Fipronil affects cockroach behavior and olfactory memory

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Abstract

Fipronil (FPL), an insecticide belonging to the class of phenylpyrazoles, is associated with the widespread mortality of pollinator insects worldwide. Based on studies carried out on residual concentrations of FPL commonly found in the environment, in this work, we evaluated the sublethal effects of FPL on behavior and other neurophysiological parameters using the cockroach *Nauphoeta cinerea* as a biological model. Sublethal doses of FPL (0.1-0.001 µg/g) increased the time spent grooming and caused dose-dependent inhibition of exploratory activity, partial neuromuscular blockade *in vivo*, and irreversible negative cardiac chronotropism. FPL also disrupted learning and olfactory memory formation at all doses tested. These results provide the first evidence that short-term exposure to sublethal concentrations of FPL can significantly disrupt insect behavior and physiology, including olfactory memory. These findings have implications for current pesticide risk assessment and could be potentially useful in establishing a correlation with pesticide effects in other insects, such as honey bees.

Key words: Phenylpyrazoles; sublethal effects; entomotoxicity**,** neurolocomotor deficits, sensorial disturbance.

Introduction

The exponential growth of the human population in the post-war period required a major expansion in crop production and resulted in a massive increase in the use of pesticides applied to crops before or after harvest to protect the commodities from deterioration (Tudi et al. 2021). The use of pesticides has increased ~50-fold since 1950 and ~2.5 million tons (corresponding to US$15 billion) are currently used worldwide every year (Gro Intelligence 2018; Zaller 2020).

Many sprayed insecticides and herbicides reach non-target species, in addition to contaminating air, water and soil (Mali et al. 2020). Pesticides are also responsible for reducing the general biodiversity and pollinator insect populations (Wells 2007), degrading habitats by reducing plant pollination, and reducing insect resources for birds (Palmer et al. 2007), and threatening endangered species (Gill et al. 2014). In addition, pesticide use can damage neighboring agricultural activity by forcing pests towards nearby crops that are free of pesticides, thereby causing harm and potential losses in crop yield (Dyck et al. 2021). Insecticides cause both lethal and sublethal effects in invertebrates and some vertebrates (Rani et al. 2021). Hence, there is a need for a better understanding of the potential risks posed by sublethal doses of insecticides on non-target insect species.

Fipronil (FPL) is a broad-spectrum insecticide, belonging to the phenylpyrazole family, that acts primarily by blocking gamma-aminobutyric acid (GABA) receptors in insects (Gupta and Anadón 2018). FPL is a systemic pesticide that distributes amply throughout plant tissues and is toxic to any insect (and, potentially, other organisms) that feed upon the plant (Simon-Delso et al. 2015). The high to moderate solubility of FPL and its persistence and leaching potential in soil means that this compound can easily contaminate aqueous environments and affect non-target invertebrates such as honey bees and other important pollinators that drink contaminated water (Bonmatin et al. 2015). FPL has been found in honey bee hives in Southern Queensland (Australia), where it has caused a massive loss of colonies involving the death of >600,000 honey bees (Robinson and Sanders, 2021). This finding agrees with evidence that exposure to sublethal doses of FPL disrupts learning, memory and orientation in gregarious insects such as honey bees (Pisa et al., 2015). Navigation and foraging behavior are also impaired since FPL reduces the proportion of active bees in the hive and causes behavioral changes, such as a deficiency in visual learning, that reduce the efficacy of foraging flights (Pisa et al., 2015).

Whereas numerous studies have investigated the neurophysiological effects of FPL in insects (Durham et al. 2001; Narahashi et al. 2010; Kostromytska et al. 2011; Gols et al. 2020), the influence of sublethal doses of this pesticide, such as commonly encountered in the environment, on insects is still poorly understood. Several studies have examined the distribution and degradation of FPL in soil and other environments (Gunasekara et al., 2007). Degradation typically ranges from 111–350 days, and in soil, concentrations ranging from

0.000636–0.0248 μg/g have usually been found (Demcheck and Skrobialowski, 2003). In view of studies on bioaccumulation and residual doses of FPL in the environment (Tingle et al. 2003; Bhatti et al. 2019; Holder et al. 2018), in this work we investigated the ability of sublethal concentrations of FPL to disrupt olfactory memory and other behavioral parameters in *Nauphoeta cinerea* cockroaches. To our knowledge, this is the first study to examine the effect of FPL on these parameters in cockroaches.

Materials and methods

Reagents and solutions

All reagents and solutions were of high purity and were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Fipronil 800 WG® (FPL) was purchased from BASF Agri-Production SAS (Borborema, São Paulo, Brazil). FPL was dissolved in 0.9% NaCl (saline solution, SS) (stock solution) and diluted (working solutions) in SS at pre-set concentrations. All solutions used in the tests were prepared daily, before use, to ensure the stability of the compound to be tested.

Cockroaches

All experiments were carried out on adult *N. cinerea* cockroaches of both sexes (3-4 months after the adult molt), with an average weight of 500 ± 30 mg measured during early morning, that were reared in polyethylene plastic boxes (20 cm x 50 cm) in sawdust substrate in a controlled environment at ± 25 °C, and a 12-h light/dark cycle (on at 7:00 am and off at 7:00 pm), with water and food *ad libitum* (basic food composition: bone and meat meal, viscera meal, animal lipids, ground corn, wheat bran, sodium chloride, flavoring, antioxidant, folic acid, iron, copper sulfate, zinc sulfate, iodine, selenium, choline chloride, potassium, magnesium, niacin, pantothenic acid, vitamin E, vitamin B6, vitamin A, vitamin D3, vitamin K3, vitamin B1, vitamin B2 and vitamin B12). The animals were kept in the above conditions, in groups, until the beginning of each experiment. In the case of the animals subjected to memory assays, the selected insects were kept in the same conditions, but partially deprived of water and food for 15 days. The purpose of this deprivation period was to increase the animals' appeal to the solutions used in the olfactory memory tests. Thus, food and water were offered only once a day, in minimal amounts that were completely consumed, to ensure the necessary resting nutrition for the well-being of the animals.

Biological assays

The effect of FPL on the biological activity of *N. cinerea* cockroaches was assessed by injecting the insects with sublethal doses of the insecticide (0.1, 0.01 or 0.001 μg/g of animal weight) directly into the hemocoel, via the third abdominal segment, using a Hamilton syringe in a final volume of 10 μl, unless otherwise specified. Insects were injected with FPL alone or only with SS in control experiments conditions.

Assay for grooming behavior

The grooming behavior of the cockroaches was monitored based on the activity of the legs and antennae which was recorded for 30 min in a controlled environment at 24-25 ºC, essentially as described by Stürmer et al. (2014). The tests were carried out individually and for each treatment, including the control saline group, a n of 30 animals were used in multiple trials and never reused again in other protocols.

Assay for locomotory activity

The locomotory activity was assessed by recording the trajectory of the cockroaches as described by Leal et al. (2018). To accomplish this, four insects each time were randomly selected and placed individually in a white polystyrene box (25 cm in length × 15 cm in width × 7 cm in height). Their exploratory behavior was recorded during 10 min by a logitech® HD WEBCAM connected to a desktop computer (Dell, São Paulo, Brazil) that recorded and retrieved the videos for posterior analysis. Animal tracking was observed using the software idTracker (Stoelting, Denver, CO, USA) and the data were analyzed using the Insect Locomotion Tracking Program ILTP®, a freely available software developed by our research group that can be downloaded at http://sites.unipampa.edu.br/gomndi. With a script specially developed for this purpose, the following were analyzed: Distance Travelled (cm); Immobile Episodes (n) and Stopped Time (%). The control and treated groups were injected with SS or FPL 10 min before the beginning of data acquisition. The tests were carried out individually in groups of 4 animals, each one in an individual box alone and for each treatment including the control saline group a n of 30 animals were used and never reused again in other protocols.

Metathoracic coxal-adductor nerve-muscle preparation

The *in vivo* metathoracic coxal-adductor nerve-muscle preparation (MCANM) was mounted as described by Carrazoni et al. (2017). For this, the cockroaches were immobilized by chilling (5-7 min) and then fixed ventral side up on a stage covered with soft rubber. After fixation, one of the third metathoracic legs was tied to an isometric force transducer (AVS Instruments, São Carlos, SP, Brazil) and indirectly electrically stimulated via the nerve fiber (0.5 Hz/5 ms). Muscle twitches were recorded for 120 min using a data acquisition and analysis system (AQCAD and ANCAD, respectively; AVS Instruments). The tests were carried out individually and for each treatment including the control saline group, a n of 6 animals were used and never reused again in other protocols.

Cockroach semi-isolated heart preparation

The activity of FPL on *N. cinerea* heart rate was evaluated as described by Leal et al. (2020). Briefly, adult cockroaches were immobilized by chilling (5–7 min) and placed ventral side up on a dissection plate. The lateral margins of the abdomen were cut along each side, and the ventral abdominal body wall was pulled out to show the viscera. After moving the viscera carefully aside, the heart was exposed still contracting, while attached to the dorsal

body wall. The heart preparations were washed by bathing it in 200µl of SS at room temperature (21–24 °C). After 5 min of heartbeat stabilization, the treatments were delivered by exchanging the bathing solution. The beats/min average in the first 5 min was taken as a reference. Heartbeat frequency was monitored for 35 min, under a 1000x Digital Microscope (Shenzhen Huishixin Technology Co., China), connected to a desktop computer (Dell, São Paulo, Brazil) that recorded and retrieved the videos for posterior analysis.The responses to saline alone or FPL were monitored from the 5th to the 30th minute, and the preparation was washed several times from the 30th to the 35th minute to remove the saline or FPL and to verify the possible reversal of the toxic effects. The tests were carried out individually and for each treatment including the control saline group, a n of 3 animals were used and never reused again in other protocols.

Memory assay

The olfactory memory assays were done as described by Matsumoto and Mizunami (2000) with a few modifications. Before the assays, the cockroaches were deprived of food and water for 15 days. The tests were subsequently done in an apparatus (dimensions: length: 29 cm; width: 14.4cm; height: 6.8cm) developed specifically for olfactory tests (Fig. 1). Initially, the cockroaches were placed in a compartment (A) and allowed to adapt to the surroundings for 5 min. Subsequently, gate (B) was lifted, giving access to the arena (C) where a rotating cylinder (D) simultaneously offered two odor sources placed inside a well (E).

The odor sources used were vanilla essence and citronella diluted 1:3 in milli-Q water. Inside each well was placed a piece of cotton (1 cm x 1 cm) soaked with 500 µl of the odor sources alternately in the cylinders. Each cylinder was capped with a circle of absorbent paper. For the memory tests, 100 µl of 40% sucrose solution was placed on the absorbent paper of cylinders with citronella odor source and 100 µl of 40% NaCl solution on the paper of cylinders with vanilla odor source. This way the animals associated the repulsive odor with the reward (40% sucrose solution). The assay began as soon as the cockroach entered the chamber (C) and involved recording the time spent probing each odor source during a 10 min period, with the position of the wells being inverted every minute. The memory test was basically divided into two phases that consisted of **preference tests** and **olfactory memory tests**. The preference tests served as positive controls to confirm the hypothesis that cockroaches feel naturally attracted to vanilla odor and repelled by citronella odor. The tests were carried out individually and for each treatment including the control saline group, a n of 30 animals were used and never reused again in other protocols.

Statistical analysis

The results were expressed as the mean ± SEM. Statistical comparisons between two experimental groups were done using Student´s t-test (Figure 6A and 6B). The statistical analysis one-way ANOVA followed by Dunnett's was used to compare treatments only with the control group (Figure 2; Figure 3A; Figure 3B; Figure 3C and Figure 6C). For comparisons when

the Y-axis had more than one variable, two-way ANOVA followed by the Bonferroni post hoc test was used, with all groups being compared with the saline control (Figure 4 and Figure 5). A p value ≤ 0.05 indicated significance. All statistical analyses were done using Prism v.7.0 software (GraphPad, San Diego, CA, USA).

Results

Grooming activity

The exposure of *N. cinerea* to sublethal doses of FPL (0.1, 0.01 and 0.001µg/g animal) significantly increased the leg grooming time but did not markedly affect the antennal grooming time (Fig. 2).

Locomotor activity

FPL caused a dose-dependent locomotory deficit (Fig. 3D), with an overall reduction of 46% in the total distance traveled by the cockroaches after injection of the highest dose (0.1 µg/g) (Fig. 3A). All three doses markedly increased the number of immobile episodes (more than 3 seconds without movement) (with a ~200% increase in this parameter at the highest dose) (Fig. 3B), in addition to causing lethargy in the cockroaches (~80% increase in stopped time (time without movement during the total experiment) at the highest dose compared to the saline control) (Fig. 3C).

Effect of FPL on cockroach metathoracic coxal-adductor nerve-muscle preparations

In cockroach metathoracic coxal-adductor neuromuscular preparations, only the highest dose of the pesticide (0.1 µg/g) significantly affected the twitch tension and caused a maximal decrease of ~45% in the contractile response in 50 min of experiment. (Fig. 4A). Incubation with FPL did not affect the baseline tension of the preparations, i.e., there was no muscle contracture independent of electrical stimulation (Fig. 4B).

Effect of FPL on semi-isolated cockroach heart preparation

In cockroach semi-isolated heart preparations, FPL (0.001-0.1 µg/200 µl) caused irreversible, dose-dependent bradycardia at all doses. In control conditions, the heart rate stabilized before the addition of FL it was considered 100%. Thus, the maximal mean heart rates were reduced to 70.3 ± 10.6%, 75.8 ± 23.4% and 70.3 ± 28.8% after 30 min for doses of 0.001, 0.01 and 0.1 µg/200 µl, respectively (Fig. 5). Extensive washing of the preparations did not lead to a recovery of heart rate, indicating the irreversible nature of the cardiac effect.

Memory test

The preference test confirmed that *N. cinerea* cockroaches naturally prefer the odor of vanilla rather than citronella (Fig. 6A). However, when citronella was offered in a 40% sucrose solution, the olfactory memory test showed that the odor preference became similar between citronella and vanilla (Fig. 6B). Treatment with sublethal doses of FPL (0.001, 0.01 and 0.1

µg/g) markedly decreased the olfactory memory for all compounds offered at all doses tested (Fig. 6C).

Discussion

The findings described here indicate that sublethal doses of FPL adversely affect several physiological systems of *N. cinerea* cockroaches and cause important behavioral changes. Exposure to FPL increased the frequency of leg grooming but not that of antennal grooming. Grooming in insects is a fundamental activity associated with body cleanliness, courtship, social signaling, movement activity and bodily excitement (Spruijt et al., 1992; Zhukovskaya et al., 2013) and is modulated primarily by octopaminergic and dopaminergic neuronal pathways (Weisel-Eichler et al., 1999; Libersat and Pflueger, 2004; Leal et al., 2018, 2020). Changes in grooming behavior are related to fundamental factors associated with the survival and persistence of insects in the environment and can influence a species´ fertility and longevity (França et al., 2017). The alterations in grooming behavior observed here suggest a direct effect of FPL on the central nervous system that involves the modulation of octopaminergic and dopaminergic neurotransmission, possibly in a similar manner to other toxins that we have studied (Sturmer et al., 2014; Barreto et al., 2020).

The influence of FPL on exploratory activity was assessed by examining the changes in the locomotor behavior of cockroaches. Specifically, FPL reduced the distance walked by cockroaches and increased the number of immobility episodes and immobility time. FPL also affected the leg grooming behavior. These findings strongly suggest that sublethal doses of FPL adversely affects the activity of the central nervous system, leading to locomotory and exploratory deficits. Alterations in the locomotion and exploratory behavior of insects can increase their susceptibility to predators (Marliére et al., 2015) since locomotion is the greatest defensive strategy used to avoid and escape predators in the environment (Adedara et al., 2016). In bees, for example, locomotion, including flight, is responsible for maintaining colony homeostasis through behavioral mechanisms and pheromones (Bortolotti and Costa, 2014). Flight activity is also responsible for recognition of the environment, including biotic factors (pheromones, diseases, stress, etc.) and abiotic factors (rain, food, temperature, etc.) that are important for colony survival, and whose failure is associated with swarming and hive abandonment. Thus, behavior and locomotion are essential for insect maintenance and survival (Mizutani et al., 2021).

Although leg grooming behavior is mostly associated with octopaminergic neurotransmission, motivation to walk is dependent on dopamine, an important excitatory neurotransmitter (Stürmer et al., 2014; Borges et al., 2020). FPL is a well-known agonist of the main inhibitory neurotransmitter, gamma-aminobutyric acid (GABA) (Byrne, 2019) and sublethal doses of this pesticide can persist in the insect neuromuscular junction (Chapman, 2012). For this reason, we cannot exclude the possibility that the mechanism behind the FPL-mediated decrease in exploratory activity may include an inhibitory effect on peripheral neuronal activity.

Indeed, experiments with neuromuscular preparations showed that even very small sublethal doses of FPL caused a reduction in the contractile activity of *N. cinerea* leg muscle. The effects observed here in cockroaches resembled those seen with sublethal doses of FPL in the behavior of gregarious insects such as bees. In the case of bees, the remaining activity of sublethal FPL doses might affect motor activity, which is important for foraging and orientation during the “dance” used to indicate the location of resources to the colony (El Hassaniet al., 2005). Thus, a FPL-induced decrease in the contractile response would disturb the exploratory activity of those pollinators, causing disorientation and interference with the maintenance of honey bee colonies (Bovi et al., 2018)

The bradycardia caused by sublethal doses of FPL suggested the involvement of octopamine, the main neurotransmitter associated with the modulation of heart rate in insects (Hillyer, 2018). High concentrations of octopamine cause tachycardia, whereas low concentrations are associated with bradycardia (Papaefthimiou and Theophilidis, 2011). In contrast, the role of other neurotransmitters such as acetylcholine (ACh), upon insect heart rate is still controversial. Some studies have shown that ACh increases heart rate, while others suggest an opposite effect (Claros-Guzmán et al., 2020). Resolution of the relative contributions of these neurotransmitters to cardiac function will require the use of pharmacological interventions to modulate the octopaminergic and cholinergic pathways, as well as an assessment of acetylcholinesterase activity.

Another important finding of this work was that sublethal doses of FPL interfered with olfactory memory learning and formation in *N. cinerea* cockroaches. Olfaction is an important sense used by insects, such as cockroaches and bees, and is closely related to behavior and physiological homeostasis. In cockroaches, which are essentially nocturnal animals, communication occurs mainly through strategies based on smell (involving pheromones and odorant bacteria) and touch (Gullan and Cranston, 2014). The insect olfactory system is one of the most developed among animals and acts by converting a chemical into an electrical signal. Physiologically, the electrical impulses generated through excitation of the system by odorous molecules are conducted from the antennal nerve to the antennal lobe (glomeruli). The information captured through this pathway is directed to a region of the animal's brain known as the “mushroom body” that is associated with learning and memory processes (Modi et al., 2020). Adult cockroaches can associate aversive odors, such as mint, with a reward such as sucrose solution, and preferential odors such as vanilla, with a punishment such as hypertonic saline solution (Watanabe et al., 2003).

Studies have shown that the reward-associated processing is mainly related to octopaminergic neuromodulation, whereas aversion behavior is related to dopaminergic neuromodulation (Gauthier and Grünewald, 2012). Glutamatergic receptors are also involved in learning processes, memory formation and retrieval (Gauthier and Grünewald, 2012; Leboulle, 2012). In the mushroom body, third-order neurons (Kenyon cells, KCs), which play critical roles in olfactory learning (Menzel et al., 2006; Liu et al., 2012), respond only to specific odors with a few spikes and result in sparse odor coding (Gupta and Stopfer, 2014; Lin et al., 2014). One

mechanism that contributes to the formation of spatially and temporally sparse odor representations in populations of KCs is a widespread and broadly tuned GABAergic inhibition that feeds signals from KCs back to KCs (Szyszka et al., 2005; Papadopoulou et al., 2011).

The precise mechanism by which sublethal FPL disturbs olfactory memory learning and formation is unclear but may be associated with GABAergic interneurons and the blockage of chloride channels in GABAergic and glutamatergic neurons. Thus, if sublethal doses of FPL cause small disturbances in GABAergic interneurons, this could create hyperexcitation, thereby preventing feedback signals to KCs, leading to a destabilization of physiological processes involved in olfaction and associated memory formation. Future experiments involving site-specific intracellular recordings in mushroom bodies and other central structures of the *N. cinerea* central nervous system may help to elucidate the mechanism involved in the FPL-induced disturbances of olfactory memory.

Conclusions

The results of this work demonstrated that sublethal doses of fipronil cause striking alterations in the physiology and behavior of *N. cinerea* cockroaches that affect the cardiovascular system together with olfactory, exploratory and locomotory behavior. These alterations involve mostly central nervous system pathways, probably by affecting octopaminergic and GABAergic neurotransmission. Overall, these findings show that the effects of sublethal doses of FPL on insect behavior should not be neglected and they reinforce the need to examine the toxicological effects of sublethal doses of insecticides prior to their approval for general and agricultural use.

Author contributions

MER: Investigation; methodology; formal analysis; writing - original draft, review and editing. LC: Data curation; formal analysis; methodology; software. BTB: Data curation; formal analysis; methodology. SS: Formal analysis; methodology. YCB: Methodology; data curation. DRA: Formal analysis; writing - review and editing. VQ: Methodology; supervision. LV: Funding acquisition; resources, supervision. SH: Writing - review and editing. CADB: Conceptualization; funding acquisition; investigation; project administration; resources; supervision; validation; visualization; writing - original draft, review and editing.

Acknowledgements

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Finance code 001), the Universidade Federal do Pampa (UNIPAMPA), and grants from Edital de Apoio a Grupos de Pesquisa-2019, PRONEM/FAPERGS/CNPq 003/2011 and CAPES 063/2010 Toxinologia. SH was supported by a research fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnologia (CNPq, grant no 313273/2018-9). DRA is supported by the European Union's Horizon 2020 Research and Innovation Programme under the Marie Skłodowska-Curie Grant Agreement No 801133. We thank Dr João Batista Teixeira

da Rocha of the Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil, for providing some of the reagents used in this work.

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Figures

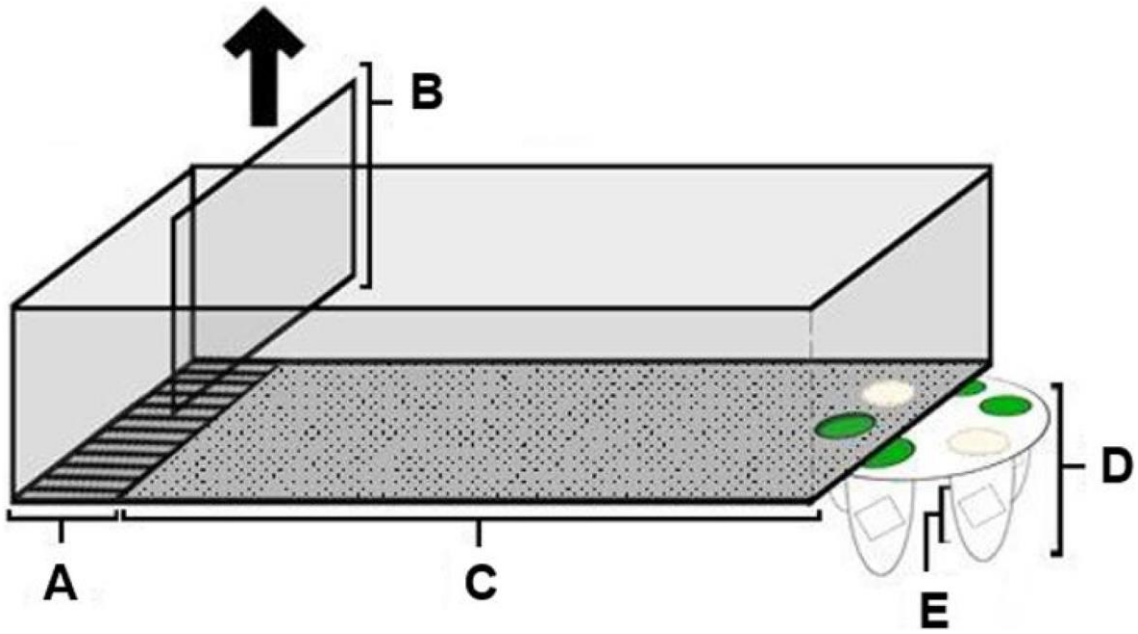


Fig. 1. Memory apparatus. (**A**) The area where the cockroaches were kept for 5 min before starting the experiment, **(B**) Gate, (**C**) The area where the experiment was done, (D) Rotating cylinder containing the odor sources, and (**E**) Odor sources.

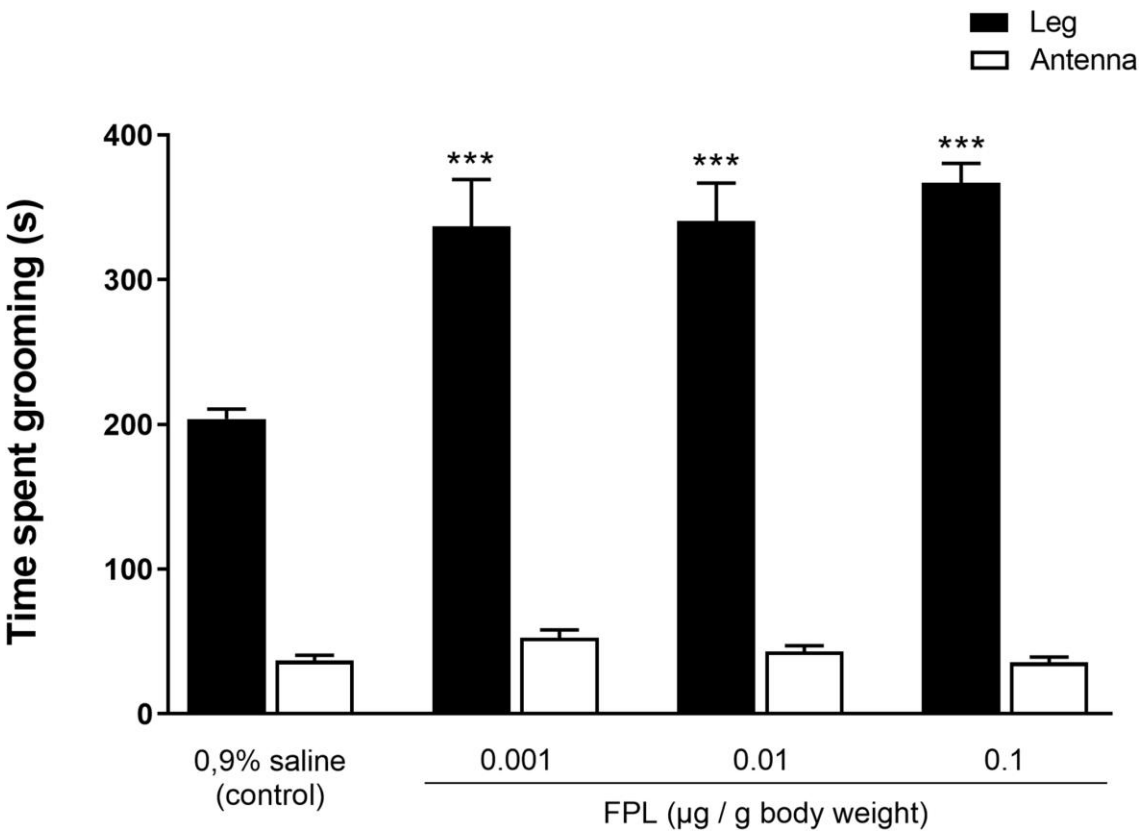


Fig. 2. The modulation of leg and antennal grooming activity in *N. cinerea* cockroaches exposed to sublethal doses of FPL (0.1, 0.01 and 0.001µg / animal weight). The compound was injected directly into the hemocoel, via the third abdominal segment, using a Hamilton syringe in a final volume of 10 µl. FPL significantly increased the leg grooming activity with minimal effect on antennal grooming activity. The columns represent the mean ± SEM (n = 30) of the total time spent grooming. *p ≤ 0.05 and ***p ≤ 0.001 compared to the corresponding saline control (one-way ANOVA followed by Dunnett's post-hoc test).

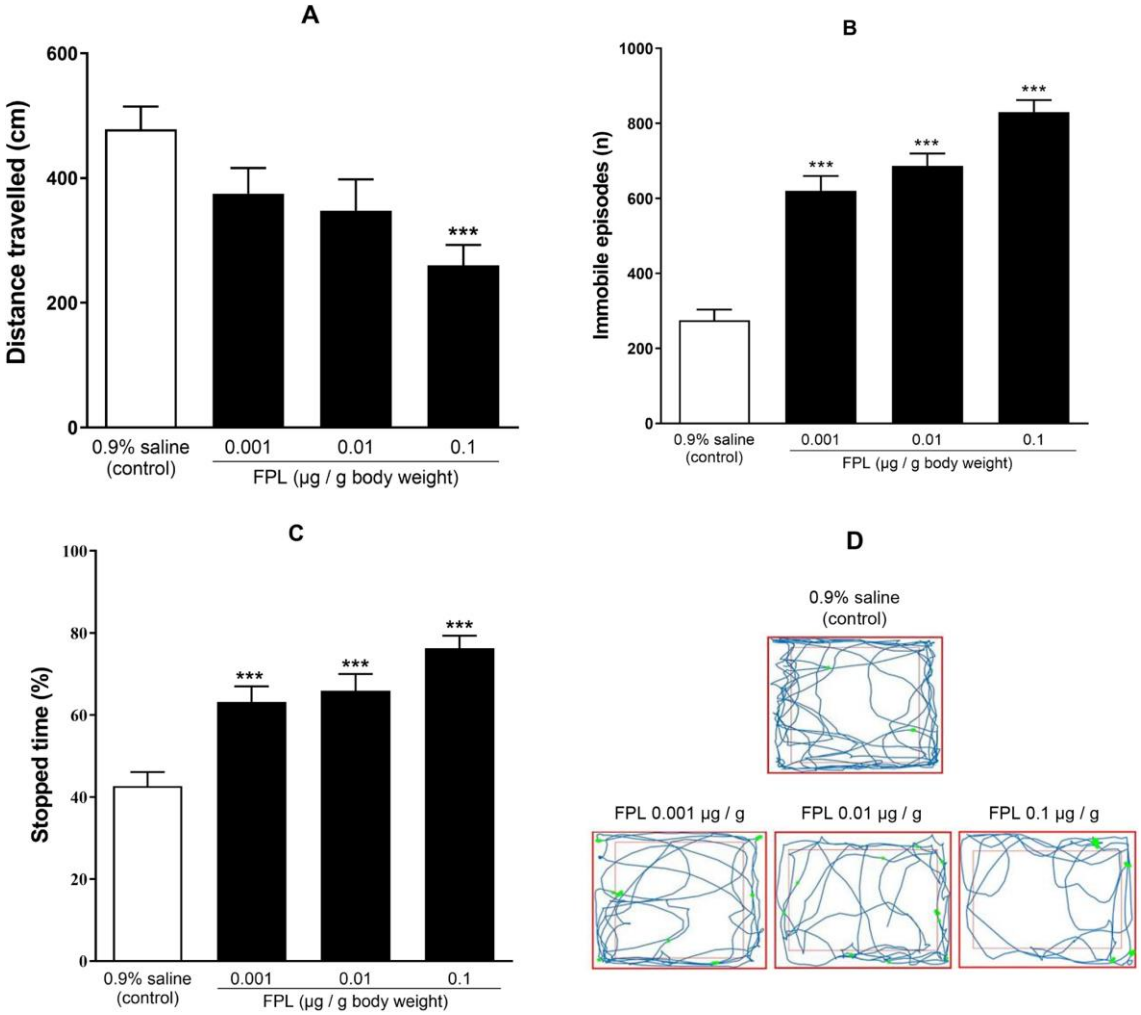
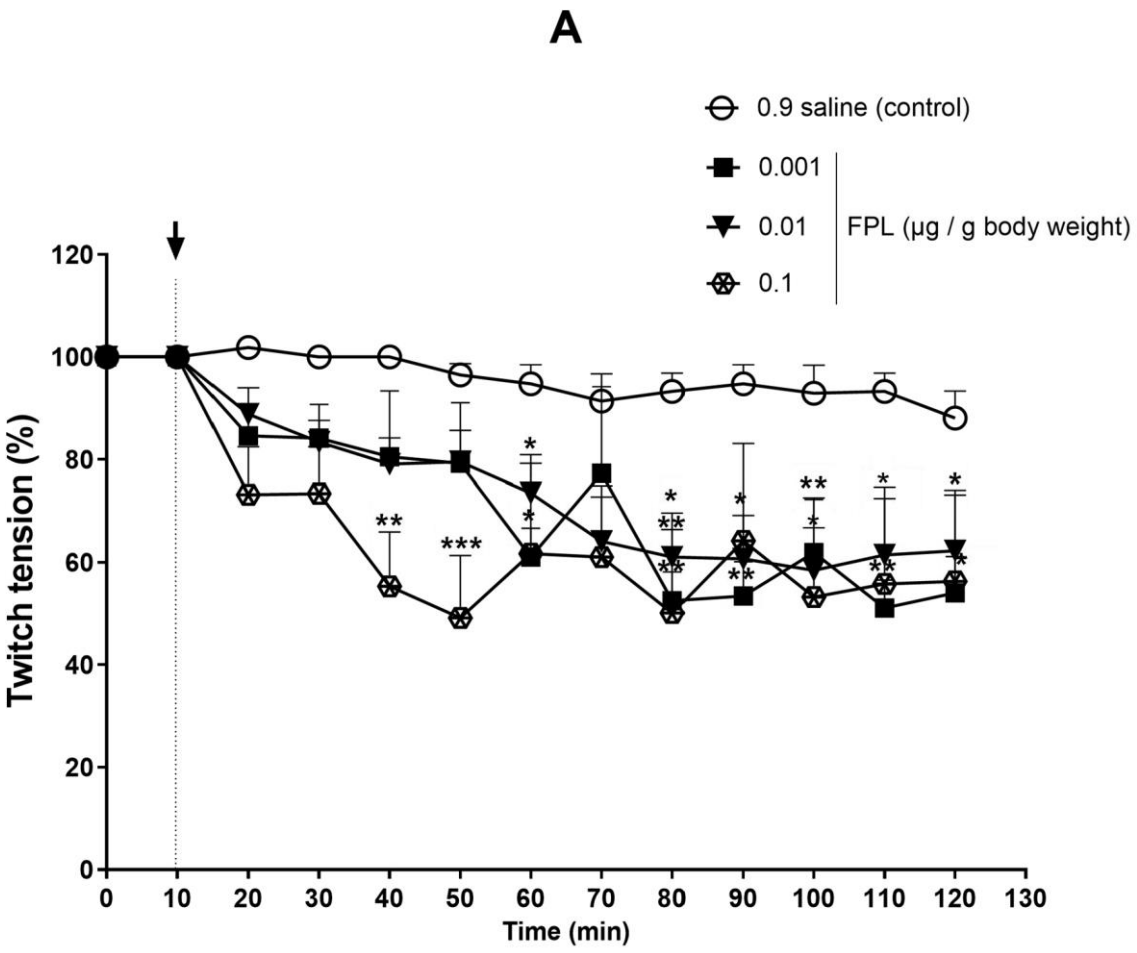


Fig. 3. Locomotory deficit induced by sublethal doses of FPL (0.1, 0.01 and 0.001 µg/animal weight) in *N. cinerea* cockroaches. The effect of FPL on locomotor activity was assessed based on: (**A**) The total distance traveled, (**B**) The number of immobile episodes, (**C**) The percentage of stopped time, (**D**) Analysis of the trajectory of the insect (blue line indicates the trajectory followed by the insect during the experiment and green dots indicate that the insect remained immobile for at least 3 seconds). The compound was injected directly into the hemocoel, via the third abdominal segment, using a Hamilton syringe in a final volume of 10 µl. The columns represent the mean ± SEM (n = 30). ***$p \leq 0.001$ (one-way ANOVA followed by Dunnett's post-hoc test.



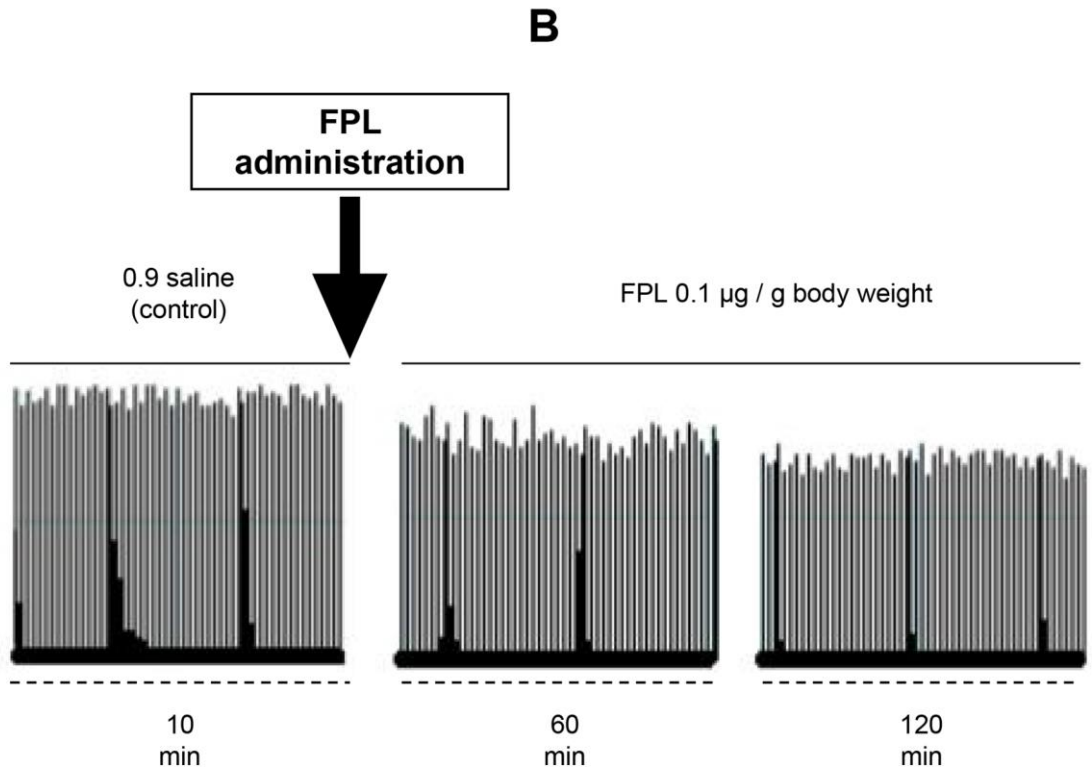


Fig. 4. Alterations in muscle twitch tension induced by sublethal doses of FPL in cockroach neuromuscular preparations. FPL (0.001, 0.01 and 0.1 mg/g) was added to the preparations and the changes in twitch tension were monitored for 2 h. (**A**) Changes in twitch tension (expressed as a percentage of the basal tension) throughout the experiment. (B) Representative traces of neuromuscular twitches. In (**A**) and (**B**), the arrows indicate the moment of FPL application. The points in (**A**) represent the mean ± SEM (n=6). *p≤0.05, **p≤0.01 and ***p≤0.001 compared to the corresponding intervals in the saline (control) group (two-way ANOVA followed by the Bonferroni post-hoc multiple comparisons test).

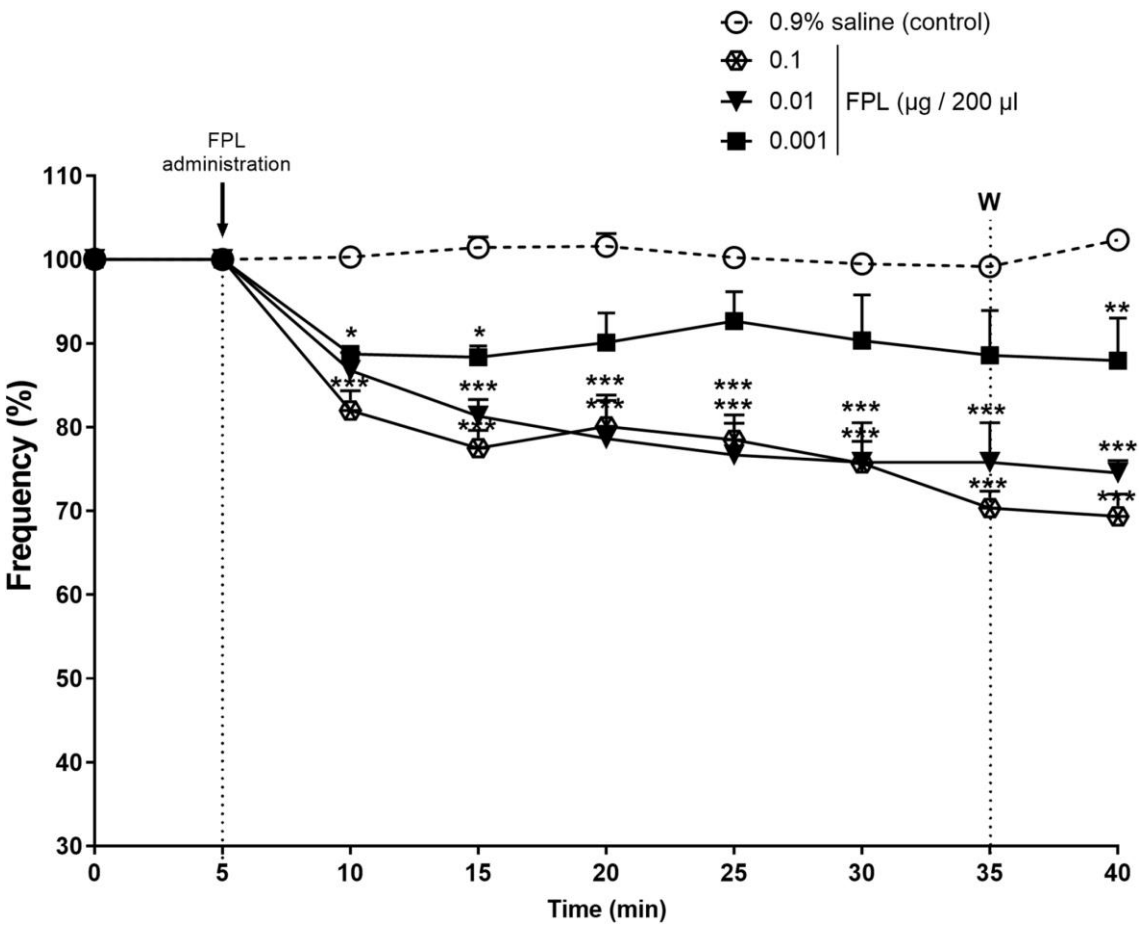


Fig. 5. Irreversible negative chronotropic effect of FPL on *N. cinerea* semi-isolated heart preparations. FPL (0.001, 0.01 and 0.1 mg/200 ml in 0.9% saline) was added to the preparations and the changes in heart rate were monitored for 30 min followed by extensive washing (**W**) that failed to reverse the bradycardia. The points represent the mean ± SEM (n=3). *p≤0.05, **p≤0.01 and ***p≤0.001 compared to the corresponding intervals in the saline (control) group (two-way ANOVA followed by the the Bonferroni *post-hoc* multiple comparisons test).

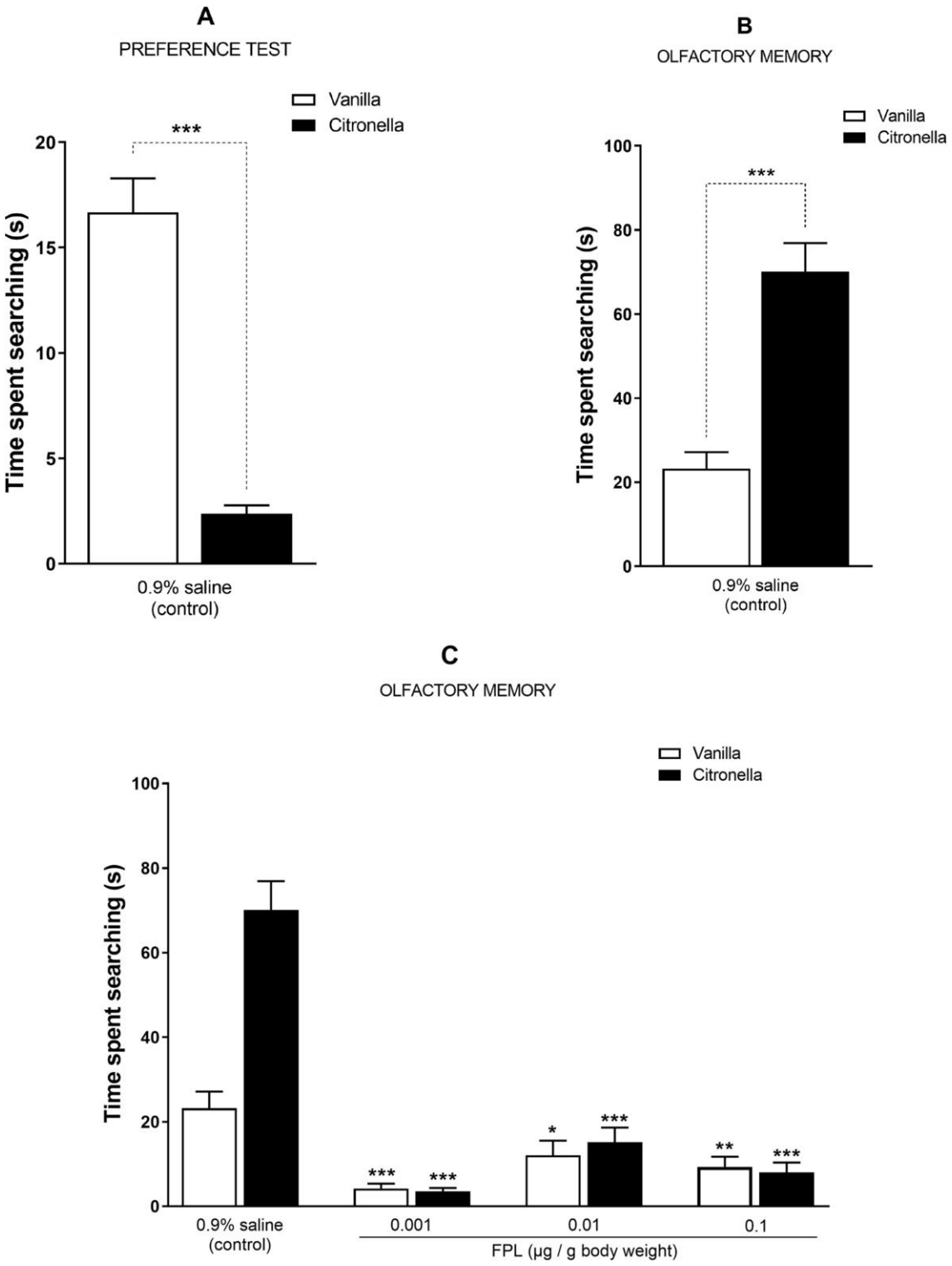


Fig. 6. Effect of sublethal doses of FPL on the olfactory memory of *N. cinerea*. (**A**) A preference test using 0.9% saline (control) showing the natural preference of the cockroaches for vanilla odor. The columns represent the mean ± SEM (n = 30) ***p≤0.001 (Student´s t-test). (**B**) The olfactory memory test with 0.9% saline (control) showing reversal of the insect's preference for citronella when the odor was associated with a reward (sucrose solution). The columns represent the mean ± SEM (n = 30) ***p≤0.001 (Student´s t-test). (**C**) Treatment with FPL (0.1,

0.01 and 0.001 µg/g) reduced the length of time (s) that cockroaches spent searching for odors associated with a reward (sucrose solution). The columns represent the mean ± SEM (n ≥ 30) *p≤0.05, **p≤0.01 and ***p≤0.001 compared to the corresponding saline control (one-way ANOVA followed by Dunnett´s *post-hoc* test).

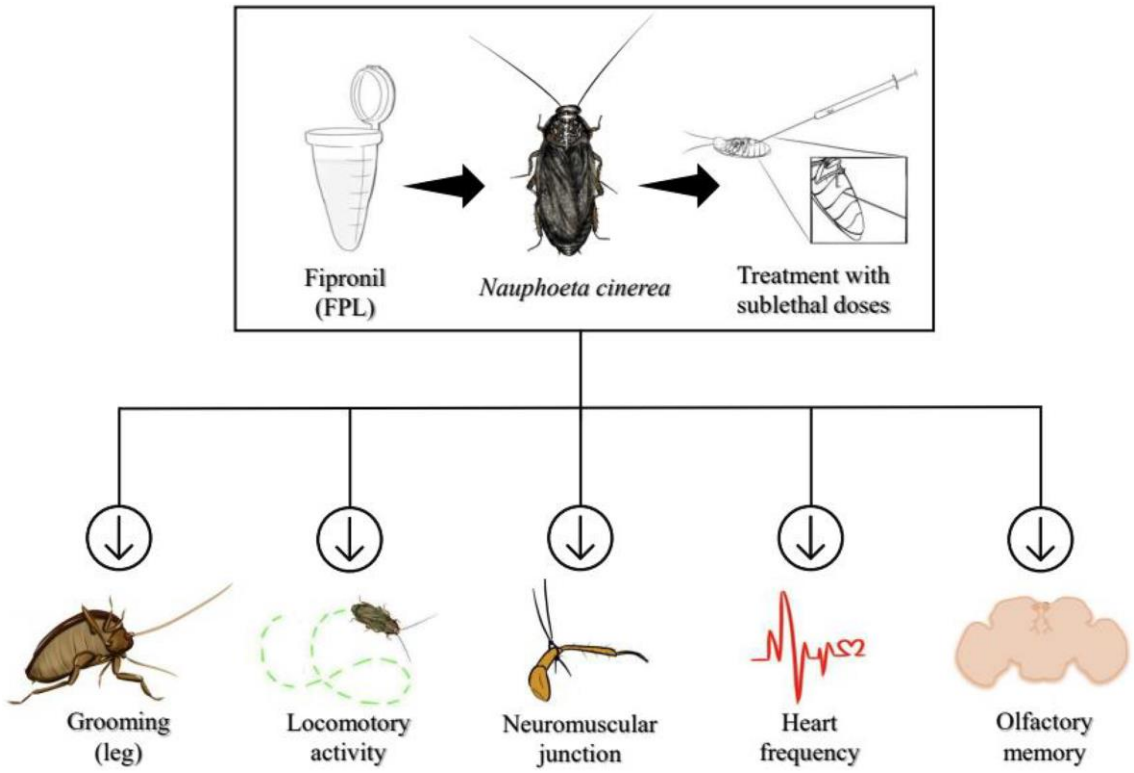


Fig. 7. Graphical Abstract. Sublethal doses of fipronil induce changes in the physiology and behavior of *N. cinerea* cockroaches through modulations in grooming and exploratory behaviors, neuromuscular twitch tension, cardiac rhythm, and olfactory memory. The figure, reinforces the need to evaluate the toxicological effects of sublethal doses of insecticides, before their approval for general use.

SUPLEMMENTARY DATA 1

A– Grooming

One-way ANOVA	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	484366	3	161455	F (3, 116) = 11.01	P<0.001
Residual (within columns)	1700429	116	14659		

Dunnett's multiple comparisons test	Mean Diff,	95% CI of diff	Significant?	Summary
Column A vs. Column C	-132.9	-207.3 to -58.52	Yes	***
Column A vs. Column E	-136.8	-211.2 to -62.42	Yes	***
Column A vs. Column G	-163.0	-237.4 to -88.62	Yes	***

Test details	Mean 1	Mean 2	Mean Diff	SE of Diff	n1	n2	q
Column A vs. Column C	203.9	336.8	-132.9	31.26	30	30	4.252
Column A vs. Column E	203.9	340.7	-136.8	31.26	30	30	4.377
Column A vs. Column G	203.9	366.9	-163.0	31.26	30	30	5.215

Data S1. Shows the details related to the statistics performed on Fig. 2 of the manuscript. **(A)**, one-way analysis of variance (ANOVA) performed for the grooming experiment: SS (sum of squares) - D.F. - MS (mean square) - F (DFn, DFd) and P value. Then, the table shows Dunnett's analysis as *post hoc* comparing *Nauphoeta cinerea* leg grooming in 0.9% saline control conditions (column A) x fipronil group 0.001µg/g animal (column C); 0.9% saline control (column A) x 0.01µg/g animal fipronil group (column E) and 0.9% saline control group (column A) x 0.1µg/g animal fipronil group (column G). Test details such as mean of each group, difference between means, n and q values are presented in the table.

SUPLEMMENTARY DATA 2

A– Distance travelled (cm)

One-way ANOVA	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	728550	3	242850	F (3, 116) = 4.89	P=0.0031
Residual (within columns)	5759185	116	49648		

Dunnett's multiple comparisons test	Mean Diff	95% CI of diff	Significant?	Summary
Column A vs. 0.001	103	-33.8 to 240	No	ns
Column A vs. 0.01	131	-6.36 to 268	No	ns
Column A vs. 0.1	218	81.5 to 355	Yes	***

Test details	Mean 1	Mean 2	Mean Diff	SE of diff	n1	n2	q
Column A vs. 0.001	478	375	103	57.5	30	30	1.79
Column A vs. 0.01	478	348	131	57.5	30	30	2.27
Column A vs. 0.1	478	260	218	57.5	30	30	3.80

B- Immobile episodes (n)

One-way ANOVA	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	4996791	3	1665597	F (3, 116) = 50.5	P<0.001
Residual (within columns)	3828826	116	33007		

Dunnett's multiple comparisons test	Mean Diff	95% CI of diff	Significant?	Summary
Column A vs. 0.001	-345	-457 to -234	Yes	***
Column A vs. 0.01	-412	-524 to -300	Yes	***
Column A vs. 0.1	-555	-667 to -444	Yes	***

Test details	Mean 1	Mean 2	Mean Diff	SE of Diff	n1	n2	q
Column A vs. 0.001	275	620	-345	46.9	30	30	7.36
Column A vs. 0.01	275	687	-412	46.9	30	30	8.78
Column A vs. 0.1	275	830	-555	46.9	30	30	11.8

C- Stopped time (%)

One-way ANOVA	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	17813	3	5938	F (3, 116) = 15.4	P<0.001
Residual (within columns)	44604	116	385		

Dunnett's multiple comparisons test	Mean Diff	95% CI of Diff	Significant?	Summary
Column A vs. 0.001	-20.5	-32.6 to -8.48	Yes	***
Column A vs. 0.01	-23.2	-35.3 to -11.2	Yes	***
Column A vs. 0.1	-33.6	-45.7 to -21.5	Yes	***

Test details	Mean 1	Mean 2	Mean Diff	SE of Diff	n1	n2	q
Column A vs. 0.001	42.7	63.2	-20.5	5.06	30	30	4.05
Column A vs. 0.01	42.7	65.9	-23.2	5.06	30	30	4.58
Column A vs. 0.1	42.7	76.3	-33.6	5.06	30	30	6.64

Data S2. Shows the details related to the statistics performed on Fig. 3 of the manuscript. Statistical analysis on exploratory behavior. In (**A**), one-way ANOVA statistical analysis for Distance travelled (cm) in the locomotion test: SS (sum of squares) - D.F. - MS (mean square) - F (DFn, DFd) and P value. Then, the Dunnett analysis as *post hoc* demonstrating the comparison between the 0.9% saline control group (column A) x fipronil in the doses (0.001; 0.01 and 0.1µg/g animal). Test details such as mean of each group, difference between means, n and q values are also presented in the table. In (**B**), one-way ANOVA statistical analysis for Immobile episodes (n) in the locomotion test: SS (sum of squares) - D.F. - MS (mean square) - F (DFn, DFd) and P value. Then, the Dunnett analysis as post hoc demonstrating the comparison between the 0.9% saline control group (column A) x fipronil in the doses (0.001; 0.01 and 0.1µg/g animal). Test details such as mean of each group, difference between means, n and q value are presented in the table. In (**C**) one-way ANOVA statistical analysis for Stopped time (%) in the locomotion test: SS (sum of squares) -D.F. - MS (mean square) - F (DFn, DFd) and P value. Then, the Dunnett analysis as *post hoc* demonstrating the comparison between the 0.9% saline control group (column A) x fipronil in the doses (0.001; 0.01 and 0.1µg/g animal). Test details such as mean of each group, difference between means, n and q values are also presented in the table.

SUPLEMMENTARY DATA 3

A– Twitch tension (%)

Two-way ANOVA	Ordinary
Alpha	0.05

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	6.63	0.72	ns	No
Row Factor	19.4	<0.001	***	Yes
Column Factor	17.2	<0.001	***	Yes

Two-way ANOVA	SS	DF	MS	F (DFn, DFd)	P value
Interaction	15426	36	428	F (36, 260) = 0.844	P=0.72
Row Factor	45164	12	3764	F (12, 260) = 7.42	P<0.001
Column Factor	40009	3	13336	F (3, 260) = 26.3	P<0.001
Residual	131942	260	507		

Bonferroni test details	Mean 1	Mean 2	Mean Diff	SE of Diff	N1	N2	t
Row 1							
Control vs. FPL 0,1µg/g	100	100	0.00	13.0	6	6	0.00
Control vs. FPL 0,01µg/g	100	100	0.00	13.0	6	6	0.00
Control vs. FPL 0,001µg/g	100	100	0.00	13.0	6	6	0.00
Row 2							
Control vs. FPL 0,1µg/g	100	100	0.00	13.0	6	6	0.00
Control vs. FPL 0,01µg/g	100	100	0.00	13.0	6	6	0.00
Control vs. FPL 0,001µg/g	100	100	0.00	13.0	6	6	0.00
Row 3							
Control vs. FPL 0,1µg/g	102	73.2	28.7	13.0	6	6	2.20
Control vs. FPL 0,01µg/g	102	88.9	13.0	13.0	6	6	0.999
Control vs. FPL 0,001µg/g	102	84.6	17.2	13.0	6	6	1.33
Row 4							
Control vs. FPL 0,1µg/g	100	73.4	26.6	13.0	6	6	2.05
Control vs. FPL 0,01µg/g	100	83.4	16.6	13.0	6	6	1.28
Control vs. FPL 0,001µg/g	100	84.2	15.8	13.0	6	6	1.22
Row 5							
Control vs. FPL 0,1µg/g	100	55.3	44.7	13.0	6	6	3.44
Control vs. FPL 0,01µg/g	100	79.1	20.9	13.0	6	6	1.61
Control vs. FPL 0,001µg/g	100	80.5	19.5	13.0	6	6	1.50

Row 6							
Control vs. FPL 0,1µg/g	96.5	49.2	47.3	13.0	6	6	3.64
Control vs. FPL 0,01µg/g	96.5	79.6	16.9	13.0	6	6	1.30
Control vs. FPL 0,001µg/g	96.5	79.3	17.2	13.0	6	6	1.32
Row 7							
Control vs. FPL 0,1µg/g	94.8	61.7	33.1	13.0	6	6	2.54
Control vs. FPL 0,01µg/g	94.8	73.5	21.3	13.0	6	6	1.64
Control vs. FPL 0,001µg/g	94.8	61.1	33.7	13.0	6	6	2.59
Row 8							
Control vs. FPL 0,1µg/g	91.4	61.1	30.4	13.0	6	6	2.33
Control vs. FPL 0,01µg/g	91.4	64.1	27.3	13.0	6	6	2.10
Control vs. FPL 0,001µg/g	91.4	77.3	14.1	13.0	6	6	1.08
Row 9							
Control vs. FPL 0,1µg/g	93.3	50.1	43.1	13.0	6	6	3.32
Control vs. FPL 0,01µg/g	93.3	61.0	32.2	13.0	6	6	2.48
Control vs. FPL 0,001µg/g	93.3	52.5	40.7	13.0	6	6	3.13
Row 10							
Control vs. FPL 0,1µg/g	94.8	64.2	30.5	13.0	6	6	2.35
Control vs. FPL 0,01µg/g	94.8	60.7	34.1	13.0	6	6	2.62
Control vs. FPL 0,001µg/g	94.8	53.5	41.3	13.0	6	6	3.18
Row 11							
Control vs. FPL 0,1µg/g	92.9	53.2	39.7	13.0	6	6	3.05
Control vs. FPL 0,01µg/g	92.9	58.5	34.5	13.0	6	6	2.65
Control vs. FPL 0,001µg/g	92.9	61.9	31.0	13.0	6	6	2.38
Row 12							
Control vs. FPL 0,1µg/g	93.3	55.8	37.5	13.0	6	6	2.88
Control vs. FPL 0,01µg/g	93.3	61.5	31.8	13.0	6	6	2.44
Control vs. FPL 0,001µg/g	93.3	51.1	42.2	13.0	6	6	3.24
Row 13							
Control vs. FPL 0,1µg/g	88.1	56.3	31.8	13.0	6	6	2.44
Control vs. FPL 0,01µg/g	88.1	62.3	25.8	13.0	6	6	1.98
Control vs. FPL 0,001µg/g	88.1	54.1	34.0	13.0	6	6	2.62

Data S3. Shows the details related to the statistics performed on Fig. 4 of the manuscript. In (**A**), statistical analysis upon cockroach leg twitch tension (%). Analysis of cockroach neuromuscular preparations during 2h recordings using two-way ANOVA, followed by the Bonferroni test as *post-hoc*. The table shows the data provided by the Prims 7 program. First, the data referring to the two-way ANOVA analysis (SS - D.F. - MS - F and P value). Following, the details of the Bonferroni test (Means - Differences between means - Value of n and t). Each row demonstrates the comparison between the 0.9% saline control group and the FPL at doses (0.001; 0.01 and 0.1µg/g animal) at every 10 min recording time.

SUPLEMMENTARY DATA 4

A-Frequency (%)

Two-way ANOVA	Ordinary
Alpha	0.05

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	16.1	<0.001	***	Yes
Row Factor	28.1	<0.001	***	Yes
Column Factor	44.7	<0.001	***	Yes

Two-way ANOVA	SS	DF	MS	F (DFn, DFd)	P value
Interaction	2228	24	92.8	F (24, 72) = 4.32	P<0.001
Row Factor	3903	8	488	F (8, 72) = 22.7	P<0.001
Column Factor	6197	3	2066	F (3, 72) = 96.1	P<0.001
Residual	1547	72	21.5		

Bonferroni test details	Mean 1	Mean 2	Mean Diff	SE of Diff	N1	N2	t
Row 1							
FPL 0.1g/g vs. Cont	100	100	-0.0504	3.78	3	3	0.0133
FPL 0.01g/g vs. Cont	100	100	-0.0504	3.78	3	3	0.0133
FPL 0.001g/g vs. Cont	100	100	-0.0504	3.78	3	3	0.0133
Row 2							
FPL 0.1g/g vs. Cont	100	100	0.00	3.78	3	3	0.00
FPL 0.01g/g vs. Cont	100	100	0.00	3.78	3	3	0.00
FPL 0.001g/g vs. Cont	100	100	0.00	3.78	3	3	0.00
Row 3							
FPL 0.1g/g vs. Cont	82.0	100	-18.3	3.78	3	3	4.84
FPL 0.01g/g vs. Cont	86.8	100	-13.5	3.78	3	3	3.58
FPL 0.001g/g vs. Cont	88.7	100	-11.6	3.78	3	3	3.06
Row 4							
FPL 0.1g/g vs. Cont	77.5	101	-24.0	3.78	3	3	6.33
FPL 0.01g/g vs. Cont	81.3	101	-20.1	3.78	3	3	5.32
FPL 0.001g/g vs. Cont	88.3	101	-13.1	3.78	3	3	3.47
Row 5							
FPL 0.1g/g vs. Cont	80.1	102	-21.5	3.78	3	3	5.68
FPL 0.01g/g vs. Cont	78.6	102	-23.0	3.78	3	3	6.07
FPL 0.001g/g vs. Cont	90.1	102	-11.5	3.78	3	3	3.03
Row 6							
FPL 0.1g/g vs. Cont	78.5	100	-21.8	3.78	3	3	5.75
FPL 0.01g/g vs. Cont	76.7	100	-23.6	3.78	3	3	6.22
FPL 0.001g/g vs. Cont	92.7	100	-7.58	3.78	3	3	2.00
Row 7							
FPL 0.1g/g vs. Cont	75.7	99.5	-23.8	3.78	3	3	6.29
FPL 0.01g/g vs. Cont	75.8	99.5	-23.7	3.78	3	3	6.25
FPL 0.001g/g vs. Cont	90.3	99.5	-9.14	3.78	3	3	2.41

Row 8							
FPL 0.1g/g vs. Cont	70.3	99.2	-28.8	3.78	3	3	7.62
FPL 0.01g/g vs. Cont	75.8	99.2	-23.4	3.78	3	3	6.17
FPL 0.001g/g vs. Cont	88.6	99.2	-10.6	3.78	3	3	2.79
Row 9							
FPL 0.1g/g vs. Cont	69.3	102	-33.0	3.78	3	3	8.72
FPL 0.01g/g vs. Cont	74.5	102	-27.8	3.78	3	3	7.35
FPL 0.001g/g vs. Cont	87.9	102	-14.4	3.78	3	3	3.[i.81

Data S4. Shows the details related to the statistics performed on Fig. 5 of the manuscript. Statistical analysis upon heart rate frequency (%). For analysis of semi-isolated cockroach heart preparations, two-way ANOVA was used, followed by the Bonferroni test as *post-hoc*. The table shows the data provided by the Prims 7 program. First, the data referring to the two-way ANOVA analysis (SS - DF -MS - F and P value). Next, the details of the Bonferroni test (Means - Differences between means - Value of n and t). Each line shows the comparison between the 0.9%saline solution control group and the FPL at doses (0.001; 0.01 and 0.1µg/g animal) at every 5 min of recording.

SUPLEMMENTARY DATA 5

A- Preference test

T test	
P value	<0.001
P value summary	***
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=8.56, df=58

How big is the difference?	
Mean of column A	16.7
Mean of column B	2.37
Difference between means (A - B) ± SEM	14.3 ± 1.67
95% confidence interval	11.0 to 17.6
R squared (eta squared)	0.558
F test to compare variances	
F, DFn, Dfd	15.5, 29, 29
P value	<0.001
P value summary	***
Significantly different (P < 0.05)?	Yes

B- Olfactory memory

T test	
P value	<0.001
P value summary	***
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=5.96, df=58

How big is the difference?	
Mean of column A	23.2
Mean of column B	70.1

Difference between means (A - B) ± SEM	-46.9 ± 7.87
95% confidence interval	-62.6 to -31.2
R squared (eta squared)	0.380

F test to compare variances	
F, DFn, Dfd	2.98, 29, 29
P value	0.004
P value summary	***
Significantly different (P < 0.05)?	Yes

C- Olfactory memory test

One-way ANOVA	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	5775	3	1925	F (3, 116) = 7.406	P<0.001
Residual (within columns)	30155	116	260.0		

Dunnett's multiple comparisons test	Mean Diff	95% CI of Diff	Significant?	Summary
Column A vs. Column C	18.97	9.057 to 28.88	Yes	***
Column A vs. Column E	11.13	1.224 to 21.04	Yes	*
Column A vs. Column G	13.83	3.924 to 23.74	Yes	**

Test details	Mean 1	Mean 2	Mean Diff	SE of Diff	n1	n2	q
Column A vs. Column C	23.20	4.233	18.97	4.163	30	30	4.556
Column A vs. Column E	23.20	12.07	11.13	4.163	30	30	2.674
Column A vs. Column G	23.20	9.367	13.83	4.163	30	30	3.323

One-way ANOVA	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	86339	3	28780	F (3, 116) = 59.52	P<0.001
Residual (within columns)	56088	116	483.5		

Dunnett's multiple comparisons test	Mean Diff	95%CI of Diff	Significant?	Summary
Column B vs. Column D	66.57	53.05 to 80.08	Yes	***
Column B vs. Column F	54.93	41.42 to 68.45	Yes	***
Column B vs. Column H	62.10	48.59 to 75.61	Yes	***

Test details	Mean 1	Mean 2	Mean Diff	SE of Diff	n1	n2	q
Column B vs. Column D	70.10	3.533	66.57	5.678	30	30	11.72
Column B vs. Column F	70.10	15.17	54.93	5.678	30	30	9.676
Column B vs. Column H	70.10	8.000	62.10	5.678	30	30	10.94

Data S5. Shows the details related to the statistics performed on Fig. 6 of the manuscript. Statistical analysis of olfactory memory tests. In (**A**), statistics of the "Preference test", using Student's "t" test for comparison between cockroach's time spent searching (s) against the scent of vanilla and citronella when in control saline 0.9%. In (**B**), Student's "t" test was used to compare the olfactory memory of *N. cinerea.* The olfactory memory was analyzed through of the cockroach's time spent searching (s) by the scent of vanilla and citronella, correlating the repulsive odor with the reward for this. In (**C**), the results of the statistical analysis of the cockroach's time spent searching (s) for the scents of vanilla and citronella with the reward system. In this set of analysis, one-way ANOVA, followed by the Dunnett test as *post-hoc* were used to compare the control group saline 0.9% with the group treated with FPL (0.001; 0.01 and 0.1µg/g animal). First, a comparison was made between the Vanilla group: 0.9% saline control (column A) x FPL 0.001µg/g animal (column C); FPL 0.01µg/g animal (column E) and FPL 0.1µg/g animal (column G). Second, the Citronella group: 0.9% saline control (column B) x FPL 0.001µg/g animal (column D); FPL 0.01µg/g animal (column F) and FPL 0.1µg/g animal (column H), were compared.