Effect of sub-lethal doses of thiamethoxam on the memory of *Apis mellifera* Linnaeus

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Honey bees forage owing to their remarkable ability to learn and memorize their cues. The effect of thiamethoxam on the memory of bees was studied through the proboscis extension reflex (PER). The bees that consumed higher doses of thiamethoxam (0.93–5.76 ng bee^{-1}) showed lesser sensitivity to sucrose than those that consumed lower doses (0.03–0.64 ng bee^{-1}). Bees sensitivity was least affected at the highest sucrose concentration (50% w/v). PER in trained and treated bees recorded after 2 and 24 h of consuming the various doses of thiamethoxam showed a significant reduction in memory (13.3–82.2% and 0.00–68.9% respectively). Field-level studies are required to validate the results and formulate strategies at the national level for safeguarding the bees.

Keywords: *Apis mellifera*, memory, proboscis extension reflex, thiamethoxam, sucrose concentration.

BEES are indispensable natural resources and are important in preserving the natural ecosystems[1,2]. They offer pollination services, and thus contribute towards ensuring plant reproduction and sustaining food security. Commercial beekeeping offers a viable system through which underprivileged sections of the society may be benefitted by earning their livelihood[3].

Honey bee colonies comprise thousands of workers, hundreds of drones and a single queen. The primary responsibility of the workers is food-gathering, for which they must explore their surroundings. Hence, worker bees must be able to learn and remember certain cues like colour, fragrance and shape of the flowers, and their location.

Large-scale honey bee colony loss has been reported worldwide. This is known as colony collapse disorder (CCD)[4,5]. Extensive use of neonicotinoids, a decline in bee forage acreage, environmental pollution, etc. are the major responsible factors[6]. According to a Government of India report[7], Punjab is the third largest user of chemical pesticides in 2021–22 after Maharashtra and Uttar Pradesh (5193 metric tonnes (MT) technical grade; neonicotinoids 716.09 MT).

The neonicotinoid group of insecticides show activity against a wide spectrum of sucking insects and some heteropterans, coleopterans and lepidopterans[8]. These chemicals once occupied a global market share of more than 25% and one representative, i.e. imidacloprid was the second most widely used agrochemical in the world[9]. Thiamethoxam is a systemic neonicotinoid[10]. It has been registered by the Central Insecticide Board and Registration Committee, Faridabad, Haryana, India for successful management of aphids infesting the major bee floral crop, i.e. mustard. This insecticide is recommended for seed treatment as well as foliar application[11]. Its residues have been reported in nectar and pollen[12]. It is highly toxic to honey bees[13]. Thiamethoxam acts as an agonist to the nicotinic acetylcholine receptors (nAChRs) of insects, predominantly abundant in the neuropil regions of the insect brain[10]. In the honey bee brain, two types of receptors, viz. α-bungarotoxin (α-BGT)-sensitive receptor and α-BGT-insensitive receptor, have been found responsible for olfactory response, learning and memory[14–16].

Olfactory memory plays an important role in bee behaviour which impacts the colony survival. Foragers learn through latent or associative learning. The former is less understood while the latter, i.e. associative learning behaviour, which is exhibited as proboscis extension reflex (PER) by a trained bee, has been widely used in apicultural research[17,18]. In this system, a stimulus is offered to the bee immediately before the reward, viz. sugar solution. Bees memorize the stimulus after a number of training events. This memory formation can be quantified through PER elicited by the scent of the stimulus. Such experiments can be conveniently conducted under laboratory conditions due to ease in selecting a stimulus and controlling stimulus-response conditions[18]. For the present study, we hypothesize that thiamethoxam may affect the memory of bees. Keeping this in view, we have evaluated the effect of its sub-lethal dosages on bee behaviour, using the PER response method.

Materials and methods

Collecting and feeding the bees

Apis mellifera ligustica Spinola foragers were collected from healthy, queen-right colonies at Punjab Agricultural University (PAU) Apiary, Ludhiana, Punjab, India. An insect net (diameter 25 cm) was used to capture the foragers at the hive entrance in the morning. These bees ($n \sim 90$/hoarding cage) were maintained in an incubator at 25° ± 2°C and 70% relative humidity. Nine (one cage for each

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concentration) wooden hoarding cages (14 × 10 × 18 cm) were maintained in this manner.

The acute median lethal dose (LD_{50} oral: 24 h) of thiamethoxam was reported as 5.20 ng bee^{-1} (ref. 19). Considering this, various concentrations of thiamethoxam in sucrose solution (50% w/v) yielding average consumption ranging from 0.03 to 5.76 ng bee^{-1} were provided *ad libitum* to the bees in the hoarding cages. Final consumption (after 4 h) in each hoarding cage corresponding to a particular concentration was calculated by subtracting final volume from initial volume. The bees show trophallaxis behaviour; therefore, each bee in a hoarding cage had a similar dose of thiamethoxam in the present experiment.

Restraining and selecting the bees

The bees in various hoarding cages were immobilized, to facilitate easy restraining, for a brief period by keeping them in freezer at –20°C for 3 min. Each bee was restrained in an individual micro-centrifuge tube based harness[20]. There were three replications, each containing ten bees. The restrained bees were tested for their sensitivity to sucrose and water separately. For each set of bees, corresponding to a particular thiamethoxam dose, sucrose solution or water was provided through separate glass capillaries and extension of proboscis in response to either sucrose solution (50% w/v) or water was recorded. A bee showing a positive response to sucrose and a negative one to the water was selected for the experiment.

Sucrose sensitivity

Response of each treated restrained bee was examined by stimulating its antennae with various concentrations of sucrose[17], including some extra concentrations (20.0%, 40.0% and 50.0% w/v). Sucrose solution was applied on the distal flagella of an antenna of a particular restrained bee for eliciting PER. Thereafter, that bee was allowed to lick the solution. An inter-trial interval of 3 min was maintained. The number of bees showing PER to a particular concentration of sucrose was recorded.

Olfactory learning

In order to evaluate PER, a temporary exhaust system consisting of a small air-exhaust fan (5.5 cm ø) was deployed (Figure 1). The individual restrained bees (a different set of bees used in the sucrose sensitivity test) were trained[21]. The sucrose solution (50% w/v) was used as an unconditioned stimulus (US), while citral (2% in acetone) was used as a conditioned stimulus (CS). US was presented through a glass capillary, while CS was presented through the odour cartridges prepared using hypodermal syringes. A 20 ml capacity syringe was found sufficient to provide a continuous puff of CS for 5 sec (ref. 20). A piece of filter paper (15 mm ø) containing 10 μl of CS was fixed on the rubber septa of the plunger with a stapler pin. The plunger was inserted back into the plastic barrel of the syringe and pulled back.

The conditioning consisted of six association trials, i.e. associating CS with a reward or US. CS was presented by targeting the antennae of the bee continually for 5 sec by pushing 20 ml of air out of an odour cartridge. This CS was coupled with US after 3 sec by touching the distal flagella of the antenna with sucrose solution. The bee was also allowed to lick the solution. An inter-trial interval of 5 min was maintained. To test the effect of thiamethoxam on the long-term memory (after 24 h) of the bees, the same trained bees were maintained in an incubator. The study was repeated three times.

Testing the memory

Short-term and long-term memories were assessed at 2 and 24 h after training[21]. These were examined through retrieval tests by exposing the trained, treated bees to a single puff of CS only, applied through an odour cartridge. The data on the number of bees showing response were recorded in a binary system, i.e. 1 for bees showing the positive response and 0 for a negative response. The per cent PER response was calculated for various sets of bees, determined on the basis of levels of consumption of thiamethoxam.

Statistical analysis

Data on sucrose sensitivity and PER were subjected to statistical analysis using analysis of variance (ANOVA) in a completely randomized design. The means and standard errors were calculated and the means were compared using least significant difference (LSD) at a 5% level of significance.

Results and discussion

The uptake of various concentrations of thiamethoxam resulted in the consumption of active ingredients ranging from

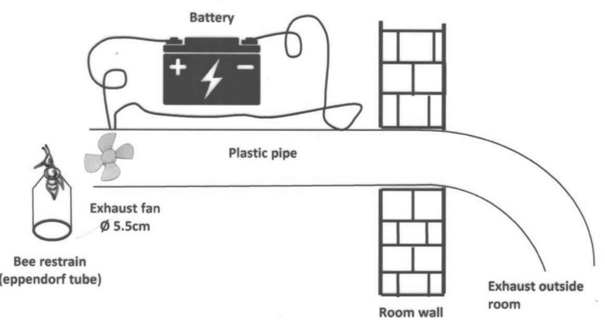

Figure 1. Diagram (not to the scale) of a temporary exhaust system for conducting experiments on PER.

Table 1. Response of *Apis mellifera* forager bees to sucrose solution following feeding on various concentrations of thiamethoxam

Consumption (ng bee^{-1}) of thiamethoxam (technical grade: purity 99.9%)	Per cent bees showing response to various concentrations (%) of sucrose solution (n = 30)									
	0.1#	0.3	1.0	3.0	10.0	20.0	30.0	40.0	50.0	Mean
5.76	0.0 ± 0.0* (0.0)	0.0 ± 0.0 (0.0)	0.0 ± 0.0 (0.0)	0.0 ± 0.0 (0.0)	36.7 ± 1.8 (37.2)	56.7 ± 0.9 (48.8)	70.0 ± 1.2 (56.8)	83.3 ± 0.9 (65.9)	96.7 ± 1.9 (80.2)	38.2 ± 13.3 (32.1)
1.62	0.0 ± 0.0 (0.0)	0.0 ± 0.0 (0.0)	6.7 ± 0.3 (14.9)	13.3 ± 0.9 (21.4)	40.0 ± 0.0 (39.2)	63.3 ± 1.9 (52.9)	76.7 ± 0.9 (61.1)	86.7 ± 0.7 (68.6)	96.7 ± 1.5 (80.2)	42.6 ± 13.1 (37.6)
0.93	0.0 ± 0.0 (0.0)	3.3 ± 0.3 (10.5)	6.7 ± 0.3 (14.9)	16.7 ± 0.3 (24.1)	46.7 ± 0.7 (42.5)	66.7 ± 0.7 (54.7)	80.0 ± 1.2 (63.4)	86.7 ± 0.3 (68.6)	96.7 ± 0.9 (79.6)	44.7 ± 12.9 (39.8)
0.64	6.7 ± 0.3 (14.9)	6.7 ± 0.3 (14.9)	10.0 ± 1.7 (18.3)	20.0 ± 1.2 (26.5)	53.3 ± 0.7 (46.9)	70.0 ± 1.2 (56.8)	83.3 ± 1.2 (65.9)	90.0 ± 1.2 (71.6)	96.7 ± 1.2 (79.9)	48.5 ± 12.7 (44.0)
0.32	6.7 ± 0.9 (14.9)	10.0 ± 1.0 (18.4)	13.3 ± 0.9 (21.4)	23.3 ± 1.7 (28.8)	60.0 ± 3.0 (50.8)	76.7 ± 0.9 (61.1)	86.7 ± 0.7 (68.6)	93.3 ± 1.2 (75.0)	100.0 ± 0.0 (89.9)	52.2 ± 12.9 (47.7)
0.16	0.0 ± 0.0 (0.0)	13.3 ± 0.7 (21.36)	16.7 ± 0.3 (24.1)	23.3 ± 1.8 (28.8)	60.0 ± 1.0 (50.8)	76.7 ± 2.4 (61.1)	86.7 ± 0.9 (68.6)	93.3 ± 0.3 (75.0)	100.0 ± 0.0 (89.9)	52.2 ± 12.9 (46.6)
0.06	6.7 ± 0.3 (14.9)	13.3 ± 1.2 (21.4)	16.7 ± 0.7 (24.1)	23.3 ± 0.3 (28.8)	60.0 ± 0.6 (50.7)	76.7 ± 1.3 (61.1)	86.7 ± 0.9 (68.6)	93.3 ± 0.3 (75.0)	100.0 ± 0.0 (89.9)	52.9 ± 12.6 (48.3)
0.03	6.7 ± 0.7 (14.9)	13.3 ± 1.2 (21.4)	16.7 ± 0.3 (24.1)	23.3 ± 2.7 (28.8)	60.0 ± 1.3 (50.7)	76.7 ± 0.7 (61.1)	86.7 ± 0.9 (68.6)	93.3 ± 1.2 (75.0)	100.0 ± 0.0 (89.9)	52.9 ± 12.6 (48.3)
Control (50% sucrose solution)	6.7 ± 0.3 (14.9)	13.3 ± 1.3 (21.4)	16.7 ± 0.9 (24.1)	23.3 ± 0.9 (28.8)	60.0 ± 2.0 (50.7)	76.7 ± 0.9 (61.1)	86.7 ± 1.2 (68.6)	96.7 ± 1.2 (79.9)	100.0 ± 0.0 (89.9)	53.3 ± 12.8 (48.8)
Mean	3.7 ± 1.2 (8.3)	8.1 ± 1.9 (14.4)	11.5 ± 2.0 (18.4)	18.5 ± 2.6 (24.0)	52.9 ± 3.2 (46.6)	71.1 ± 2.5 (57.6)	82.6 ± 1.9 (65.6)	90.7 ± 1.5 (75.8)	98.5 ± 0.6 (85.5)	

LSD (p = 0.05); A (sucrose concentration) = 0.9; B (thiamethoxam consumption) = 0.9; $A \times B$ = 2.6.
*Mean of three sets of bees having 10 individuals each (mean ± SEm); #Per cent sucrose concentration in distilled water.
Figures in parentheses are the means of arcsine $\sqrt{\text{percentage}}$ transformation.

Table 2. Proboscis extension reflex (PER) elicited by *A. mellifera* after feeding on various concentrations of thiamethoxam

Consumption (ng bee^{-1}) of thiamethoxam (technical grade: purity 99.9%)	*Mean per cent *A. mellifera* bees (n = 30) bees eliciting positive PER to 50% sucrose solution		
	After 2 h of treatment	After 24 h of treatment	Mean
0.64	13.3 ± 0.0 (21.4)	0.0 ± 0.0 (0.0)	6.7 ± 6.7 (10.7)
0.32	28.9 ± 2.2 (32.5)	13.3 ± 0.0 (21.4)	21.1 ± 7.8 (26.9)
0.16	42.2 ± 2.2 (40.5)	28.9 ± 2.2 (32.5)	35.66 ± 6.7 (36.5)
0.06	57.8 ± 2.2 (49.5)	42.2 ± 2.2 (40.5)	50.0 ± 7.8 (44.9)
0.03	75.6 ± 2.2 (60.4)	62.2 ± 2.2 (52.1)	68.9 ± 6.7 (56.2)
Control	82.2 ± 2.2 (65.1)	68.9 ± 2.2 (56.1)	75.6 ± 6.7 (60.6)
Mean	50.00 ± 11.0 (44.9)	35.9 ± 11.1 (33.8)	

*Mean of three sets of bees having 10 individuals each (mean ± SEm).
Figures in parentheses are the means of arcsine $\sqrt{\text{percentage}}$ transformation.
LSD (p = 0.05); A (time) = 1.1; B (thiamethoxam consumption) = 1.9; $A \times B$ = 2.6.

5.76 to 0.03 ng bee^{-1}, i.e. LD_{50} and LD_{50}/192 respectively (Table 1). The thiamethoxam concentrations resulting in a consumption range of as low as LD_{50}/9 and lower, i.e. 0.64–0.03 ng bee^{-1} were selected as sub-lethal, the doses which do not have statistically significant mortality than control, for conducting the experiment (Table 2). Higher doses of thiamethoxam, e.g. 1 ng bee^{-1}, i.e. LD_{50}/5 and lower, were considered sub-lethal for conducting the experiment[22]. The variation can be attributed to a difference in the administration, i.e. chronic feeding, while in the present study, the toxic dose was provisioned only once for 4 h.

Sucrose sensitivity

The bees that consumed a thiamethoxam dose of 5.76 ng bee^{-1} (equivalent to the median lethal dose) did not exhibit PER up to 3% sucrose solution. However, at this dose PER was exhibited at 10% concentration (36.7 ± 1.8%), which increased to 96.7 ± 1.9% at 50% sucrose solution. At lower thiamethoxam consumption (0.64 ng bee^{-1}; 1/9th of LD_{50}), a low proportion of bees (6.7 ± 0.3%) responded to the lowest test concentration of sucrose, i.e. 0.1%, though this response increased with the increase in sucrose concentration and more than 90% of the bees were registered responding to 40% sucrose solution. The sensitivity evaluated at the lowest thiamethoxam consumption, i.e. 0.03 ng bee^{-1}, revealed more than 80% bees responding to 40% sucrose solution. The bees that consumed thiamethoxam between 0.64 and 0.03 ng bee^{-1} responded to the lowest tested sucrose concentration, hence chosen for the PER study.

The dose 0.64 ng bee^{-1} of thiamethoxam was the discriminatory dose since at all sucrose concentrations, this was the lowest dose that resulted in a reduction in the response of the bees (Table 2). The dose of 0.32 ng bee^{-1} did not affect PER in the bees at any sucrose concentration. Further, the maximum discriminatory response was observed at 10% sucrose concentration, as the response variation between no response dose (5.76 ng bee^{-1}) and maximum response dose (0.32 ng bee^{-1}) was not only the maximum (23.3%), but between every two successive doses too (3.0% and 10.0%) the response variation was maximum. For higher sucrose concentrations, the test insecticide dose up to 5.76 ng bee^{-1} resulted in a lesser response effect. Even the untreated bees were less responsive (6.7–23.3%) at sucrose concentrations lower than 10%.

Overall, the sensitivity of bees to sucrose concentration decreased as their intake of thiamethoxam increased. Chronic oral exposure (11 days) of newly emerged honey bees to thiamethoxam (1 ng bee^{-1}) was registered to have reduced response to 3% and 10% sucrose concentration, while 0.1 ng bee^{-1} feeding of thiamethoxam did not affect the response[22].

Associative learning

Classical conditioning paradigm, an associative learning paradigm in which an animal learns to associate two unrelated stimuli[23], showed short-term memory (2 h after training) to be more affected compared to long-term memory (24 h after training). The highest and lowest thiamethoxam consumption doses showed PER values of 13.3% (0.64 ng bee^{-1}) and 75.6% (0.03 ng bee^{-1}) after 2 h respectively. In untreated control, 82.2 ± 2.2% of bees exhibited PER.

In assessing long-term memory, 68.9 ± 2.2% of the bees showed PER after the lowest consumption of thiamethoxam, while for the highest consumption, PER was zero. Since the consumption of thiamethoxam as low as 0.03 ng bee^{-1} resulted in PER value significantly lower than the untreated control, the no observed effect level (NOEL) will be <0.03 ng bee^{-1} for *A. mellifera* foragers. It was reported that bees upon feeding thiamethoxam (0.1 and 1 ng bee^{-1}) showed a slight to non-significant decrease in performance while learning and in retrieval tests[22].

Standard calibration curves under both testing conditions revealed the coefficient of determination as 0.851 and 0.832 between insecticide consumption quantities and PER percentage (Figure 2 *a* and *b*). Similar regression values

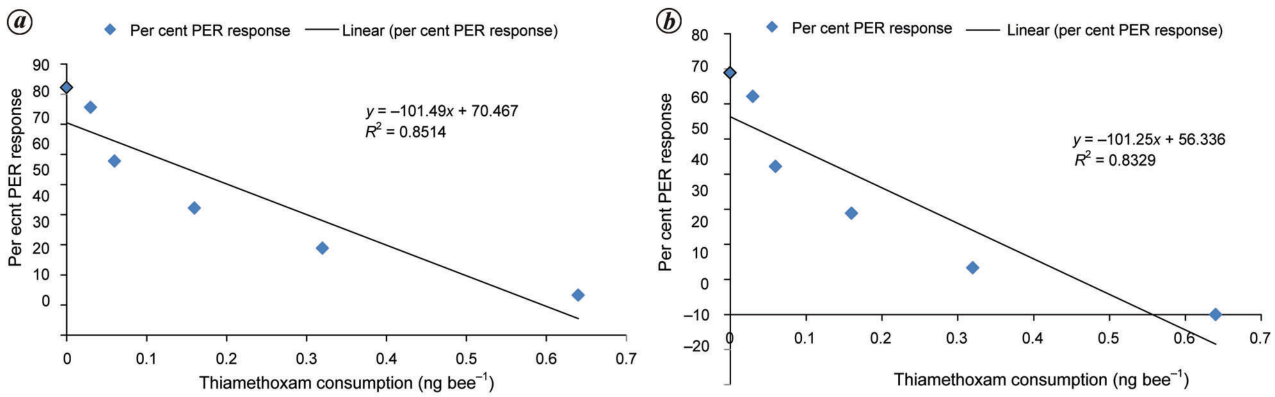

Figure 2. Calibration curve showing the per cent proboscis extension reflex (PER) at (***a***) 2 h and (***b***) 24 h after exposure to thiamethoxam.

($y = –101.4x + 70.46$ for short-term (2 h) memory and $y = –101.2x + 56.33$ for long-term (24 h) memory) imply that the influence of thiamethoxam-feeding persisted unfazed at least till 24 h. No difference among the treated and untreated bees with respect to sucrose sensitivity and memory formation upon topical application or oral feeding of thiamethoxam (0.1, 0.5 and 1 ng bee^{-1}) was recorded[24]. The insecticide was reported to have neither a direct-acting agonist nor an antagonist, but was converted into clothianidin[25]. These results explain the cause, in addition to acute mortality of foragers, for decrease in the number of active foragers in colonies placed in such an ecosystem where thiamethoxam has been applied. The effect on long-term memory formation proteins, i.e. protein kinase A (pka) and cAMP response element binding (creb) may have affected memory formation[26]. In addition, the effect on *CSP3* gene (chemosensory protein) and *Obp21* gene (odorant-binding proteins) reduced the chemosensory ability of such bees[27].

Conclusion

The present study shows the negative effect of sub-lethal doses of thiamethoxam on sucrose sensitivity of bees. Negative effects on long-term and short-term memories have also been registered. These influences would impact the overall colony development. Thus, beekeepers must take care of their apiary by maintaining contact with the farmers to avoid exposure of bees to thiamethoxam. The apiaries need to be shifted to safer places if the application of this insecticide is inevitable.

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