloprid in a 15% sugar water. Two more solutions, of 0.1 µg/L and 0.01 µg/L in a 15% sugar water, were made in the same way. Such as for the ordinary 15% sugar water solution which served as control, these thiacloprid solutions were provided to the ants in the usual small cotton-plugged tubes. The plugs were refreshed every 2 - 3 days and the entire content of the tubes was renewed each week.

## 3. Methods

### 3.1. Experimental Timing

Experiments on the effect of thiacloprid were done by replacing the 15% sugar water tubes of their ordinary diet by tubes filled with a 15% sugar water solution containing a known concentration of thiacloprid.

The timeline of control and experimental tests for each of the colonies, with the duration of exposure to the three concentrations of thiacloprid, is showed in **Figure 1**. Three experimental series were carried out, in the order (A and B), then (C and D) and then E. Control measurements on colonies fed with their ordinary normal diet always preceded the experiments with thiacloprid because if proceeded in reverse, there was a theoretical possibility that some amount of the insecticide remained in the ants' body. However, the experiment on the remaining effect of thiacloprid after its consumption was stopped showed that the control value was reached after already some 30 hours after weaning (see Results, "Decrease of the effect of thiacloprid after its consumption was stopped").

A whole series of control measurements took 5 or 6 days, depending on the number of experiments to be done on thiacloprid (Figure 1). Each series of experiments with a given concentration of thiacloprid began after the ants had had this solution of insecticide for 24 hours, which solution then remained available to them for the duration of the experimental work. Each day, it was checked if ants drunk the solution of thiacloprid, what they did. The experiments and their timing followed the same time order (daily and hourly time) as their respective controls, with the experiment measuring locomotion speed repeated five or six days later, depending on the colony used. Environmental variables were stable and invariant during the whole experimental process.

**Colonies A and B:**

**Normal diet:**
food and activity
day 1 -- linear and angular speed
day 2 -- orientation
day 3 -- audacity, speed on roughness
day 4 -- brood caring
day 5 -- social relationships, escaping
day 6 -- moving through twists and turns

**2µg/L diet:** day 8 ---- (normal diet replaced by 2µg/L)
food and activity
day 9 ---- linear and angular speed
day 10 -- orientation, conditioning
day 11 -- audacity, speed on roughness, conditioning
day 12 -- brood caring, conditioning
day 13 -- social relationships, escaping, conditioning
day 14 -- moving thr. twists and turns, conditioning
day 15 -- linear and angular speed, conditioning
day 16 -- dependence
day 17 -- (resting day without sugar water)
day 18+ -12 h under 2µg/L, then weaning

**Colony E:**

**Normal diet:** day 1 -- linear and angular speed
**0.01µg/L diet:** day 2 -- (normal diet replaced by 0.01µg/L)
day 3 -- linear and angular speed
day 4 -- resting under 0.01µg/L
day 5 -- resting under 0.01µg/L
day 6 -- resting under 0.01µg/L
day 7 -- resting under 0.01µg/L
day 8 -- linear and angular speed

**Colonies C and D:**

**Normal diet:** day 1 -- linear and angular speed
day 2 -- orientation
day 3 -- audacity, speed on roughness
day 4 -- brood caring
day 5 -- social relationships

**0.01µg/L diet:** day 6 ---- (normal diet replaced by 0.01µg/L)
day 7 ---- linear and angular speed
day 8 ---- orientation
day 9 ---- audacity, speed on roughness
day 10 -- brood caring
day 11 -- social relationships
day 12 -- linear and angular speed

**0.1µg/L diet:** day 13 --(0.01µg/L replaced by normal diet)
day 14 -- (normal diet replaced by 0.1µg/L)
day 15 -- linear and angular speed
day 16 -- orientation
day 17 -- audacity, speed on roughness
day 18 -- brood caring
day 19 -- social relationships
day 20 -- linear and angular speed

Figure 1. Experimental timeline. Daily timing of the behavioral traits measured on ants. Control measurements are those made on ants fed with their ordinary (normal) sugar water. Thereafter, the effects of three thiacloprid concentrations in the ants' sugar water were tested on these traits. With the exception of social relationships, traits on which 2 µg/L had no effect were not tested again on the lower 0.01 and 0.1 concentrations. For each colony, the numbering of experimental days began with the first trait measured on a normal diet.

The 2 µg/L solution was provided to colonies A and B for an initial assessment of the adverse effects of thiacloprid. This assessment covers the broadest spectrum of behavioral traits carried out at all three concentrations. Experiments that were carried out only with the 2 µg/L concentration were conditioning and memorization, moving through twists and turns, thiacloprid dependence and the loss of thiacloprid's effects on ant angular velocity after consumption of the product ceased (Figure 1).

To assess if the insecticide still induced adverse effects at lower concentrations

(0.1 and 0.01 µg/L), two other colonies, C and D, were used. They were controlled for the same traits as those controlled for the 2 µg/L concentration, with the exception of traits that showed no or few harmful effects under this 2 µg/L solution (*i.e.*, escaping and moving through twists and turns) and with the exception of behaviors measuring conditioning, dependence and decrease of the effect after removing the thiacloprid solution. However, social relationships, a trait showing no harmful effect under 2 µg/L, was nevertheless assessed again under the lower concentrations. Although the results for the two lowest concentrations are presented in descending order of concentration, the lower of the two concentrations was the first to be presented to the ants after they had been fed on their normal diet only, so as to avoid any prior presence of insecticide residues. Thus, after 5 days of control experiments under normal diet, then one resting day under a 0.01 µg/L thiacloprid diet, colonies C and D were tested during 6 days under this concentration, followed by a resting day under normal diet and another one under a 0.1 µg/L concentration, then tested during 6 days under this last concentration. In order to verify the results obtained about linear and angular speeds on colonies C and D with the 0.01 µg/L solution, a supplementary, checking experiment with its own control was conducted on colony E after one and six days with such a solution (**Figure 1**).

The results obtained using the three concentrations are reported in each of the subsections below. They are summarized in **Table 2** and **Table 3**. The numbering and counting of the experimental days refer to **Figure 1**.

### 3.2. Statistical Methodology

Being limited by the number of colonies available at the time of the study, controls and experiments had to be done on the same colonies. To obtain a sufficient number of measurements, results from colonies A and B were pooled, along with those from colonies C and D. Moreover, the ants were tested at the colony, not at the individual level. Repeated testing of some of the same individuals was therefore possible, but the variability of the responses from one measurement to the next was wide, suggesting that there was a significant turnover of individuals in the harvesting area. Besides, and more relevant, one of the experiments showed that the effects of thiacloprid were entirely lost within 33 hours. This is fast compared with the interval between two measurements of the same behavioral trait, which is around a week. As the time elapsed between the experiments and their respective controls was at least 7 days (colonies A + B) or 5 or 6 days (colonies C + D), we can therefore assume that the ants reacted sufficiently independently from one test to the next. Non-parametric statistics were used not only because some speed and orientation measurements were non-normal and their variances sometimes unequal (Levene's test: $P < 0.001$), but also because of the relatively small sample sizes we may expect that parametric tests, even if perfectly specified (*i.e.*, even if we knew the exact distribution of the variables), would not readily detect differences.

For each speed and orientation variable, the values obtained for ants consuming thiacloprid were thus statistically compared to those obtained some days earlier for ants normally fed, by using the Mann-Whitney (M-W) test, with the Z values adjusted for ties. An exception to strict independence of data sets could have occurred between measurements made at the time of weaning and at the successive three-hour times that followed, as some of the same individuals could have been included in samplings timely close to each other. However, the more distant, the more independent will be the samples. A Kruskal-Wallis (K-W) ANOVA test for multiple comparisons of measurements was nevertheless used. The obtained P values are those of a normal distribution corresponding to the calculated Z normal deviates. They were adjusted for multiple comparisons by using the Benjamini-Hochberg procedure [37], the false discovery rate being set at 0.05, a procedure valid in case of positive regression dependency [38]. The polynomial model best describing the decrease of the effect of thiacloprid on the ants' sinuosity was selected using the curve fitting stepwise procedure described in Zar [39].

The Wilcoxon paired-samples test was used when two samples were compared along the same gradation of extraneous conditions, such as a succession of days. A $\chi^2$ goodness-of-fit test was used to assess whether two distributions of ant numbers, with the same starting numbers, were identical within the limits of sampling error. The control distribution was the one expected under normal diet. When the starting numbers were different, the $\chi^2$ test for two independent samples was used to test the difference or similarity in shape of the two distributions. When appropriate, comparisons between colonies were also made using the Fisher's test for independent samples.

All tests were performed using Statistica® v.10 software, also used to calculate the polynomial curve describing the decrease of the effect. For easy reading in the text, P values issued from the normal deviate Z and well below 0.0001 are shown as P < 0.001.

### 3.3. Meat and Sugar Water Consumption Activity

The ants sighted on the meat, at the entrance of the sugar water tube, and being active elsewhere where separately and punctually counted (*i.e.*, during ca one second) when living under ordinary diet and after that, while they consumed a 2 µg/L thiacloprid solution.

These counts were conducted four times per day, two of them during the day, two others during the night (4 counts × 2 colonies = 8 daily counts), during six days. For each kind of diet, the six daily means were calculated (**Table 1**, lines I to VI) and those obtained for ants under thiacloprid diet were compared to the six daily means obtained for ants normally fed. For information purpose, the means of the six daily means are shown for each kind of diet (**Table 1**, last line I-VI).

### 3.4. Linear and Angular Speeds on the Foraging Area; Orientation to a Tied Nestmate

Linear speed (mm/s) is the length of a trajectory divided by the time spent to travel it. Angular speed (ang.deg./cm) is the sum of the angles made by successive adjacent segments, divided by the length of the trajectory. Orientation (ang. deg.) to a location is the sum of successive angles made by the direction of the trajectory and the direction towards the location, divided by the number of measured angles.

Linear and angular speeds were measured without stimulating the ants. Orientation was measured while stimulating the ants with a nestmate tied to a piece of paper (**Figure 2(A)**). Such a tied ant emits its attractive mandible glands alarm pheromone, what induces the ants located at 1 to 8 cm from it to rapidly walk in its direction [40]. When the orientation value is lower than 90˚, the animal tends to orient itself towards the attractive pheromone location. When it is higher than 90˚, the animal tends to avoid the location.

These traits were first assessed on ants walking on the rather smooth floor of their foraging area (traits 1 and 2 in **Table 2** and **Table 3**). For that, in each two colonies (A and B as well as C and D) the trajectories of 20 foragers, chosen as randomly as possible, were followed by tracking them above a glass sheet during about 5 s each. In total, 40 trajectories were so recorded for each measured variable. For colony E, after having followed the first 20 ants, they were temporarily kept in a small cup outside the foraging area, while 20 other ants were followed. These trajectory chunks were then analyzed using self-developed software named OVS [41].

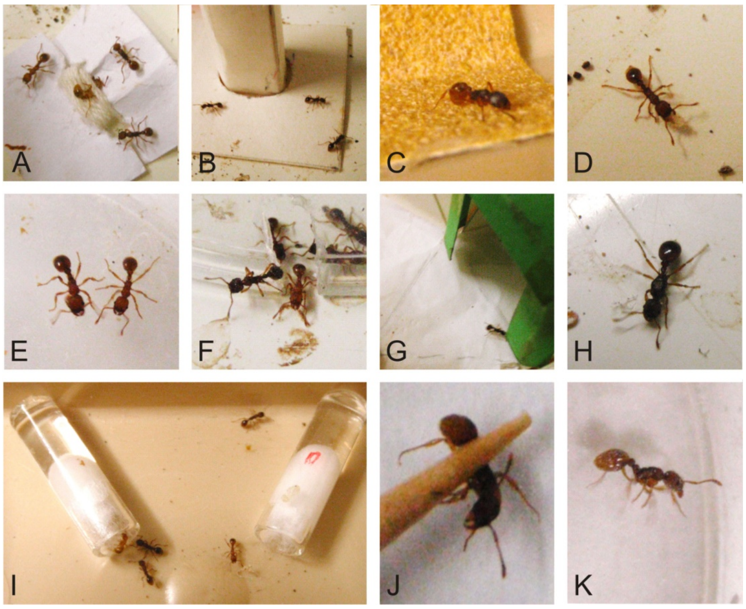


**Figure 2.** Some of the experiments conducted to assess the effect of thiacloprid on the ant *Myrmica sabuleti*. A: attractiveness of a tied nestmate. B: moving near an unfamiliar device. C: walking on a rough substrate. D: returning a larva to the nest after it was removed by the experimenter. E: social relationships during a dyadic encounter between nestmates. F: escaping out an enclosure. G: response to a green hollow cube to which the ant was conditioned. H: A queen unaffected by the presence of thiacloprid in her colony's diet. I: assessment of thiacloprid dependence by means of a choice test between a sugar solution containing thiacloprid (in the right-hand tube) and a solution without. J: a worker ant brought into physical contact with the thiacloprid solution and K: stumbling and falling a few seconds later. The photos are at different scales.

### 3.5. Audacity

This trait intended to quantify audacity or curiosity towards a new situation and was assessed through the ants' tendency to come onto an unfamiliar device. A cylinder (height = 4 cm; diameter = 1.5 cm), vertically tied to a platform (9 $cm^2$), both in Steinbach® white paper, was deposited in the ants' foraging area, and the ants coming onto it were counted 20 times over 10 minutes (**Figure 2(B)**). The mean and the extremes of the counted numbers were established (trait 3 in **Table 2** and **Table 3**). The numbers obtained for each two tested colonies and for each two successive minutes were correspondingly added, providing ten successive numbers. These ten numbers obtained for ants having consumed a thiacloprid solution during 3 days were paired with the ten ones obtained 7 or 14 days earlier (depending on the colony and the thiacloprid concentration used) for ants normally fed and were compared by using the Wilcoxon test.

To compare the effect of one concentration with that of another, measured on a different colony, the difference in distribution of the number of ants under and from 5 individuals present on the device was assessed using Fisher's test for independent samples.

### 3.6. Speed on a Rough Surface (Referred as Speed on Roughness)

We observed that while ants walked easily, rapidly and straight fully on a rather smooth substrate such as that of their foraging area, they walked slowly and sinuously when on a rough substrate and more often touched this substrate with their antennae (**Figure 2(C)**).

For assessing the effect of thiacloprid on the ants' walking speed on a rough surface, their linear and angular speeds were quantified while they walked on a rough substrate, in the way they had been quantified 6, 7 or 14 days earlier (depending on the colony and the concentration used) when walking on the smooth floor of their foraging area (see the subsection relative to linear and angular speeds).

An experiment on a colony consisted in transferring (using a cup) about 15 ants at a time to the first area of a small tray (15 cm × 7 cm × 4.5 cm) divided in a starting smooth area of 3 cm long, followed by a second 3 cm long area covered with a piece of 280 emery paper, and a third final and smooth area of 9 cm long. Ten trajectories of workers walking on the emery paper were recorded. These first 15 workers were temporarily kept outside the colony while 15 other workers of the same colony were tested in the same way. Twenty trajectories per colony were so recorded, making a total of 40 trajectories for 2 colonies. The values for linear and angular speeds (trait 4 in **Table 2** and **Table 3**) obtained for ants having consumed thiacloprid during three days were compared to those obtained 6 or 7 days before (depending on the colony used) on ants normally fed.

### 3.7. Brood Caring Behavior

This behavior takes place inside the nest and was thus not easy to observe and quantify. Therefore, for each colony, about twenty larvae were gently taken out of the nest and laid down near the nest entrance. The normal behavior of a worker was then to immediately bring back the larvae into the nest. For each colony, the workers' behavior towards five of the removed larvae was observed during five minutes (n˚ of observations = 10). Only five larvae of each colony were observed because they had to be followed at the same time during five minutes. The larvae that were not brought back in the nest after 30 seconds, 1, 2, 3, 4 and 5 minutes (**Figure 2(D)**) were counted. The six numbers obtained for each two colonies were correspondingly summed (trait 5 in **Table 2** and **Table 3**). The six sums obtained for ants consuming thiacloprid were compared to the six sums obtained 6, 7 or 14 days earlier (depending on the colony and concentration used) for ants normally maintained. The experiment was conducted only once per kind of diet and colony because it greatly perturbed the ants' society.

### 3.8. Social Relationships

Ants pertaining to the same colony are not aggressive towards each other (**Figure 2(E)**). For examining the impact of thiacloprid on this usual peaceful social behavior, five dyadic encounters were conducted for each colony (total n˚ of encounters = 10). Each of these encounters was performed in a small cup (diameter = 2 cm, height = 1.6 cm) the borders of which having been slightly talked. During an encounter, one ant of the pair was observed for 5 minutes, and the numbers of times this ant did nothing (level 0 of aggressiveness), touched the other ant with its antennae (level 1), opened its mandibles (level 2), gripped and/or pulled the other ant (level 3), and tried to sting or stung the other ant (level 4) were registered. For each of these aggressiveness levels, the numbers obtained for each ant and each colony were correspondingly added (trait 6 in **Table 2** and **Table 3**). The distribution of the values obtained on this aggressiveness scale for ants consuming thiacloprid was compared to that obtained 6, 7 or 14 days earlier (depending on the colony and the thiacloprid concentration) for ants normally fed by using the $\chi^2$ two-sample test, as it is the difference in shape of the two distributions of aggressive acts that matters to us. In addition, for each kind of diet, the ants' aggressive behavior was assessed by an aggressiveness variable labelled "a" and equaling the number of behaviors observed for the aggressiveness levels 2 + 3 + 4 divided by the number of behaviors observed for the aggressiveness levels 0 + 1 (trait 6, var "a" in **Table 2** and **Table 3**).

### 3.9. Escaping Ability

Six ants of each colony were enclosed under a reversed polyacetate cup (height = 8 cm, bottom diameter = 7 cm, ceiling diameter = 5 cm, the inner walls having been slightly talked) deposited in the foraging area. An exit hole (3 mm height, 2 mm broad) was managed in the rim of the bottom of the cup (**Figure 2(F)**). For each colony, the ants escaped after 2, 4, 6, 8, 10 and 12 minutes were counted and the numbers obtained for each two colonies were correspondingly added (**Table**

2, trait 7). The six numbers obtained for ants having thiacloprid at their disposal were compared to those obtained seven days earlier for ants normally fed.

### 3.10. Progression along a Twists and Turns Path

Two folded pieces of paper (Steinbach®, 12 cm × 4.5 cm) were inserted into a small tray (15 cm × 7 cm × 4.5 cm) making a path with 4 twists and turns between a 2 cm long area in front of this path and an 8 cm long area beyond it (a photo of this device is shown in e.g., [42]. Such a device was built for each colony. To conduct an experiment, 15 ants were transferred into the area lying in front of the twists and turns path, and the ants being still there as well as those being in the area lying beyond the "difficult" path were counted after 2, 4, 6, 8, 10 and 12 minutes. The numbers obtained for the two colonies were correspondingly added (Table 2, line 8). The numbers of ants observed under thiacloprid in the course of time were compared with those of ants normally fed and observed seven days earlier, assuming that the tested workers were not the same after a week's delay.

### 3.11. Adaptation to the Effect of Thiacloprid

Adaptation, in the sense of habituation, is here defined as the functional (physiological) adjustment of an individual to the effect of a product. It occurs when, over its use, an individual is less and less impacted by this product [43]. For examining this adaptation, at last one trait impacted by the product must be assessed soon after the individuals consume the product, then again after a long consumption period, and the results of the two assessments compared. In the present work, the ants' locomotion appeared to be largely impacted by thiacloprid. Therefore, the ants' linear and angular speeds were assessed after having been one day under thiacloprid diet and later on, after 7 days (colonies A + B) or 6 days (colonies C + D and E) under this diet. For each kind of speed, the median and quartiles of the recorded values were established (Table 2, trait 9 and Table 3, trait 7). The values obtained after one and more days were compared.

### 3.12. Conditioning Acquisition, Memory

Once an individual is conditioned to a stimulus, it keeps its conditioning during several days and even after having lost it, it can again acquire it more rapidly than initially [44] [45]. Therefore, it can no longer be used for quantifying its conditioning acquisition. Consequently, for the present conditioning, the control was previously done on a similar, but different colony of *M. sabuleti* collected on the same site and maintained under normal diet. For the colonies under a 2 µg/L thiacloprid diet, at a given starting time, a green hollow cube made in strong paper (Canson®) was deposited above the entrance of the tube containing the sugared solution of the insecticide along with mealworm pieces transferred aside that cube. Since that starting time, the ants underwent operant visual conditioning. Along the ants' conditioning acquisition, then along their possible loss of conditioning after the removal of the green cube, 10 ants of each colony were tested in an own

Y-apparatus (made of strong white paper, with its sides slightly talked) set in a separated tray. A green hollow cube had been inserted randomly in the left or the right branch of these Y-apparatus. To conduct a test on a colony, 10 workers were one by one transferred in the area lying in front of the two branches of the Y-maze, and for each ant, its first choice of one or the other branch of the Y-maze was recorded (**Figure 2(G)**). Choosing the branch provided with the green cube was considered as giving the correct response. After having been tested, each ant was kept in a glass until 10 ants of its colony were tested, this to avoid testing twice the same ant. When the 10 ants had been tested, all of them were set back into their foraging area. For each successive time period of conditioning, the 10 responses obtained during the control experiment were used as they were, while the 10 responses obtained for the two colonies having thiacloprid at their disposal were added (n˚ of responses: $10 \times 2 = 20$). This allowed calculating the percentages of correct responses obtained during the control and during the test experiments (**Table 4**). The scores of ants that had consumed thiacloprid were compared with those of ants that had been fed normally.

## 3.13. Dependence on Thiacloprid Consumption

Dependence on a product occurs when an individual enjoys consuming this product, tries to have it permanently at its disposal, uses it even if suffering from its adverse effect, and can finally no longer live without using it [46]. Dependence on thiacloprid was examined through the ant's preference between normal sugar water and sugar water containing a 2 µg/L thiacloprid solution, this after the ants had this insecticide solution at their disposal during 8 days. On the ninth day, 15 ants of each colony were transferred into an own tray (15 cm × 7 cm × 5 cm) into which two cotton-plugged tubes (h = 2.5 cm, diam. = 0.5 cm) had been set. One of them contained sugar water, the other contained the sugared solution of the insecticide. The tube containing thiacloprid was deposited on the right in one tray and on the left in the other tray (**Figure 2(I)**). For each two colonies, the ants sighted at the entrance of each tube were counted 15 times over 15 minutes, and for each tube, the 15 obtained numbers were added. The sums obtained for the two colonies were correspondingly added, this allowing calculating the proportion of ants having visited each kind of tube. The obtained totals were compared to the numbers expected if ants had randomly gone onto each tube, by using the binomial test.

## 3.14. Decrease of the Effect of a 2 µg/L Thiacloprid Solution after Its Consumption Was Stopped

This decrease was investigated after the ants consumed during nine days a sugar solution containing 2 µg/L of thiacloprid.

For assessing the exact loss of thiacloprid activity after weaning, we gave not that sugared solution to the ants on the 10th day, at the end of which we provided the ants with a fresh sugared solution of 2 µg/L thiacloprid. On the 11th day, after 12 hours under this insecticide diet, the ants' angular speed (a trait affected by thiacloprid) was assessed (see below). Immediately after that, at a time named t =

0, the tubes containing the sugared solution of thiacloprid were replaced by tubes filled of pure sugared water, what was the start of the weaning. Since that time, the ants' angular speed was assessed each three hours until it became similar to that recorded for ants under normal diet. Note that, although the ants' sinuosity was assessed in the usual manner, only 20 instead of 40 ants' trajectories were recorded and analyzed. This reduction of the sample size was done to have enough time for quantifying the ants' angular speed between each data recording and thus to be able evaluating over time the state of decay of the effect of the product.

For each angular speed record, the median and quartiles of the 20 obtained values were established (Table 5), and the distributions of the values were compared to those obtained at t = 0 h and to the control one. As these sample values were of unequal variances (Bartlett's test: $\chi^2 = 49.76$; df = 12; P = 0.000002) and non-normally distributed, they were compared using the non-parametric Kruskal-Wallis's test.

## 4. Results

### 4.1. Food Intake, Activity

Under a 2 µg/L thiacloprid diet, the ants eat less meat, drunk less sugar water and were far less active than when normally maintained (Table 1). These three differences were statistically significant (Wilcoxon test: N = 6, T = −21, P = 0.016). It might be tempting to attribute the low consumption of sugar water containing thiacloprid to a repellent taste or smell of this insecticide, but these potential properties have not been statistically demonstrated by a choice test (see section 4.12). The lower number of workers present on the food as well as active elsewhere else is more simply explained by the negative action of thiacloprid on their mobility (see next section, 4.2).

**Table 1.** Effect of a 2 µg/L thiacloprid concentration in the ants' sugar water diet, on their food intake and general activity.

| | **Normal diet: number[a] of ants** | | | **Diet with 2 µg/L thiacloprid: number[a] of ants** | | |
|---|---|---|---|---|---|---|
| Days | on meat | on sugar water | in activity | on meat | on sugar water | in activity |
| I | 1.00 | 1.50 | 12.00 | 0.00 | 0.25 | 4.00 |
| II | 0.75 | 1.38 | 9.50 | 0.25 | 0.00 | 3.50 |
| III | 1.75 | 1.75 | 17.00 | 0.25 | 0.25 | 3.50 |
| IV | 1.50 | 1.75 | 14.63 | 0.00 | 0.13 | 3.00 |
| V | 1.63 | 2.25 | 15.50 | 0.25 | 0.38 | 3.25 |
| VI | 1.75 | 2.50 | 14.25 | 0.13 | 0.13 | 2.50 |
| mean I-VI | 1.39 | 1.85 | 15.31 | 0.15 | 0.13 | 3.29 |

a. Mean number of ants of colonies A + B punctually counted four times a day over six days, on their meat, on their sugar water as well as being active at any place (lines I to VI). Mean of these means.

### 4.2. Linear and Angular Speeds on the Foraging Area

Observation showed that while having a thiacloprid diet at their disposal, the ants presented difficulties to walk on the ordinary smooth floor of their foraging area: they often felt down, could not correctly move their legs, and stopped. These observations were confirmed by numerical data.

In presence of a 2 µg/L thiacloprid concentration for just one day (colonies A and B, day 9), the ants walked far more slowly and more sinuously than while under normal diet (Table 2, trait 1), the difference being statistically significant (linear speed, 3.3 *vs* 8.8 mm/s; M-W: $Z = 7.70$, $P < 0.001$; angular speed, 261 *vs* 96 ang.deg./cm; M-W: $Z = -7.67$, $P < 0.001$). Similarly, after one day in presence of a concentration of 0.1 µg/L of thiacloprid (Table 3, trait 1), the ants moved at a slower speed (3.9 *vs* 8.9 mm/s; M-W: $Z = 7.70$, $P < 0.001$) and more sinuously (264 *vs* 97 ang.deg./cm; M-W $Z = 7.64$, $P < 0.001$) than under a normal diet.

Table 2. Effect of a 2 µg/L thiacloprid concentration in the ants' sugar water diet, on some of their ethological and physiological traits (colonies A and B).

| Trait | Under normal diet | Under diet with thiacloprid |
|---|---|---|
| **(1)** Moving in the foraging area[a]: | | |
| linear speed (mm/s) | 8.8 (7.7 - 9.9) | 3.3 (2.9 - 3.7) |
| angular speed (ang.deg./cm) | 96 (83 - 112) | 261 (217 - 289) |
| **(2)** Orientation (ang.deg.) | 25.8 (20.5 - 40.9) | 53.8 (45.0 - 69.6) |
| **(3)** Audacity (number of ants) | 2..7 [1 - 3] | 1.10 [0 - 23] |
| **(4)** Moving on a rough surface[b]: | | |
| linear speed (mm/s) | 2.8 (2.6 - 4.1) | 2.1 (1.8 - 2.3) |
| angular speed (ang.deg./cm) | 259 (222 - 285) | 392 (347 - 457) |
| **(5)** Brood caring: over time number | 30s 1' 2' 3' 4' 5' | 30s 1' 2' 3' 4' 5' |
| of larvae not re-entered among 12 | 8 6 4 2 0 0 | 10 10 9 7 5 4 |
| **(6)** Social relationships: number of | 0 1 2 3 4; var "a" | 0 1 2 3 4; var "a" |
| behaviors on an aggressiveness scale; variable "a" | 65 58 11 0 0; 0.09 | 48 60 9 0 0; 0.08 |
| **(7)** Number of ants among 12, escaped over | 2' 4' 6' 8' 10' 12' | 2' 4' 6' 8' 10' 12' |
| time from an enclosure | 2 5 9 10 12 12 | 1 3 6 10 11 12 |
| **(8)** Progress through a twists and turns path: | 2' 4' 6' 8' 10' 12' | 2' 4' 6' 8' 10' 12' |
| over time number of ants still in front of path | 18 15 13 9 6 6 | 27 21 17 13 11 10 |
| over time number of ants reaching beyond path | 0 2 6 8 11 15 | 0 0 3 10 12 18 |
| **(9)** Adaptation after 7 days of thiacloprid diet[a]: | | |
| linear speed (mm/s) | 8.8 (7.7 - 9.9) | 4.7 (4.2 - 5.1) |
| angular speed (ang.deg./cm) | 96 (83 - 112) | 181 (149 - 230) |

**a**. Locomotion speeds were assessed on the smooth floor of the ants' foraging area under normal diet (control), then after one day of thiacloprid diet and, in order to highlight a possible physiological adaptation, again after seven days under the latter diet. **b**. Speed on a rough surface inserted in a small distinct tray was assessed under normal diet (control) and after three days under thiacloprid diet. Median (and quartiles) of locomotion speeds and orientation of 40 individuals. Mean [and extremes] of ant numbers counted on an unfamiliar device (audacity measurement).

**Table 3.** Effect of 0.01 and 0.1 µg/L thiacloprid in the ants' sugar water diet (colonies C and D).

| Trait | Under normal diet | Under diet with thiacloprid |
|---|---|---|
| **(1)** Moving in the foraging area[a]: | | |
| linear speed (mm/s) | 8.9 (7.6 - 10.9) | 0.1 µg/L: 3.9 (3.2 - 4.3) |
| *id.* | | 0.01 µg/L: 5.5 (4.9 - 5.9) |
| angular speed (ang.deg./cm) | 97 (81 - 117) | 0.1 µg/L: 264 (206 - 288) |
| *id.* | | 0.01 µg/L: 183 (160 - 200) |
| **(2)** Orientation (ang.deg.) | 30.6 (24.7 - 43.4) | 0.1 µg/L: 39.1 (28.3 - 64.2) |
| *id.* | | 0.01 µg/L: 40.4 (31.3 - 52.9) |
| **(3)** Audacity (number of ants) | 2.23 [1 - 3] | 0.1 µg/L: 1.25 [0 - 2] |
| *id.* | | 0.01 µg/L: 1.43 [1 - 3] |
| **(4)** Moving on a rough surface: | | |
| linear speed (mm/s) | 3.3 (3.0 - 3.5) | 0.1 µg/L: 3.0 (2.6 - 3.3) |
| *id.* | | 0.01 µg/L: 3.2 (2.8 - 3.4) |
| angular speed (ang.deg./cm) | 283 (240 - 343) | 0.1 µg/L: 330 (299 - 366) |
| *id.* | | 0.01 µg/L: 315 (268 - 346) |
| **(5)** Brood caring: over time number | 30s 1' 2' 3' 4' 5' | 30s 1' 2' 3' 4' 5' |
| of larvae not re-entered among 12 | 9 8 5 4 1 0 | 0.1 µg/L: 10 10 9 6 5 3 |
| *id.* | | 0.01 µg/L: 9 8 7 5 1 0 |
| **(6)** Social relationships: number of | 0 1 2 3 4; var "a" | 0 1 2 3 4; var "a" |
| behaviors on an aggressiveness scale; variable "a" | 64 55 6 0 0; 0.05 | 0.1 µg/L: 51 59 8 0 0; 0.07 |
| *id.* | | 0.01 µg/L: 60 44 6 0 0; 0.06 |
| **(7)** Adaptation after 6 days of thiacloprid diet[b]: | (linear) 8.9 (7.6 - 10.9) | (linear) 0.1 µg/L: 4.6 (3.9 - 5.0) |
| linear speed (mm/s) | | 0.01 µg/L: 4.2 (3.8 - 4.5) |
| angular speed (ang.deg./cm) | (angular) 97 (81 - 117) | (angular) 0.1 µg/L: 240 (213 - 265) |
| | | 0.01 µg/L: 266 (239 - 299) |

**a.** Locomotion speeds were assessed on the smooth floor of the ants' foraging area under normal diet (control), then after one day of thiacloprid diet and, in order to highlight a possible physiological adaptation, again after six days under the latter diet. The experiments were firstly made using the 0.01 µg/L concentration. Then, after a resting day under normal diet and another day under a 0.1 µg/L concentration, they were assessed using the latter concentration. These dilutions still impacted the ants' locomotion, orientation, audacity and larvae re-entering, but contrary to the concentration of 2 µg/L they did not affect (for 0.01 µg/L) or not so much (for 0.1 µg/L) their speed on a rough surface. **b.** As measured by their locomotion speed in their foraging area, the ants did not adapt themselves to the concentration of 0.01 µg/L and very slightly to the 0.1 µg/L one. Median (and quartiles) of locomotion speeds and orientation of 40 individuals. Mean [and extremes] of ant numbers counted on an unfamiliar device (audacity measurement).

After one day in presence of a concentration of 0.01 µg/L of thiacloprid (Table 3, trait 1), the ants walked somewhat faster, but still at a slower linear speed (5.5 *vs* 8.9 mm/s; M-W: $Z = 7.40$, $P < 0.001$) and more sinuously (183 *vs* 97 ang.deg/cm; M-W: $Z = -7.04$, $P < 0.001$) than under a normal diet.

The impact of the 0.1 µg/L concentration on the linear speed was somewhat lower than that of the 2 μg/L concentration (3.9 *vs* 3.3 mm/s; M-W: Z = 3.34, P = 0.0008) and larger than that of the 0.01 μg/L concentration (3.9 *vs* 5.5 mm/s; M-W: Z = 6.11, P < 0.001) (compare trait 1 in **Table 2** and **Table 3**). Its impact on the angular speed was similar to that of the 2 μg/L concentration (264 *vs* 261 ang.deg./cm; M-W: Z = −0.64, P = 0.52) and higher than that of the 0.01 μg/L concentration (264 *vs* 183 ang.deg./cm; M-W: Z = −5.14, P < 0.001).

Thiacloprid thus impacted the ants' locomotion, the more at the highest of the three tested concentrations. Such a difficulty to walk could affect other ethological and physiological traits, a presumption investigated thanks to the following experiments.

### 4.3. Orientation to a Tied Nestmate

Under normal maintenance, the ants quickly went onto a tied nestmate (**Figure 2(A)**). Those under a 2 μg/L thiacloprid diet for two days poorly did so (**Table 2**, trait 2), the difference being statistically significant (M-W: Z = −5.85, P < 0.001).

This behavioral trait was still affected when thiacloprid was presented at each of the two lower concentrations (**Table 3**, trait 2). In the presence of the 0.1 μg/L and 0.01 μg/L concentrations, the ants oriented themselves similarly (39.1 *vs* 40.4 ang.deg.) to a tied nestmate, but less well than under normal diet (respectively, M-W: Z = −2.29, P = 0.02 and M-W: Z = −2.58, P = 0.01). This effect was not as strong as that induced by the concentration of 2 μg/L (compare **Table 2** and **Table 3**: 0.1 *vs* 2 μg/L: 39.1 *vs* 53.8 ang. deg.; M-W: Z = −2.68, P = 0.0068 and 0.01 *vs* 2 μg/L: 40.4 *vs* 53.8 ang. deg.; M-W: Z = −3.12, P = 0.0018).

The impact of thiacloprid on the ants' orientation behavior is possibly due to that on locomotion, but it could also result from an impact on the ants' sensory perception (in this case, particularly the olfactory one), a presumption checked by a further experiment (see subsection 4.5, Speed on Roughness).

### 4.4. Audacity

Ants fed a solution of 2 μg/L thiacloprid came onto the unfamiliar device (**Figure 2(B)**), even climbed on the tower, but were not numerous to do so (**Table 2**, trait 3), due to their difficulty in walking. Consequently, the numbers of counted ants on the apparatus statistically differed between the ants maintained with and without thiacloprid (Wilcoxon test: N = 9, T = 45, P = 0.002).

In presence of the 0.1 and of the 0.01 μg/L solutions, meanly 1.25 and 1.43 ants climbed on the unfamiliar device (**Table 3**, trait 3), the difference with the control diet (2.23 ants) remaining significant (Wilcoxon test, respectively N = 10, T = 55, P = 0.001 and N = 7, T = 28, P = 0.0078). The ants were thus still less inclined to come onto the newly exposed device.

Under a 0.1 μg/L solution, the mean number of ants (1.25) was intermediate between that (1.43) under a 0.01 μg/L solution and that (1.10) under a 2 μg/L solution (compare with **Table 2**), but the distributions of the numbers of ants did not significantly differ between them (Fisher's tests: all comparisons with P ≥ 0.65).

## 4.5. Speed on Roughness

While under normal diet the ants walked far more slowly and sinuously on a rough substrate than on the rather smooth surface of their foraging area. The difference is such as to make any statistical test unnecessary (just compare traits 1 and 4 in Table 2 and Table 3) and can be attributed to the nociceptive effect caused by contact with the roughness of the substrate. It is therefore the effect of thiacloprid on ant movement on rough surface that matters.

Under a concentration of 2 µg/L of thiacloprid, the ants walked more slowly (2.1 *vs* 2.8 mm/s, M-W: Z = 6.07, P < 0.001) and more sinuously (392 *vs* 259 ang.deg./cm, M-W: Z = −6.29, P < 0.001) than under normal diet (Table 2, trait 4; Figure 2(C)).

Under a 0.1 µg/L diet (Table 3, trait 4), the ants walked at a slightly slower linear speed (3.0 *vs* 3.3 mm/s: M-W: Z = 2.56, P = 0.01) and a somewhat higher angular speed (330 *vs* 283 ang.deg./cm: M-W: Z = −2.99, P = 0.003) than under normal diet. They showed an intermediate behavior between the 2 µg/L and the 0.01 µg/L diet.

Under a 0.01 µg/L thiacloprid concentration (Table 3, *idem*), the ants walked on the rough surface as if they walked on this substrate when under normal diet, the effect of this low concentration becoming negligible, with a linear speed of 3.2 *vs* 3.3 mm/s (M-W: Z = 0.89, P = 0.37) and an angular speed of 315 *vs* 283 ang.deg./cm (M-W: Z = −1.68, P= 0.09).

The fact that on a rough substrate, whether with or without thiacloprid in their food, the ants' speed remained reduced compared to that of their movement on a smooth substrate shows that the ants' sensory perception was not affected by this insecticide, as without tactile perception they would have moved as fast as on a smooth surface. Consequently, as thiacloprid did not appear to alter sensory perception, the ants' poor orientation towards a tied nestmate in the presence of a diet containing this insecticide (section 4.3) appeared to be essentially due to an impairment of their locomotion faculties. However, the impact on orientation could also have resulted from an effect of thiacloprid on the ants' social relationships, a presumption examined thanks to the two following experiments (subsections 4.6 and 4.7).

## 4.6. Brood Caring

Under normal diet, the ants soon found the larvae removed from the nest, took them in their mandibles and transported them inside the nest (Figure 2(D)). Under a 2 µg/L thiacloprid diet, the ants also found the larvae, took them in their mandibles, but had difficulties to hold them, then, even if being able to do so, had difficulties to transport them towards the nest. Consequently, though the ants' brood caring was not largely impacted by thiacloprid, the numbers of not-reentered larvae over time significantly differed between the ants maintained with and without thiacloprid (Table 2, trait 5) (goodness-of-fit $\chi^2$ = 63.34, df = 2, P < 0.001). Motile impairments, in the broadest sense of the term, could explain this result.

In presence of a 0.1 μg/L solution, the ants had also difficulties to transport the larvae, the numbers of these larvae not re-entered over time being statistically higher than those obtained for ants normally fed (goodness-of-fit $\chi^2 = 16.61$, df = 3, $P < 0.001$). In fact, under this 0.1 μg/L diet, the same slowness in larvae transport was observed as on under a 2 μg/L diet (compare **Table 2** and **Table 3**). This slowness was obviously due to locomotion difficulties and seemingly not to the ants' care instinct itself because they duly tried to take and transport the larvae, but poorly succeeded to do so.

The difference between the numbers of over time not yet re-entered larvae for the ants normally fed and those under a thiacloprid 0.01 μg/L diet was not significant (**Table 3**, trait 5: goodness-of-fit $\chi^2 = 1$, df = 3, $0.75 < P < 0.90$). Thus, a concentration of 0.01 μg/L of thiacloprid did not affect the transport of brood by the ants.

Thiacloprid thus appears to reduce larval transport efficiency by impairing worker motility, particularly at higher insecticide concentrations, and not by affecting social relations between worker and brood.

## 4.7. Social Relationships

Ants under normal diet or under a diet with a 2 μg/L thiacloprid concentration never attacked one another, but stayed near each other (**Figure 2(E)**), making nothing or doing antennal contacts, and seldom slightly opening their mandibles. The variable "a" assessing their aggressiveness equaled 0.08 for ants normally fed and 0.09 for those having the insecticide at their disposal (**Table 2**, trait 6). The difference in distribution of the numbers of observed levels of aggressiveness between the two diets was not significant: $\chi^2$ two-sample test = 1.64, df = 2, $0.30 < P < 0.50$.

Under concentrations of 0.1 and 0.01 μg/L of thiacloprid, the ants also developed no aggressiveness towards their nestmates (compare trait 6 in **Table 2** and **Table 3**). The distribution of their behaviors on an aggressiveness scale did not statistically differ from those of ants normally maintained (respectively $\chi^2 = 0.85$, df = 2, $0.50 < P < 0.70$ and $\chi^2 = 1.66$, df = 2, $0.30 < P < 0.50$, with variable "a" equaling 0.06 and 0.07). The experiments made using each three concentrations showed thus that thiacloprid had no impact on the ants' social behavior, a trait that may be linked to cognition.

This subsection and the previous ones tend to support the view that it is the impairment of the ant's motility, in the broadest sense of the term, which may explain the negative action of thiacloprid on their speed, orientation, moving on an unfamiliar device and brood caring behavior.

However, in the (unproven) case where thiacloprid could have induced aggression, it cannot be ruled out that the appearance of agonistic behavior between nestmates could have been counterbalanced by a calming effect resulting from the reduction in their motility, which would have explained the absence of aggression observed.

### 4.8. Escaping Ability

A 2 μg/L thiacloprid concentration did not impact this trait (**Table 2**, trait 7; **Figure 2(F)**). Under normal diet, the enclosed ants walked erratically during a short time, then calmly along the rim of the enclosure, found the exit and went out. While consuming thiacloprid, the ants walked with difficulty, sinuously, but without stress and soon walked along the rim of the enclosure. They found the exit, and succeeded to go out after some time. Therefore, thiacloprid did not appear to have induced stress, and the ants' cognitive ability seemed to be intact. Since the ants having the insecticide at their disposal moved with difficulty, the numbers of ants escaped over time were somewhat lower than those of ants under normal diet.

The results concerning social relations between worker nestmates and the ability to escape from an enclosure appear to support the view that thiacloprid does not induce stress or impair the cognitive abilities of ants (but see in section 6, Discussion, a possible interpretation of the cognition outcomes by the reaction time left available to the ants due to the impairment of their locomotor and motile faculties).

### 4.9. Progression along a Twists and Turns Path

Regardless of when their numbers were counted, under normal diet or under a diet with a 2 μg/L thiacloprid concentration, there were always more ants still present in the starting area of the twists and turns path (**Table 2**, trait 8; goodness-of-fit test: $\chi^2 = 23.94$, df = 5, $P < 0.001$). However, the distribution pattern of these numbers as a function of elapsed time was identical for both types of diet (two-sample test: $\chi^2 = 0.38$, df = 5; $P = 0.995$), showing that the slower progress of the ants in these bends was due to the slowing effect of thiacloprid on their speed of movement. As for the ants that reached the end of the path, as expected, the number of those under a 2 μg/L thiacloprid diet was lower than those under normal diet during the first 6 minutes of the count. After that, however, the opposite was true. The explanation lies in the observation: ants under thiacloprid diet were less likely to turn back than those under normal diet. The small difference was however not significant (goodness-of-fit test: $\chi^2 = 4.32$, df = 3, $0.10 < P < 0.25$).

### 4.10. Adaptation to the Effect of Thiacloprid

The ants physiologically adapted to the effects of a 2 μg/L thiacloprid concentration on their locomotion (**Table 2**, trait 9). After having been under this insecticide diet during 7 days (from day 9 to day 15), the ants' locomotion speed tended towards that of an ant under normal diet. They walked somewhat more rapidly and less sinuously than after having been during 1 day (*i.e.* day 9) under this diet, a difference statistically significant (**Table 2**, compare trait 1 with trait 9: linear speed, M-W: $Z = -6.20$, $P < 0.001$; angular speed, M-W: $Z = 4.99$, $P < 0.001$). However, this adaptation was partial: after 7 days under thiacloprid, the ants still walked more slowly and more sinuously than under normal diet (day 1), the difference being still significant (linear speed, M-W: $Z = 7.70$, $P < 0.001$; angular speed, M-W: Z = −6.69, P < 0.001).

After 6 days (day 20) under a 0.1 μg/L concentration, the ants also presented some adaptation, walking a little more rapidly, but not significantly less sinuously than after 1 day (**Table 3**, compare traits 1 and 7): their linear speed went from 3.9 to 4.6 mm/s (M-W: Z = −3.37, P = 0006) and their angular speed from 264 to 240 ang.deg./cm (M-W: Z = 0.71, P = 0.48).

After 6 days under a 0.01 μg/L thiacloprid solution (day 12), the ants were statistically significantly more affected than after 1 day in presence of that concentration (**Table 3**: *idem*): their linear speed went from 5.5 to 4.2 mm/s (M-W: Z = 6.09, P< 0.001) and their angular speed from 183 to 266 ang.deg./cm (M-W: Z = −6.89, P < 0.001). The ants showed thus no physiological adaptation at the 0.01 μg/L concentration.

The discovery that the ants did not restore their usual (*i.e.* under normal diet) locomotor behavior under a 0.01 μg/L concentration, but partly under a more concentrated solution (0.1 and 2 μg/L) may sound strange. Therefore, and proceeding in the usual way, we verified this result on a separate and previously unused colony (E). These results, here reported, are not in a table. Under a diet without the insecticide, the ants of this colony E had a linear speed of 9.0 mm/s (7.7 - 9.5) and an angular speed of 91 ang.deg./cm (79 - 111). After one day with 0.01 μg/L thiacloprid, their linear speed equaled 5.4 mm/s (4.9 - 6.3) and their angular speed 167 ang.deg./cm (143 - 204). Six days later, these speeds equaled 4.3 mm/s (3.6 - 4.6) and 253 ang.deg./cm (239 - 294). These values did not statistically differ from those previously obtained for colonies C and D: *i.e.*, under normal diet, the comparison gave M-W: Z = 0.92, P = 0.35 for the linear speed and M-W: Z = 0.72, P = 0.47 for the angular speed. After one day under 0.01 μg/L thiacloprid, the values were Z = −0.29, P = 0.77 for the linear speed and Z = 0.84, P = 0.40 for the angular speed and after six days under 0.01 μg/L thiacloprid, there was also no difference for the linear speed (M-W: Z = 0.83, P = 0.41) and the angular speed (M-W: Z = 0.68, P = 0.49). The results obtained on colonies C and D were thus confirmed by those on colony E. Furthermore, as for colonies C and D, after 6 days under 0.01 μg/L, the linear speed was significantly lower and the angular speed significantly higher than those after only one day under this insecticide concentration (M-W: Z = 5.88, P < 0.001 and Z = −6.80, P < 0.001). A Kruskal-Wallis analysis comparing the successive samples represented in **Figure 3** showed that all speed differences were highly significant (P < 0.001), except between one and six days when the ants were under the 0.1 μg/L thiacloprid concentration (P= 0.072 for linear speed and 1 for angular speed), a result intermediate between the effect of the 2 μg/L and the 0.01 μg/L concentrations. To summarize, an obvious individual physiological adaptation to thiacloprid occurred for the 2 μg/L concentration, a slighter adaptation occurred for the 0.1 μg/L concentration, and no adaptation was observed for the 0.01 μg/L concentration (**Figure 3**). This should be taken into account for the agricultural use of thiacloprid, even if the physiological mechanism that could explain these observations is still to be understood.

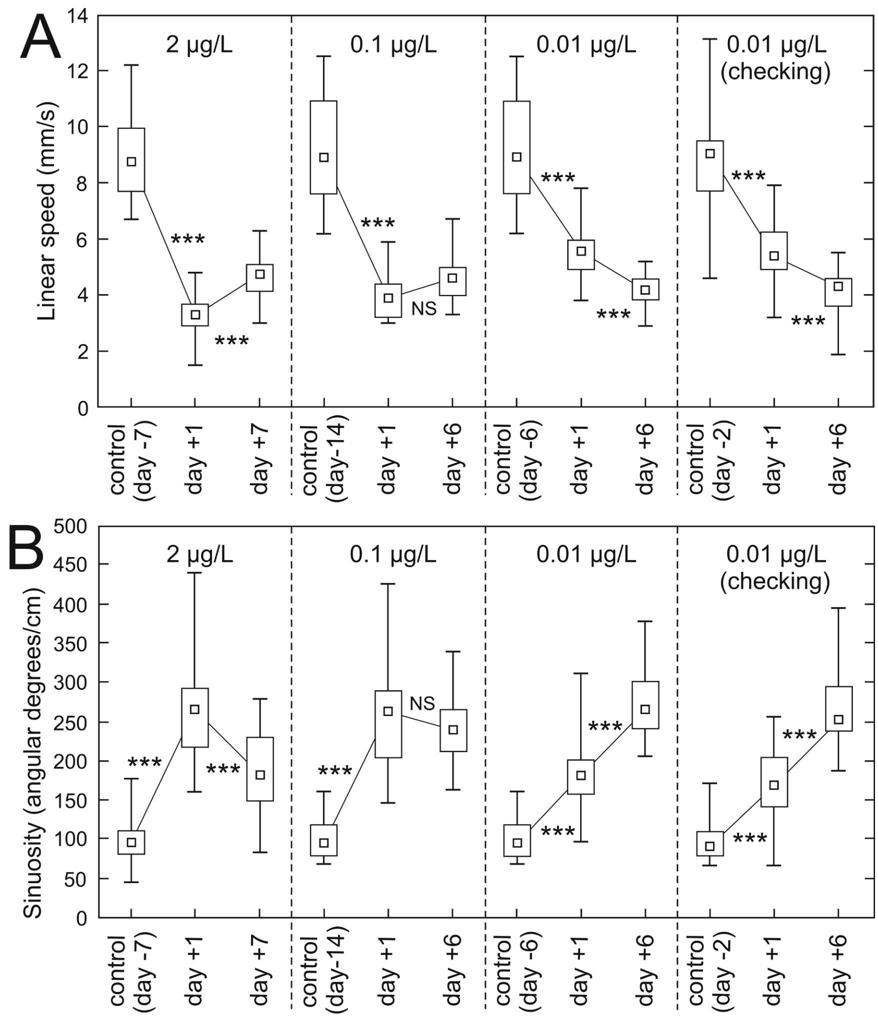


Figure 3. Adaptation to the effect of three concentrations of thiacloprid (2, 0.1, 0.01 µg/L) provided in the sugar water diet of the ant *M. sabuleti*. The effect on the ants' linear (A) and angular (B) speeds was assessed before (*i.e.*, control = diet without the insecticide) or after the diet containing thiacloprid was left at their disposal during one day (=at day + 1) or six to seven days. Median, inter-quartile range and extremes of speeds of 40 foragers walking in their foraging area. ***: significant difference at P < 0.001 (Kruskal-Wallis test with Benjamini-Hochberg correction).

## 4.11. Conditioning Acquisition, Memory

A 2 µg/L thiacloprid concentration did not affect the ants' conditioning acquisition (Table 4, upper part; Figure 2(G)). However, while having this insecticide at their disposal, the ants moved more slowly in the Y-apparatus than ants under normal diet and took their time for choosing the correct response. Since during the test time was not taken into account, their slowness gave them some advantage, and their conditioning scores were somewhat significantly higher than those of ants normally maintained (Wilcoxon test: N = 5, T = 15, P = 0.031). After the cue removal, the ants living in the presence of thiacloprid kept their learning as well as those normally maintained (Table 4, lower part; N = 5, T = 9, P = 0.406). These results seem to support the non-alteration of memory, but as the treated ants moved slower, they had more time to choose the correct path in the Y-maze.

**Table 4.** Impact of a 2 µg/L thiacloprid concentration on the ants' conditioning acquisition and memory.

| | Normal diet | Diet with 2 µg/L thiacloprid | | |
|---|---|---|---|---|
| | | Number of correct *versus* wrong responses | | |
| Time (hours) | Conditioning scores | Colony A | Colony B | Conditioning scores |
| 7 h | 60% | 7 *vs* 3 | 6 *vs* 4 | 65% |
| 24 h | 60% | 7 *vs* 3 | 7 *vs* 3 | 70% |
| 31 h | 70% | 7 *vs* 3 | 8 *vs* 2 | 75% |
| 48 h | 70% | 8 *vs* 2 | 8 *vs* 2 | 80% |
| 55 h | 80% | 8 *vs* 2 | 9 *vs* 1 | 85% |
| 72 h | 85% | 9 *vs* 1 | 8 *vs* 2 | 85% |
| Cue removal | | | | |
| 7 h | 85% | 8 *vs* 2 | 8 *vs* 2 | 80% |
| 24 h | 80% | 7 *vs* 3 | 8 *vs* 2 | 75% |
| 31 h | 80% | 8 *vs* 2 | 9 *vs* 1 | 85% |
| 48 h | 80% | 8 *vs* 2 | 9 *vs* 1 | 85% |
| 55 h | 80% | 8 *vs* 2 | 9 *vs* 1 | 85% |
| 72 h | 80% | 8 *vs* 2 | 8 *vs* 2 | 80% |

### 4.12. Dependence on Thiacloprid Consumption

During the choice experiment, ants of colony A came onto the 2 µg/L thiacloprid solution 14 times and 16 times on this product-free solution. The ants of colony B went to the thiacloprid solution 17 times and to the insecticide-free solution 24 times. In total, 40 (56.3%) of the ants' visits were for the sugared water solution and 31 (43.7%) ones for the thiacloprid solution (**Figure 2(I)**). While a slight preference appeared for the usual sugar water, these numbers statistically did not differ from those (35.5, 35.5) expected if the ants had randomly visited the two solutions ($Z = -0.95$, $P = 0.17$). It is obvious that the ants did not develop any dependence on thiacloprid consumption.

### 4.13. Decrease of the Effect of Thiacloprid after Its Consumption Was Stopped

Numerical and statistical results are given in **Table 5**, and graphically illustrated in **Figure 4**. The action of a 2 µg/L thiacloprid solution on the ants' sinuosity of locomotion did not significantly differ from its initial effect during 6 hours after weaning. Then, it began to significantly differ from 9 hours after weaning ($P < 0.05$), having thus stayed active during about 8 hours. Eighteen hours after weaning, the effect of thiacloprid became highly different from its initial one ($P < 0.001$). Its effect differed significantly from the control until 24 hours after weaning (P = 0.0583). From that point on, it gradually became similar to the control and indistinguishable from the latter 33 hours after weaning.

**Table 5.** Decrease of the effect of a 2 µg/L thiacloprid concentration in the ants' sugar water diet after its consumption was stopped by substituting this solution with a standard diet of sugar water.

| Time | Angular speed | K-W test, B-H adjustment | |
|---|---|---|---|
| (hours) | (ang.deg./cm) | P values *versus* t = 0 h | P values *versus* control |
| 0 h | 255 (220 - 310) | - | <0.0001 |
| 3 h | 255 (234 - 292) | 0.4396 | <0.0001 |
| 6 h | 210 (192 - 251) | 0.1033 | <0.0001 |
| 9 h | 204 (169 - 230) | 0.0260 | <0.0001 |
| 12 h | 179 (173 - 208) | 0.0080 | <0.0001 |
| 15 h | 179 (163 - 201) | 0.0016 | <0.0001 |
| 18 h | 130 (94 - 183) | 0.0002 | 0.0088 |
| 21 h | 130 (108 - 138) | <0.0001 | 0.0356 |
| 24 h | 115 (101 - 136) | <0.0001 | 0.0583 |
| 27 h | 116 (86 - 143) | <0.0001 | 0.1898 |
| 30 h | 109 (96 - 121) | <0.0001 | 0.3133 |
| 33 h | 101 (79 - 112) | < 0.0001 | 0.4325 |
| control | 96 (83 - 112) | <0.0001 | - |

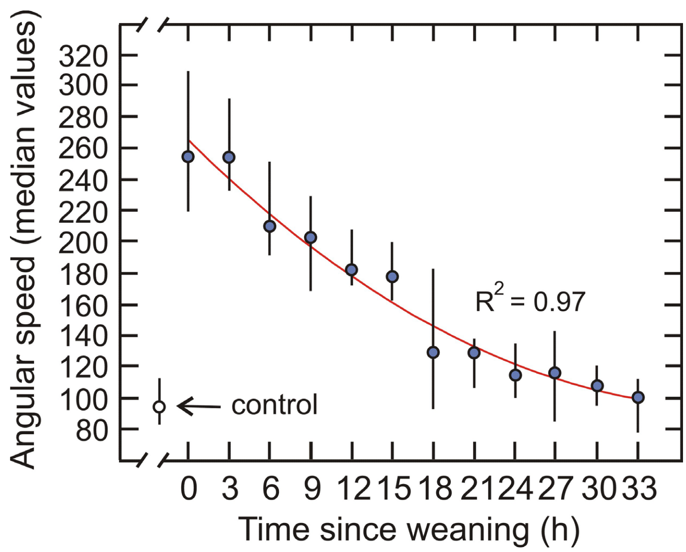


**Figure 4.** Decrease of the effect of thiacloprid after its use was stopped. The effect of thiacloprid statistically differed from its initial one at about 9 hours after weaning, differed from the control situation until about 24 after weaning, and became indiscernible from the control 33 hours after its use was stopped. The graph shows the median values and inter-quartile ranges of the records of angular speeds (in ang.deg./cm). Numerical and statistical results are given in Table 5.

To summarize, the effect of thiacloprid became very different from its initial one 12 hours after weaning (P = 0.008), and statistically no longer distinct from that of a normal diet from 24 hours after weaning. It vanished in a total of 33 hours according to a quadratic function which equation was: angular speed (in ang.deg./cm, which values range from 83 to 310) = 264.61 − 8.46t + 0.10t$^2$, with t being time in weaning hours. $R^2$ = 0.97.

## 5. Supplementary Observations

Thiacloprid may also act by contact, a presumption we checked thanks to a simple handling. As soon as the observer touched an ant worker with a small wooden stick imbibed with 2 µg/L thiacloprid, the ant immediately walked with difficulties and tumbled (**Figure 2(J)**, **Figure 2(K)**). It did not die but had its locomotion impacted all day long. Furthermore, after the end of the entire experimental work, contrary to other similar behavioral studies conducted with harmful substances, the inside of the nest tubes contained several dead ants which were not initially present. These dead ants were perhaps those that drunk the thiacloprid solution. However, this increase of the number of ant corpses was not assessed.

Contrary to the foraging workers, the five ant queens present in the used colonies appeared unaffected by thiacloprid (**Figure 2(H)**). It may be because they were never directly in contact with this insecticide, but more probably because the queens detoxify more efficiently than the workers, what is the case with the ant *Lasius niger* [26].

## 6. Discussion

Thiacloprid and other neonicotinoids are known to have deleterious effects on the central nervous system (CNS) of rats [17], on the foraging and social communication of bees [28]-[31], on the growth and survival rate of their brood [24], on the soil fauna [18] [19] and on freshwater insects [7]. Here we investigated about other physiological and ethological adverse effects of thiacloprid on an ant, using a not too high, sublethal concentration (*i.e.* 2 µg/L). Schläppi *et al.* [26] estimated that 4.5 µg/L was a low concentration for thiamethoxam, and 10 µg/L was used as the highest thiacloprid concentration by Barmentlo *et al.* [7]. We also examined the effects of two lower concentrations, 0.1 and 0.01 µg/L, to assess if they still had toxic effects, in which case they could be efficiently used by growers while limiting the impact on other living organisms, knowing that the 0.1 µg/L pesticide concentration is the limit authorized by the European legislation in water intended for human consumption [16] [47].

We found that a concentration of 2 µg/L negatively impacted the ants' locomotion, including their speed on a rough substrate and their progression in a twists and turns path, as well as other motor-related behavioral traits, *i.e.* orientation towards a source of pheromone, moving on an unfamiliar device and ability to transport larvae (brood caring). The mobility impairment caused by thiacloprid may also explain the lower number of active workers and of those present on the food.

We should be aware that moving on an unfamiliar device may also express a cognition-related behavior, *i.e.*, the exploratory tendency. Moreover, under thiacloprid diet the ants move slower and when they perform cognition-related behaviors or traits relating in some way to it, such as ability to escape from an enclosure, learning and memory, they have more time to find the way to escape or to choose the right way in a Y-maze. Thus, the behavioral results concerning these traits under thiacloprid diet cannot be entirely disentangled from locomotor impairment. Social relationships between nestmates could perhaps also have benefited from a slowing of the ants' motility. Sensory perception, for its part, appeared unaffected.

When a 0.1 µg/L and even a 0.01 µg/L concentration were tested, they induced the same side effects as a 2 µg/L concentration, but at a significant lower level. The exception, for a 0.01 µg/L concentration, concerns physiological adaptation.

As shown by a choice experiment, the ants developed no dependence on a 2 µg/L thiacloprid solution, showing statistically equal taste interest in thiacloprid and in their usual sugar water. Based on the choice of 71 workers, it is likely that the 44% of them that choose the thiacloprid solution were not overly repelled by the taste or smell of this insecticide.

At a concentration of 2 µg/L, the effect of this product on ant's angular velocity completely ceased 33 hours after weaning. This slow decrease allowed the ants adapting themselves to the impact of thiacloprid (see next paragraph) and avoided them to develop a dependence on this product [46].

Adaptation at the individual physiological level partially occurred to the 2 µg/L concentration, weaker to the 0.1 µg/L concentration and not to the 0.01 µg/L one. This result suggests that the ants detoxified thiacloprid when ingesting it at the higher concentration here used, detoxified it less at a lower concentration and not at all at the lowest used concentration, as if the detoxification process required the presence of a minimal amount of this insecticide. An adaptation, even partial, is beneficial for the ants which, in the course of time, are less impacted by thiacloprid, but this is not what growers would expect, their goal being the elimination of crop pests. An adaptation to thiacloprid would oblige farmers to use more and more amounts of it over time, what would accentuate the adverse effects of this pesticide on the non-targeted fauna as well as posing a risk to human health. It is thus suggested to use thiacloprid at a low concentration *i.e.*, one that does not induce adaptation but still produces harmful effects on insects (e.g., here shown to be in the range of 0.01 µg/L), and to repeat the application if and when necessary. In our study it was not tried to further precise the value of this low concentration because the ant used in the present study is only a biological model and that, for an agricultural use, the appropriate concentration depends on the targeted crop pest species.

The adverse effect of thiacloprid on the locomotion of insects is probably that of any neonicotinoid insecticide, the agonistic action on nicotinic acetylcholine receptors [48]. These insecticides "take" the place of acetylcholine and cannot be breached down by acetylcholinesterase. This blocking induces a constant activation of the nicotinic acetylcholine receptors which disturbs the neural aspect of behavior and ultimately leads to paralysis and death. Another effect of thiacloprid on behavior may be that on the energy metabolism of the mitochondria [28], resulting in a decrease of activity, such as that of locomotion speed (linear speed) studied in the present work.

We observed that thiacloprid can act through physical contact on the *M. sabuleti* ant workers, and this in a few seconds, albeit without killing them immediately. We have also shown that it can act through ingestion, but apparently more slowly, and caused the effects revealed in the present work. On other hymenopterans such as honeybees and bumblebees, thiacloprid has also a topical toxicity, but this toxicity was found to be somewhat lower than its oral toxicity, as indicated by its higher $LD_{50}$ value [21]. The queens of *M. sabuleti* were not affected by thiacloprid, maybe because they were never in contact with this product, but more probably because ant queens are known to detoxify more efficiently than workers, as it was shown in *Lasius niger* under thiamethoxam diet [26].

Besides for the usual self-precautions needed when farmers use thiacloprid, attention must also be taken for not contaminating the surrounding land as well as the aquatic environment. Sewage stations should be equipped to brough degradation processes of thiacloprid and other neonicotinoid residuals into play. Using less toxic insecticides (e.g., pyrethroids) for small cultivated areas is advised. Anyway, whenever possible, natural alternatives should be preferred for controlling crop insects.

## 7. Conclusions

To conclude, *M. sabuleti* worker ants feeding on sugar water containing a 2 µg/L thiacloprid concentration had their motor-linked behaviors negatively impacted. Cognition-related traits appeared unaffected, but we cannot rule out that their observed behavioral outcomes were affected by the reduction in the ants' locomotor faculties. Sensory perception, for its part, appeared unaffected. Sugar water solutions containing 0.1 and 0.01 µg/L thiacloprid also affected the ants' motor-linked behaviors, the less at the lesser concentration.

As measured by the ants' linear and angular velocities and contrary to what might have been expected, some physiological adaptation to the insecticide occurred at a concentration of 2 µg/L, less so at 0.1 µg/L and none at 0.01 µg/L. So, for an agronomic use that would be the least damaging for the environment, and according to the plague insect targeted, we need to look for the lowest effective concentration of thiacloprid that nevertheless produces significant behavioral damage on this insect and which, in order to avoid the development of resistance, does not result in physiological adaptation.

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## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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