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

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ABSTRACT

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

Key Words: *Apis mellifera*, memory, proboscis extension reflex, thiamethoxam.

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INTRODUCTION

Bees are indispensible natural resources and are important in preserving the natural ecosystems[1,2]. Bees offer pollination services, and thus contribute towards ensuring plant reproduction and in sustaining food security. Commercial beekeeping offers a viable system through which under-privileged section of the society may also be benefitted for earning livelihood[3] .

Honey bee colonies comprise of thousands of workers, hundreds of drones and headed by a single queen. The primary responsibility of the workers is food gathering, for which they must

explore their surroundings. Hence, worker bee must have an excellent ability to learn and remember certain cues like colour, fragrance and shape of the flowers and the location.

Large scale honey bee colony loss has been reported worldwide. The syndrome has been named as Colony Collapse Disorder (CCD)[4,5]. . Extensive use of neonicotinoids , decline in bee forage acreage, environmental pollution, etc. are the major responsible factors[6]. As per Government of India report[7], Punjab was the third largest user of chemical pesticides after Maharashtra and Uttar Pradesh (5193 MT tech. grade during 2021-22; neonicotinoids 716.09 MT).

Neonicotinoid group of insecticides have activity against a wide spectrum of sucking insects and some Heteropterans, Coleopterans and Lepidopterans[8]. At a certain time, these chemicals occupied a global market share of more than 25 per cent and its 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 Central Insecticide Board and Registration Committee, Faridabad, India for successful management of aphids, infesting major bee floral crop i.e. mustard. It 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]. It acts as an agonist to the insect's nicotinic acetylcholine receptors (nAChR) predominantly abundant in the neuropil regions of insect brain[10] . In honey bee brain, two types of receptors- α-bungarotoxin (α-BGT)-sensitive receptor and the α-BGT-insensitive receptor[14] have been found responsible for olfactory response, learning and memory[15,16].

Olfactory memory plays an important role in bee behaviour which impacts the colony survival. Foragers learn through latent or associative learning. The former is little 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 a bee immediately before the reward - 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 and keeping that point in view, we have attempted to study 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. An insect net (diameter 25 cm) was used to capture the foragers at the hive entrance in the morning. These bees (n~90/hoarding cage) were maintained in incubator at 25 ± 2°C and 70% relative humidity. This way nine (1 cage for each concentration) wooden hoarding cages (14x10x18 cm) were maintained.

The acute median lethal dose (LD_{50} oral: 24h) of thiamethoxam was reported as 5.20 ng bee^{-1} [19]. Considering this, various concentrations of thiamethoxam in sucrose solution (50% w/v) yielding average consumption ranging from 0.03-5.76 ng bee^{-1} were provided *ad libitum* to bees in 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 have trophallaxis behaviour, therefore, each bee in a hoarding cage got 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 ºC for 3 min. Each bee was restrained in individual micro-centrifuge tube based harness[20]. There were 3 replications each containing 10 bees. Restrained bees were tested for their sensitivity to sucrose and water separately. 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 +ve response to sucrose and –ve 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 the PER. Thereafter, that bee was allowed to lick some quantity. An inter-trial interval of 03 minutes was maintained. The number of bees showing PER to a particular concentration of sucrose was recorded.

Olfactory learning

In order to evaluate the PER, a temporary exhaust system consisting of a small air-exhaust fan (5.5 cm ∅) was deployed (Plate 1). The individual restrained bees (a different set of bees as used in sucrose sensitivity test) were trained[21]. The sucrose solution (50% w/v) was used

as unconditioned stimulus (US) while the citral (2% in acetone) was used as conditioned stimulus (CS). The US was presented through a glass capillary while the CS was presented through the odour cartridges prepared by using hypodermal syringes. A 20 ml capacity syringe was found sufficient to provide a continuous puff of CS for 5 seconds[20]. A piece of filter paper (15 mm ∅) having applied with 10 µL of CS was fixed on the rubber septa of plunger with a stapler pin. The plunger was inserted back in the plastic barrel of the syringe and pulled back.

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

Testing the memory

Short term and long term memories were examined at 2 and 24 h after training[21]. These were tested through retrieval test by exposing the trained treated bees to a single puff of CS only, applied through odour cartridge. The data on the number of bees showing the response were recorded in a binary system i.e. 1 for bees showing the +ve response and 0 for –ve 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 Completely Randomized Design. The means and standard errors were calculated and the means compared using LSD at 5 per cent level of significance.

RESULTS AND DISCUSSION

The uptake of various concentrations of thiamethoxam resulted in consumption of active ingredient ranging from 5.76-0.03 ng bee^{-1} i.e. LD_{50} and LD_{50}/192, respectively (Table 1). The thiamethoxam concentrations resulting in 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 concentrations, the doses which do not have statistically significant mortality than control, for conducting the experiment. A little higher dose of thiamethoxam, 1 ng bee^{-1} i.e. LD_{50}/5 and lower were considered as sub-lethal dose for conducting experiment[22]. The variation can be attributed to difference in administration process i.e. chronic feeding while in the present study, toxic dose was provisioned only once for 4 h.

Sucrose sensitivity

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

144 thiamethoxam between 0.64 and 0.03 ng bee^{-1} responded to the lowest tested sucrose
145 concentration, which is why it was chosen for PER study.

146 The dose 0.64 ng bee^{-1} of thiamethoxam was discriminatory dose since at all sucrose
147 concentrations, this was the lowest dose that resulted in reduction in bees' response (Table 1).
148 The dose of 0.32 ng bee^{-1} did not affect the PER in bees at any of sucrose concentration. Further,
149 the maximum discriminatory response was observed at 10 per cent sucrose concentration as the
150 response variation between no response dose (5.76 ng bee^{-1}) and the maximum response dose
151 (0.32 ng bee^{-1}) was not only the maximum (23.3%), but between every two successive doses too
152 (3.0% and 10.0%), the response variation was the maximum. For higher sucrose concentrations,
153 the test insecticide's dose upto 5.76 ng bee^{-1} resulted in lesser response effect. Even the untreated
154 bees were less responsive (6.7-23.3%) at sucrose concentrations lower than 10 per cent.

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

159 **Associative learning**

160 Classical conditioning paradigm, an associative learning paradigm in which an animal
161 learns to associate two unrelated stimuli[23], showed the short term memory (2h after training) to
162 be more affected as compared to long term memory (24h after training). The highest and lowest
163 thiamethoxam consumption dose showed PER values as 13.3 per cent (0.64 ng bee^{-1}) and 75.6
164 per cent (0.03 ng bee^{-1}) after 2 h, respectively. In untreated control, 82.2$\pm$2.2 per cent bees
165 exhibited PER.

In assessing the long term memory, 68.9±2.2 per cent of the bees showed PER after the lowest consumption of thiamethoxam while that at the highest consumption, the PER was 0.0. Since the consumption of thiamethoxam as low as 0.03 ng bee^{-1} resulted in PER value significantly lower than 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 during learning and in retrieval tests[22].

Standard calibration curves (Fig. 1& 2) under both the testing conditions revealed coefficient of determination as 0.851 and 0.832 between insecticide consumption quantities and PER percentage. Similar regression values ((y = -101.4x + 70.46 for short term (2h) memory and y = -101.2x + 56.33 for long term (24h) memory) imply that the influence of thiamethoxam feeding persisted unfazed atleast till 24 h. No difference among the treated and untreated bees w.r.t. 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]. Thiamethoxam was reported to have neither a direct-acting agonist nor an antagonist but is converted into clothianidin[25]. These results explain the cause, in addition to acute mortality of foragers, for decrease 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 the memory formation[26]. In addition to this, effect on *CSP3*gene (Chemosensory protein) and *Obp21* gene (Odorant-binding proteins) do reduce chemosensory ability of such bees[27].

CONCLUSIONS

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

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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 concentrations)=(0.9) B (Thiamethoxam consumption)= (0.9) A x B (2.6)

***** Mean of three sets of bees having 10 individuals each (mean$\pm$S.E.$_m$); # Per cent sucrose concentration in distilled water
Figures in parentheses are the means of arc sine $\sqrt{\text{percentage}}$transformation

Table 2: Proboscis extension reflex (PER) elicited by *Apis mellifera* bees after feeding on various concentrations of thiamethoxam

Consumption (ng bee^{-1}) of thiamethoxam (Technical grade: purity 99.9%)	***Mean per cent *Apis mellifera* (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±S.E.$_m$)

Figure in parentheses are the means of arc sine$\sqrt{\text{percentage}}$ transformations

LSD (p=0.05): A (Time)= (1.1) B (Thiamethoxam consumption)= (1.9) A x B= (2.6)

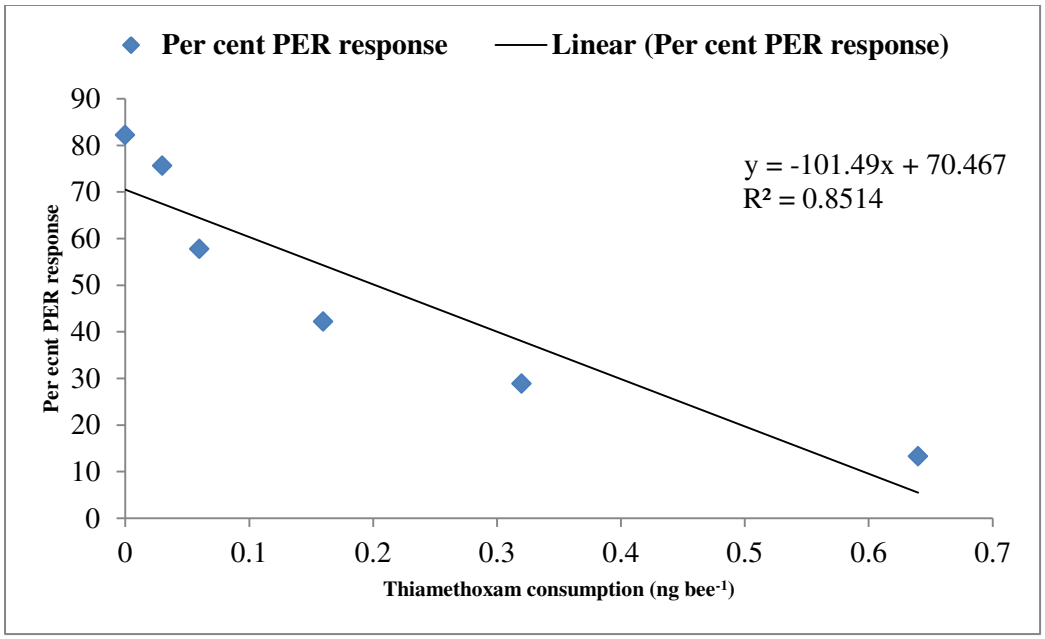

Fig 1. Calibration curve showing the per cent proboscis extension reflex at 2 h after exposure to thiamethoxam

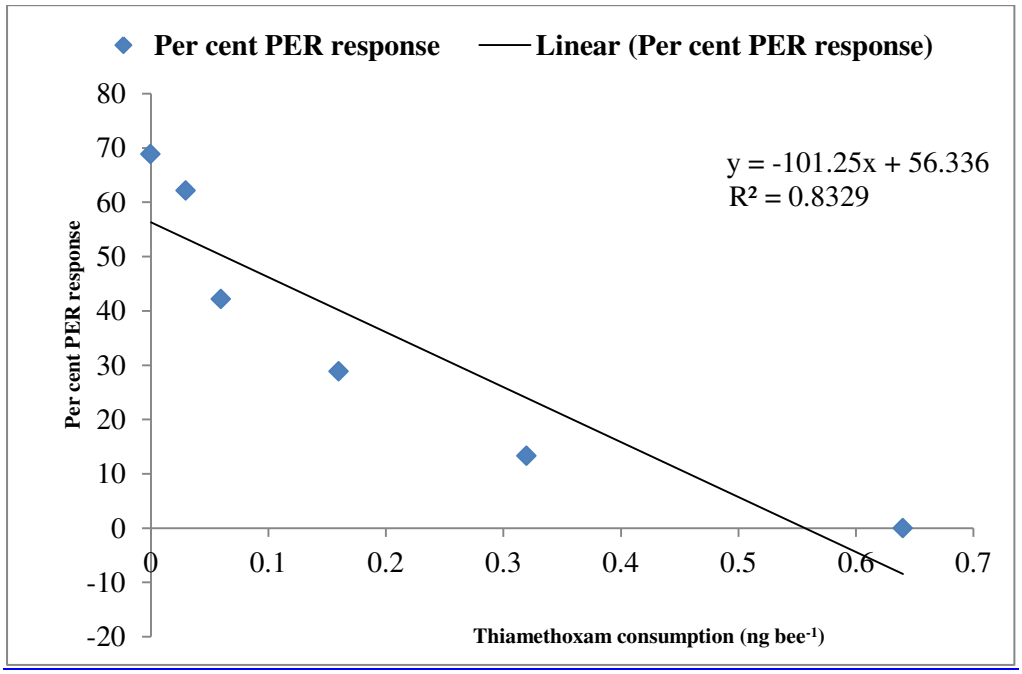

Fig 2. Calibration curve showing the per cent proboscis extension reflex at 24 h after exposure to thiamethoxam

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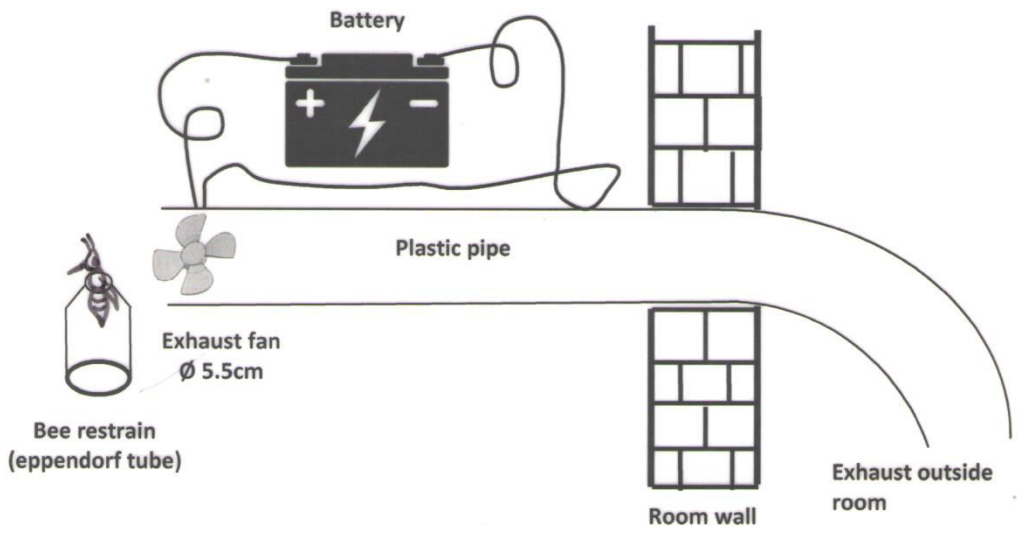

Plate 1: Diagram (not to the scale) of a temporary exhaust system for conducting experiment on Proboscis Extension Reflex