The flavonoid rutin protects against cognitive impairments by imidacloprid and fipronil

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ABSTRACT

The ongoing decline of bee populations and its impact on food security demands integrating multiple strategies. Sublethal impairments associated with exposure to insecticides, affecting the individual and the colony levels, have led to insecticide moratoria and bans. However, legislation alone is not sufficient and remains a temporary solution to an evolving market of insecticides. Here, we ask whether bees can be prophylactically protected against sublethal cognitive effects of two major neurotoxic insecticides, imidacloprid and fipronil, with different mechanisms of action. We evaluated the protective effect of the prophylactic administration of the flavonoid rutin, a secondary plant metabolite, present in nectar and pollen, and known for its neuroprotective properties. Following controlled or *ad libitum* administration of rutin, foragers of the North American bumble bee *B. impatiens* received oral administration of the insecticides at sublethal realistic dosages. Learning acquisition, memory retention and decision speed were evaluated using olfactory absolute conditioning of the proboscis extension response. We show that the insecticides primarily impair acquisition but not retention or speed of conditioned response. We further show that the administration of the flavonoid rutin successfully protects the bees against impairments produced by acute and chronic administration of insecticides. Our

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results suggest a new avenue for the protection of bees against sublethal cognitive effects of insecticides.

KEY WORDS: Flavonol, Neonicotinoid, Bee decline, Pollinator.

INTRODUCTION

The decline of populations of managed and wild bees is a global concern because of their services provided through pollination (Bailes et al., 2015; Peixoto et al., 2022; Wood et al., 2020). Among the causes for the decline, insecticides have been the major culprits due to their lethal and sublethal effects. Sublethal impacts are particularly critical because of the subtle impairment of individuals, later translated into the deterioration of colony performance (Aliouane et al., 2009; Crall et al., 2018). For example, neuroactive insecticides alter neuronal activity (Dupuis et al., 2012; Palmer et al., 2013), lead to oxidative stress (Gregorc et al., 2018; Martelli et al., 2020), impair mitochondrial function (Moffat et al., 2015), and eventually lead to neurodegeneration (Peng and Yang, 2016). Consequently, bees exhibit impairments in motor control (Moffat et al., 2016; Williamson et al., 2014), sensory sensitivity (Démares et al., 2016; Démares et al., 2018; Eiri and Nieh, 2012; Kessler et al., 2015), learning and memory (Muth et al., 2019; Wright et al., 2015; Zhang and Nieh, 2015), and navigation abilities (Henry et al., 2012), even at low field-realistic doses.

Addressing the deceptively subtle effects on insect pollinators, the European Union (EU) banned and imposed restrictions on the use of major neuroactive insecticides, notably fipronil and the neonicotinoids imidacloprid, thiamethoxam, and clothianidin (Butler, 2018; Gross, 2013; IPBES, 2016). Yet despite the legislation, concerns remain as insecticides are widely relied upon elsewhere, even within certain EU countries for non-flowering crops (Sonne and Alstrup, 2019) and alternative products, such as pyrethroids, may be prone to evolution of resistance, opening the door to pest outbreaks (Carreck, 2017; Gong et al., 2021; Hanson et al., 2017; Kathage et al., 2018; Lundin, 2021). Moreover, legislation alone may lead to an arms race between the development of new insecticides, the long-term scientific evaluation of sublethal effects, and the path toward new political action (Asher, 2018; Siviter and Muth, 2020; Siviter et al., 2018).

Hence, future food security in the face of pollinators' decline and potential pest outbreaks urgently calls for multilevel strategies.

Recently, bee nutrition has surged as a key factor in bee resilience against insecticides and other environmental stressors (Bernklau et al., 2019; Brodschneider and Crailsheim, 2010; Liao et al., 2017; Mao et al., 2013; Mitton et al., 2020; Negri et al.). Specific nutrients such as caffeine, p-coumaric acid, quercetin, kaempferol and casein, increase resilience against insecticides while enhancing pathogen tolerance and supporting the immune system (Bernklau et al., 2019; Folly et al., 2021; Liao et al., 2017; Mao et al., 2013; Mitton et al., 2020). Interestingly, some of these secondary metabolites from plants, such as flavonoids, are also intensely studied for their potential as remedies against human neurodegenerative diseases including Parkinson's and Alzheimer's disease (Maan et al., 2020; Maher, 2019; Pu et al., 2007; Sabogal-Guáqueta et al., 2015). Flavonoids are known for their antioxidant activity, mitochondrial stabilization, neural protection, and regulation of multiple cellular pathways (Heim et al., 2002; Maan et al., 2020; Nkpaa and Onyeso, 2018; Punithavathi et al., 2010; Xu et al., 2019), and they are mass-produced as nutraceuticals and thus readily available. Might flavonoid supplements protect not just humans, but also bees against neurocognitive impairments produced by insecticides?

Here, our goal was threefold. First, we evaluated whether the acute administration of a commercial form of imidacloprid, a neuroactive insecticide, impairs performance in learning and memory tasks in the North American bumble bee *Bombus impatiens*. We selected a commercial form of the insecticide because of the recognized effect of adjuvants, thus providing us with a more realistic, worst-case, scenario for impairment and potential protection. Imidacloprid is a first-generation neonicotinoid that acts as a partial agonist of nicotinic acetylcholine receptors (nAChRs) and its sublethal effects in bees have been thoroughly assessed (IPBES, 2016). We selected bumble bees because they are relevant as managed and wild pollinators and are more sensitive to certain neuroactive insecticides relative to the European honey bee *Apis mellifera* (Cresswell et al., 2012; IPBES, 2016; Moffat et al., 2016), thus being particularly well suited as an indicator for subtle deleterious effects. Sublethal effects of neuroactive insecticides on bumble bees include low reproduction (Whitehorn et al., 2012), altered thermoregulation (Crall et al., 2018; Potts et al., 2018), and cognitive impairment (Siviter et al., 2021a; Smith et al., 2020).

Also, the consequences of exposure to insecticides extend beyond the individual, affecting colony function (Crall et al., 2018; Gill et al., 2012; Siviter et al., 2021b).

Most importantly, as part of our first goal, we investigated whether a controlled prophylactic administration of the flavonol rutin, a glycoside form of quercetin, would confer any protection against the impairment observed. Rutin and quercetin, as well as many other potentially beneficial flavonoids, are present in nectar and pollen across many plant species (De-Melo et al., 2018; Gullón et al., 2017; Kostić et al., 2019). The amount of rutin varies greatly among plant species and preparations as well as across different authors (Gullón et al., 2017). Establishing rutin's potential role as protectant against insecticide effects on pollinators would support the relevance of enhancing plant diversity for conservation of bees and other pollinators and explain some of the negative effects of monocultures on bee health (Obregon et al., 2021). We selected rutin because it is associated with neural protection induced by multiple causes, including impact on AChRs (Pu et al., 2007; Richetti et al., 2011) and, in the presence of Beta-glucosidases, can hydrolyze to quercetin, another flavonoid associated with expression of detoxification enzymes (Zhang et al., 2012) and antagonism of apoptosis (Miao et al., 2021). Moreover, our previous results showed that partial protection of Africanized honey bees against imidacloprid was better after administration of rutin than quercetin, probably due to a combined effect of rutin and its hydrolyzed form (García, L.M., Sutachan, J.J., Morantes-Ariza, C.F., Caicedo-Garzón, V., Albarracín, S.L., Riveros, A.J., unpublished).

As a second goal, we set out to test whether the protection induced by rutin was independent of the mechanism of action of the insecticide and the controlled administration of the flavonoid. We selected an acute administration of fipronil, an insecticide primarily acting as an antagonist of the Gamma-Aminobutyric acid (GABA) receptors. GABA is a neurotransmitter involved in motor control and information processing through inhibitory feedback within neural networks (El Hassani et al., 2005; El Hassani et al., 2009; Gauthier and Grünewald, 2012; Mustard et al., 2020). In this case, we also allowed *ad libitum* administration of rutin, which enabled us to test its innocuity. Finally, as a third goal, we aimed to test the limits of protection, by exposing the bees to *ad libitum* administration of the flavonoid followed by *ad libitum* administration of fipronil and imidacloprid.

Our results show that the acute and *ad libitum* administration of imidacloprid and fipronil impair learning acquisition and that the prophylactic administration of rutin led to protection against the deleterious effects. We conclude that the prophylactic administration of rutin generally induces protection against learning and memory impairments induced by two insecticides with different mechanisms of action.

MATERIALS AND METHODS

Collection and maintenance of bees

We acquired three colonies of the bumble bee *Bombus impatiens* from Koppert Biological Systems Inc., MI, USA. Colonies were maintained under laboratory conditions with an *ad libitum* supply of pollen (inside the nest) and 1M sucrose solution [external feeder attached to the supplied colony box through short (15cm) transparent tubes]. For all experiments we relied exclusively on forager bees collected from the external feeder. Bees from only a single colony were used for each of the three respective experiments described below to control for the potential variation among bees originating from different colonies.

Dosages of insecticides

We determined the dose of insecticides based on the $LD_{50,}$ the so-called realistic (field reported) concentrations, the maximum volume imbibed by a forager, and the number of required training trials (to avoid satiation during training). Reports on LD_{50} concentrations of imidacloprid in bumble bees vary between 1-4 ng/bee (Riaño and Cure, 2016; Marletto et al., 2003). Based on this, we determined a low sublethal dose of 0.03ng/bee, approximately 1/100 of the intermediate values reported for the LD_{50} in bumble bees. We have also previously established impairment of learning and memory in Africanized honey bees using such low doses (Garcia, L.M., Sutachan, J.J., Morantes-Ariza, C.F., Caicedo-Garzón, V., Albarracín, S.L., Riveros, A.J., unpublished). A bumble bee drinks about 150μL of sugar water per foraging trip (Pattrick et al., 2020) or before satiation (personal observations) and we aimed to provide the insecticide and conduct the experiments without satiating the bees. For training, we estimated 10μL ingested per trial so that by the end of the training the total amount consumed approximated 110μL (after eleven trials, see below). Then, we determined a volume of 20μL (minimum collected by a bumblebee; Pattrick et al., 2020) of a 5nM solution of imidacloprid (1.3ppb) to administer 0.03ng/bee. This

concentration is within the range of the field reported values reaching up to 64.58ng/g (65ppb) in nectar and 1.8ng/g (1ppb; Jiang et al., 2018) in pollen. Hence, a bee receiving a controlled dose of imidacloprid (Experiments 1-2) ingested in total 130µL of liquid. Thus, satiation was not expected to affect performance.

For the dose of fipronil the information is scarcer and whereas concentrations are reported, individual dosages (ng/bee) are not generally available for cognitive impairment. Thus, we followed our empirically determined dose of 1 ng/bee based on honey bees (Garcia, L.M., Caicedo-Garzón, V., Riveros, A.J., unpublished), which is below the reported LD_{50} of 4.2 ng/bee in honey bees (Pisa et al., 2015) and previous results indicated behavioral impairment with this dose (Garcia, L.M., Caicedo-Garzón, V., Riveros, A.J., unpublished; El Hassani et al., 2005). We provided this dose in the form of 20µL of a 0.11µM solution (Experiment 2) unless the administration was *ad libitum* (Experiment 3). Importantly, the concentration of 0.11µM (48ppb) is within the reported ranges of fipronil in nectar (2.3ppb-70ppb; reviewed by Bonmatin et al., 2015).

Training apparatus

The training apparatus has been previously described (Jernigan et al., 2014; Riveros and Gronenberg, 2009; Riveros et al., 2020). Briefly, the apparatus consists of 12 individual chambers, each hosting a bee restrained with a yoke in a plastic pipette tip. Each chamber is connected to a vacuum that cleans the air after odor stimulation. In front of the setup, there is a glass tube directed at the bees. The glass tube is connected to two currents of air originating from the same source. A first current is an ongoing stream of clean air whereas the second is controlled by valves and enables an overlapping flow of scented air used as a conditioning stimulus. Relying on two streams that originate from the same source guarantees that the overall flow is constant during a trial and that bees are not conditioned to the changes in pressure.

Training procedure

General protocol: Bees were exposed to a presentation of an odor for 10s. The odor stimulus consisted of a piece of filter paper loaded with 5µL of 1-nonanol (Alfa Aesar A12510) incorporated into the air current tube. Seven seconds after the onset of the odor presentation, the

antennae were stimulated with 1.5M sucrose solution and, following a reflexive extension of the proboscis (PER), the bee was allowed to drink for 3s while the odor current was still ongoing. We excluded the bees that did not respond to the sucrose solution with a PER. Each paired presentation was considered a training trial and each individual received eleven training trials with an average intertrial interval of 10 minutes. After training, all bees were fed 20µL of 1M sucrose solution and maintained in plastic boxes with wet cotton until the retention test. 24h after the last training trial, we presented the bees with the conditioned odor. We recorded whether a bee exhibited a conditioned PER. Bees not exhibiting a conditioned PER were stimulated with 1M sucrose solution to test motivation. For each trial (acquisition and retention), we recorded whether a conditioned PER was exhibited and its latency in seconds using a metronome (2Hz).

Experiment 1. Protection of rutin against an acute exposure to imidacloprid

We collected bees from Colony 1 while on the feeder, anesthetized them on ice and yoke-restrained them in plastic pipettes. Bees were maintained in the pipettes for the entire duration of the experiment (five days). During the first day, we randomly assigned each bee to one of two treatments: i. 20µL of 1M sucrose solution twice a day or ii. 20µL of a 1µM rutin (600ppb; 12 ng/bee; Sigma-Aldrich R5143) solution dissolved in 1M sucrose solution twice a day. This dosage of rutin (determined after Garcia, L.M., Sutachan, J.J., Morantes-Ariza, C.F., Caicedo-Garzón, V., Albarracín, S.L., Riveros, A.J., unpublished) is within or below the reported concentrations from the field, although there is enormous variation among plant species (1180ppb in nectar reported by (Guffa et al., 2017; Gullón et al., 2017). Bees received a total of six doses across three consecutive days. On the fourth day, we randomly assigned each bee to one of two treatments: i. 20µL of 1M sucrose solution or ii. 20µL of 5nM (1.3ppb) solution of imidacloprid (Prime Source LLC, IN, USA) in 1M sucrose solution. Thus, each bee belonged to one of four treatments (Table 1): Control (sucrose for three days), Rut (rutin for three days), Imid (sucrose solution for three days and then imidacloprid before training), Rut+Imid (rutin for three days and then imidacloprid before training). Two hours after the administration of the solution, an experimenter blind to the treatments trained the bees using olfactory conditioning of the PER (see above). For the administration of insecticides in Experiment 1-3, we stimulated the antennae with 1M sucrose solution to induce a PER and fed the insecticides directly to the tongue. Thus, the antennae were not contaminated with insecticides.

Experiment 2. Protective effect of ad libitum self-administration of rutin against an acute exposure to imidacloprid and fipronil

We collected bees from Colony 2 while on the feeder and randomly assigned each bee to one of two treatments: i. 1M sucrose solution or ii. 1µM rutin (Sigma -Aldrich R5143) solution diluted in 1M sucrose solution. Bees were maintained in groups of 10 bees in plastic containers where they could freely walk and had *ad libitum* access to the feeding solutions (two vials with 1mL were provided daily). On the night of the third day, we removed the feeders to starve the bees. On the fourth day, we ice-anesthetized and yoke-restrained the bees in plastic pipettes. Bees were maintained in the pipettes for the rest of the experiment (two days). One hour after harnessing, we randomly assigned the bees to one of three treatments: i) 20µL of 1M sucrose solution, ii) 20µL of 5nM imidacloprid in 1M sucrose solution (Prime Source LLC, IN, USA), iii) 20µL of 0.11µM fipronil (Taurus SC, Control Solutions Inc, TX, USA) in 1M sucrose solution. Thus, each bee belonged to one of six treatments (Table 1): Control (*ad libitum* sucrose for three days), Rut (*ad libitum* rutin for three days), Imid (*ad libitum* sucrose for three days and then acute imidacloprid before training), Rut+Imid (*ad libitum* rutin for three days and then acute imidacloprid before training), Fip (*ad libitum* sucrose for three days and then acute fipronil before training), Rut+Fip (*ad libitum* rutin for three days and then acute fipronil before training). Two hours after the administration of the solution, an experimenter blind to the treatments trained the bees using olfactory conditioning of the PER (see above).

Experiment 3. Protective effect of ad libitum self-administration of rutin against ad libitum exposure to imidacloprid and fipronil

We collected bees from Colony 3 while on the feeder and randomly assigned each bee to one of two treatments: i. 1M sucrose solution or ii. 1µM rutin (Sigma -Aldrich R5143) solution diluted in 1M sucrose solution. Bees were maintained in groups of 10 bees in plastic containers and had *ad libitum* access to the feeding solutions as in Experiment 2. Starting on the fourth day, we replaced one of the vials with one of three solutions: 1M sucrose solution, ii) 5nM imidacloprid in 1M sucrose solution, iii) 0.11µM fipronil (Taurus SC, Control Solutions Inc, TX, USA) in 1M sucrose solution. Feeders were refilled daily for three additional days, providing a self-administered chronic exposure to insecticides. Thus, each bee belonged to one of six treatments (Table 1): Control (*ad libitum* sucrose for six days), Rut (*ad libitum* rutin for three days and then

ad libitum sucrose and rutin for three days), Imid (*ad libitum* sucrose for three days and then *ad libitum* sucrose and imidacloprid for three days), Rut+Imid (*ad libitum* rutin for three days and then *ad libitum* rutin and imidacloprid for three days), Fip (*ad libitum* sucrose solution for three days and then *ad libitum* sucrose and fipronil for three days), Rut+Fip (*ad libitum* rutin for three days and then *ad libitum* rutin and fipronil for three days). On the night of the third additional day, we anesthetized, and yoke-restrained the bees in plastic pipettes. Bees were maintained in the pipettes for the rest of the experiment (two days). The following day, an experimenter blind to the treatments trained the bees using olfactory conditioning of the PER.

Data analyses

For analyses we included only bees exhibiting a response to the sucrose solution across all training trials and the memory retention test. We calculated for each bee a learning score between 0 (no conditioned PER across the ten trials) and 10 (conditioned response across all ten trails). The first training trial was not considered in the score and was used to determine that bees did not have a spontaneous response to the conditioned stimulus. The scores within the planned comparisons were compared using a Wilcoxon test (one or two-sided p-values depending upon predictions). Based on previous evidence, we predicted a decrease in performance of the bees exposed to insecticides and an improvement in bees prophylactically fed with rutin (relative to bees fed with insecticide and not fed with rutin). Of interests were the following comparisons: Control vs. Fip/Imid to evaluate the effect of the insecticides, Control vs. Rut to evaluate the innocuousness of the rutin, Rut vs. Rut+Imid/Rut+Fip, to test whether there was full protection, and Imid vs. Rut+Imid and Fip vs. Rut+Fip to test whether there was any significant protection. Differences in retention after 24h were recorded as a nominal variable and compared relative to the last training trial using an Exact Fisher's test. At the population level, we constructed and analyzed learning curves by relying on a Generalized Linear Mixed Model (GLMM). For the GLMM, we used a binomial structure with a Logit link function; also, we included Treatment (feeding schedule) and training Trial as fixed effects and the individual as a random effect. Latency of response was recorded as a continuous variable between 0.5s and 10s. We averaged at least two conditioned responses (i.e. bees with only a single conditioned PER were not included in the latency analyses). Comparisons of response latencies and body size (head width) were evaluated using an ANOVA if the distribution of data was normal (tested using a Shapiro-

Wilk W test). In all cases, error due to multiple comparisons was controlled using the False Discovery Rate one stage method (Pike, 2011; Verhoeven et al., 2005) and the corrected p-values (q-values) are presented. All the analyses were done using JMP v.16.1.0 (SAS Institute).

RESULTS

Experiment 1. Protection of rutin against an acute exposure to imidacloprid

We collected and maintained 200 bees. Some bees were excluded due to escaping (N=8), dying during the three-day maintenance period before the exposure to pesticide (Control: N=5; Rut: N=2; Imid: N=2), not exhibiting a PER before training (Control: N=1; Rut: N=3; Imid: N=4; Rut+Imid: N=5) or at least once during training (Control: N=6; Rut: N=7; Imid: N=3; Rut+Imid=6), not exhibiting a PER during the retention test or dying during the 24h period before the retention test (Control: N=4; Rut: N=4; Imid: N=1; Rut+Imid=3). Moreover, we conducted a screening of outliers for memory and learning score using Mahalanobis distances (0 bees excluded). Thus, we conducted our final tests using 136 bees distributed across four treatments: Control (N=34), Rut (N=35), Imid (N=36), Rut+Imid (N=31). Mean body size (head width) did not significantly differ across groups (Control: Mean±SE=3.6±0.02 mm; Rut: Mean±SE=3.5±0.02 mm; Imid: Mean±SE=3.6±0.03 mm; Rut+Imid: Mean±SE=3.6±0.03 mm; ANOVA: $F_{3,132}$=2.26, P=0.09).

Overall, we found that the administered compounds affected the level of performance of bees among treatments as indicated by the learning score (Kruskal-Wallis test: χ_3=15.81, P=0.0012). We found that the administration of imidacloprid significantly impaired the performance of bees. Relative to Controls, the bees exposed to imidacloprid (Imid) exhibited significantly lower learning scores (Mean±SE: Control=5.1±0.49; Imid=2.6±0.42; Wilcoxon-test: Z=-3.37, P=0.0016; Fig. 1A) yet no significant differences in response latencies (Mean±SE: Control=2.1s±0.17; Imid=2.6s±0.21; t_{105}=2.0, P=0.1; Fig. 1B). In contrast, the administration of rutin was innocuous such that the bees in the Rut group exhibited learning scores (Mean±SE: Rut=5.3±0.55; Wilcoxon-test: Z=0.40, P=0.69; Fig. 1A), and response latencies (Mean±SE: Rut=1.9s±0.19; t_{105}=-0.67, P=0.51; Fig. 1B) that did not significantly differ from Control bees. Most importantly, the bees in the Rut+Imid group exhibited learning scores (Mean±SE:

Rut+Imid=4.2±0.57; Fig. 1A) that were significantly higher than bees in the Imid group (Wilcoxon-test: Z=2.0, P=0.046) but were not significantly different from Rut bees (Wilcoxon-test: Z=1.41, P=0.21; Fig. 1A). Moreover, response latencies of bees in the Rut+Imid group (Mean±SE: 2.44s±0.17; Fig. 1B) were not significantly different from bees in the Rut group (Rut+Imid vs. Rut t_{105}=-1.89, P=0.12) or in the Imid group (Rut+Imid vs. Imid t_{105}=-0.65, P=0.35).

Like the analysis focused on individual scores, we found that acquisition at the population level was significantly affected by the training protocol and the feeding treatment (Fig. 1C). We observed an increase in the percentage of conditioned responses across training trials (Trial: GLMM: F=314.04, P<0.0001), and a significant variation in the level of response across treatments (Treatment: GLMM: F=103.1, P=0.0018). We did not find a significant interaction between the treatment and the training trial (Treatment*Trial: GLMM: F=1.4, P=0.26), indicating that the overall pattern of acquisition across trials were not different. Thus, we conducted a new analysis on the reduced model. We found that bees were significantly affected by the training trial (Trial: GLMM: F=311.9, P<0.0001) and the treatment (Treatment: GLMM: F=5.78, P=0.001). After detailed analyses on our planned comparisons on the Least Square Means, we found that the bees administered with imidacloprid (Imid) exhibited significantly lower acquisition than bees receiving only sucrose (Control; one-sided t=-3.5, P=0.0003). In contrast, we did not find significant differences in learning acquisition between bees exposed to rutin (Rut) or bees receiving only sucrose (Control; t=0.19, P=0.85). Most importantly, we found that the bees prophylactically fed with rutin and then exposed to the insecticide exhibited significantly higher levels of acquisition relative to bees exposed to imidacloprid (Imid) and (Rut+Imid; one-sided t=2.14, P=0.017). Similarly important, we did not find differences between the learning acquisition of bees exposed only to rutin (Rut) and bees prophylactically fed with rutin and then exposed to imidacloprid (Imid; t=-1.46, P=0.15).

After 24h, we found that the overall pattern of response was maintained relative to the last training trial, with bees in the Imid group exhibiting the lowest percentage of conditioned responses (Fig. 1D). Bees in the Imid group exhibited significantly lower memory retention than Control bees (Adjusted Wald test: Proportion difference= 0.46, P=0.0001) and the odds of remembering were estimated to be 9.1 times higher (95% CI=2.8-29.1) for Control bees relative

to Imid bees. In contrast, bees in the Rut group exhibited a level of memory retention that did significantly differ from Control bees (Adjusted Wald test: Proportion difference=-0.05, P=0.590). Importantly, bees in the Rut+Imid group exhibited levels of memory retention that were significantly higher than Imid bees (Adjusted Wald test: Proportion difference=0.35, P=0.001) but did not significantly differ from Rut bees (Adjusted Wald test: Proportion difference=0.06, P=0.590). Also, the odds of remembering were estimated to be 4.5 times higher (95% CI=1.6-12.9) for Rut+Imid bees relative to Imid bees. However, retention itself was not generally affected by the administration of the treatments as shown by the comparison, within each treatment, of the probability of exhibiting a conditioned PER during the last training trial and during the 24h retention test (Fisher's exact test: Control: P=0.15; Imid: P=1.0; Rut+Imid: P=0.18; Rut: P=0.19; Fig. 1D).

Experiment 2. Protective effect of rutin ad libitum self-administration against an acute exposure to imidacloprid and fipronil

We collected and maintained 223 bees. Some bees were excluded due to not exhibiting a PER before training (Control: N=5; Rut: N=7; Imid: N=2; Rut+Imid: N=5; Fip: N=2; Rut+Fip: N=7) or at least once during training (Control: N=2; Rut: N=0; Imid: N=2; Rut+Imid=0; Fip: N=0; Rut+Fip: N=0), not exhibiting a PER during the retention test (Control: N=0; Rut: N=4; Imid: N=2; Rut+Imid=3; Fip: N=2; Rut+Fip: N=3) or dying during the 24h period before the retention test (Control: N=0; Rut: N=0; Imid: N=1; Rut+Imid=0; Fip: N=0; Rut+Fip: N=0). We excluded 29 additional bees following a screening of outliers for memory and learning score using Mahalanobis distances. Thus, we conducted our final tests using 147 bees distributed across six treatments: Control (N=30), Rut (N=22), Imid (N=23), Rut+Imid (N=21), Fip (N=26), Rut+Fip (N=25). Mean body size (head width) was slightly larger in the Fip group but overall did not significantly differ across groups (Control: Mean±SE=3.6±0.03 mm; Rut: Mean±SE=3.6±0.04 mm; Imid: Mean±SE=3.6±0.05 mm; Fip: Mean±SE=3.7±0.04 mm; Rut+Imid: Mean±SE=3.6±0.05 mm; Rut+Fip: Mean±SE=3.6±0.04 mm; ANOVA: $F_{5,141}$=1.98, P=0.09).

Overall, we found that the administered compounds affected the level of performance as indicated by the learning score (Kruskal-Wallis test: χ_5=40.03, P<0.0001). We found that the administration of imidacloprid and fipronil significantly impaired the performance of bees.

Relative to Controls, the bees exposed to imidacloprid (Imid) and fipronil (Fip) exhibited significantly lower learning scores (Mean±SE: Control=6.8±0.3; Imid=2.4±0.46; Fip= 4.8±0.6 Wilcoxon-test Control vs. Imid: Z=-5.25, P=0002; Wilcoxon-test Control vs. Fip: Z=-2.5, P=0.014; Fig. 2A), but not significantly different response latencies (Mean±SE: Control=2.1s±0.2; Imid=2.6s±0.3; Fip=2.3s±0.2; Control vs. Imid t_{126} =1.50, P=0.96; Control vs. Fip t_{126} =0.73, P=0.96; Fig. 2B). In contrast, the bees in the Rut group exhibited learning scores (Mean±SE: Rut=6.1±0.35; Wilcoxon-test Control vs. Rut: Z=-1.61, P=0.15; Fig. 2A), and response latencies (Mean±SE: Rut=2.3s±0.2; Control vs. Rut t_{126} =-0.71, P=0.96; Fig. 2B) that did not significantly differ from Control bees.

Remarkably, bees in the Rut+Imid and the Rut+Fip groups exhibited learning scores (Mean±SE: Rut+Imid=5.5±0.3; Mean±SE: Rut+Fip=6.5±0.6) that were significantly higher than bees in the Imid and Fip groups respectively (Wilcoxon-test for Rut+Imid vs. Imid: Z=4.21, P=0.0002; Wilcoxon-test for Rut+Fip vs. Fip: Z=2.16, P=0.027; Fig. 2A) but were not significantly different from Rut bees (Wilcoxon-test for Rut+Imid vs. Rut: Z=0.98, P=0.33; Wilcoxon-test for Rut+Fip vs. Rut: Z=-1.18, P=0.28; Fig. 2A). Also, the response latencies of bees in the Rut+Imid group (Mean±SE: 2.8s±0.27) were not significantly different from bees in the Rut group (Rut+Imid vs. Rut t_{126}=-1.55, P=0.21; Fig. 2B) or from bees in the Imid group (Rut+Imid vs. Imid t_{126}=0.58, P=0.96; Fig. 2B). Finally, the response latencies of bees in the Rut+Fip group (Mean±SE: 1.7s±0.2) was not significantly different from bees in the Rut group (Rut+Fip vs. Rut t_{126}=1.80, P=0.96; Fig. 2B) or than bees in the Fip group (Rut+Fip vs. Fip t_{126}=-1.81, P=0.21; Fig. 2B).

Like the analyses at the individual level, we found that, at the population level, bees in all groups were significantly affected by the training protocol and the feeding treatment (Fig. 2C). We observed an increase in the percentage of conditioned responses across training trials (Trial: GLMM: F=131.5, P<0.0001), but a significant variation in the level of response across treatments (Treatment: GLMM: F=11.0, P<0.0001). We did not find a significant interaction between the treatment and the training trial (Treatment*Trial: GLMM: F=1.6, P=0.17), indicating that the overall patterns across trials were not different. Thus, we conducted a new analysis of the reduced model. We found that bees were significantly affected by the training trial (Trial: GLMM: F=135.9, P<0.0001) and the treatment (Treatment: GLMM: F=11.9,

P<0.0001). After detailed analyses on our planned comparisons on the LS Means, we found that the bees administered with the insecticides (Imid, Fip) exhibited significantly lower acquisition than bees receiving only sucrose (Control; Imid vs. Control: one-sided t=-6.91, P<0.0001; Fip vs. Control: one-sided t=-3.21, P=0.0009). In contrast, we did not find significant differences in learning acquisition between bees exposed to rutin (Rut) or bees receiving only sucrose (Control; t=-1.14, P=0.26). Most importantly, we found that the bees prophylactically fed with rutin and then exposed to the insecticides exhibited significantly higher levels of acquisition relative to bees exposed to imidacloprid (Imid vs. Rutin+Imid: one-sided t=4.77, P<0.0001) and fipronil (Fip vs. Rut+Fip: one-sided t=2.76, P=0.0033). Also, the bees prophylactically fed with rutin and then exposed to imidacloprid (Rut+Imid vs. Rut: t=0.70, P=0.48) or fipronil (Rut+Fip vs. Rut: t=-0.81, P=0.42), exhibited acquisitions that were not different from bees fed only with rutin (Rut).

After 24h, we found that the overall pattern of response was maintained relative to the last training trial, with bees in the Imid and Fip groups exhibiting the lowest percentages of conditioned response (Figure 2D). Bees in the Imid and the Fip groups exhibited significantly lower memory retention than Control bees (Adjusted Wald test for Imid vs. Control: Proportion difference=0.52, P=0.0004; Adjusted Wald test for Fip vs. Control: Proportion difference=-0.31, P=0.035) and the odds of remembering were estimated to be 12.7 times higher (95% CI=3.0-53.2) for Control bees relative to Imid bees and 3.6 times higher relative to Fip bees (95% CI=1.2-10.9). In contrast, bees in the Rut group exhibited a level of memory retention that did not significantly differ from Control bees (Adjusted Wald test: Proportion difference=-0.16, P=0.39).

Importantly, bees in the Rut+Imid group exhibited levels of memory retention that were significantly higher than Imid bees (Adjusted Wald test: Proportion difference=0.30, P=0.035; Fig. 2D) but did not significantly differ from Rut bees (Adjusted Wald test: Proportion difference=0.07, P=0.65; Fig. 2D). The odds of remembering were estimated to be 5.0 times higher (95% CI=1.12-22.2) for Rut+Imid bees relative to Imid bees. In the case of the protection against fipronil, the bees in the Rut+Fip group exhibited levels of memory retention (62.5%) that were clearly higher than the bees in the Fip group (37.5%; Fig. 2D), yet the difference was not

statistically significant after the one-stage FDR correction (Adjusted Wald test: Proportion difference=0.25, P=0.06; a two-stage FDR rendered a P=0.038). However, the levels of memory retention of Rut+Fip bees did not significantly differ from Rut bees (Adjusted Wald test: Proportion difference=0.1, P=0.59), suggesting an intermediate level of memory protection.

However, retention itself was not generally affected by the administration of the treatments as shown by the comparison, within each treatment, of the probability of exhibiting a conditioned PER during the last training trial and during the 24h retention test (Fisher's exact test: Control: P=1.0; Imid: P=1.0; Rut+Imid: P=1.0; Fip: P=0.26; Rut+Fip: P=1.0; Rut: P=0.36; Fig. 2D).

Experiment 3. Protective effect of rutin ad libitum self-administration against chronic exposure to imidacloprid and fipronil.

We collected and maintained 360 bees. During the maintenance period some of the bees died (Control: N=2; Rut: N=4; Imid: N=3; Fip: N=2; Rut+Imid: N=0; Rut+Fip: N=5). Due to constraints in time for yoke-restraining, training, and maintenance, we could prepare for evaluation only 192 bees. Before training, some bees were excluded due to not exhibiting a PER (Control: N=4; Rut: N=1; Fip: N=2; Imid: N=5; Rut+Imid: N=3; Rut+Fip: N=3). After training, bees were excluded due to not exhibiting a PER during the retention test (Control: N=0; Rut: N=0; Fip: N=3; Imid: N=1; Rut+Imid: N=0; Rut+Fip: N=1) or dying during the 24h period before the retention test (Control: N=1; Rut: N=2; Fip: N=1; Imid: N=0; Rut+Imid: N=2; Rut+Fip: N=2). We excluded 30 additional bees following a screening of outliers for memory and learning score using Mahalanobis distances. Thus, we conducted our final tests using 131 bees distributed across six treatments: Control (N=24), Rut (N=24), Imid (N=15), Rut+Imid (N=24), Fip (N=25), Rut+Fip (N=19). Mean body size (head width) did not significantly differ across groups (Control: Mean±SE=3.5±0.04mm; Rut: Mean±SE=3.5±0.04mm; Imid: Mean±SE=3.5±0.04mm; Fip: Mean±SE=3.5±0.05 mm; Rut+Imid: Mean±SE=3.5±0.04mm; Rut+Fip: Mean±SE=3.5±0.04mm; ANOVA: $F_{5,125}$=0.384, P=0.859).

We found that bees generally consumed all the compounds provided, but consumption varied across the compounds added to the sucrose solution. Sucrose solution consumption was highest in Control bees but similar between bees also consuming insecticide (Control: Mean±SE=195.5

±3.5µL; Fip: Mean±SE=150.5 ±8.5µL; Imid: Mean±SE=155.3 ±10µL). Similarly, consumption of rutin was estimated to be higher in Rut bees but similar in bees exposed to rutin and the insecticide (Rut: Mean±SE=192.1 ±7.7µL; Rut+Fip: Mean±SE=162.6 ±8.8µL; Rut+Imid: Mean±SE=151.9 ±9.6µL). Finally, estimated volumes of ingested insecticide were similar across treatments including the same chemical (Fip: Mean±SE=93.7 ±5.9µL; Rut+Fip: Mean±SE=97.3 ±5.7µL; Imid: Mean±SE=103.5 ±3.6µL; Rut+Imid: Mean±SE=88.6 ±4.1µL). These estimated ingested volumes clearly suggest higher dosages per bee (Imid: 0.13ng/bee; Rut+Imid: 0.11ng/bee; Fip: 4.5ng/bee; Rut+Fip: 4.7ng/bee) compared with our treatments where the dosage was controlled (Experiments 1 and 2).

Overall, we found that the administered compounds affected the level of performance as indicated by the learning scores (Kruskal-Wallis test: χ_5=75.89, P<0.0001). We found that *ad libitum* administration of imidacloprid and fipronil impaired the performance of bees. Relative to Controls, the bees exposed to imidacloprid (Imid) and fipronil (Fip) exhibited significantly lower learning scores, although the effect was barely significant in the case of fipronil after the FDR correction (Mean±SE: Control=7.0±0.7; Imid=0.0±0.0; Fip= 5.2±0.8 Wilcoxon-test Control vs. Imid: Z=-5.37, P=0.0003; Wilcoxon-test Control vs. Fip: Z=-1.78, P=0.05; Fig. 3A) while response latencies did not differ significantly (Mean±SE: Control=2.3s±0.3, N=20; Fip=2.6s±0.2, N=18; Control vs. Fip t_{84} =0.95, P=0.44). In contrast, the administration of rutin was innocuous such that the bees in the Rut group exhibited learning scores (Mean±SE: Rut=8.8±0.3; Wilcoxon-test Rut vs. Control: Z=1.45, P=0.175) and response (Mean±SE: Rut=1.9s±0.2, N=24; Control vs. Rut t_{84} =-1.0, P=0.44) that did not significantly differ from Control bees.

Remarkably, bees in the Rut+Imid and the Rut+Fip groups exhibited learning scores (Mean±SE: Rut+Imid=1.3±0.3; Mean±SE: Rut+Fip=8.4±0.4) that were significantly higher than bees in the Imid and Fip groups, respectively (Wilcoxon-test for Rut+Imid vs. Imid: Z=3.35, P=0.0009; Wilcoxon-test for Rut+Fip vs. Fip: Z=2.69, P=0.007; Fig. 3A). However, bees in the Rut+Imid group exhibited learning scores that were significantly lower (Wilcoxon-test for Rut+Imid vs. Rut: Z=5.97, P=0.0003; Fig. 3A) and response latencies significantly longer than bees in the Rut

group (Mean±SE: 3.9s±0.5, N=9; Rut+Imid vs. Rut t_{84} =-4.17, P=0.0005; Fig. 3B). In contrast, bees in the Rut+Fip group exhibited learning scores that were not significantly different from bees in the Rut group (Wilcoxon-test for Rut+Fip vs. Rut: Z=0.43, P=0.67; Fig. 3A) but latencies that barely significantly differed from Rut bees after the FDR correction (Rut+Fip: Mean±SE: 2.8s±0.3, N=18; Rut+Fip vs. Rut t_{84} =-2.31, P=0.05; Fig. 3B). Also, bees in the Rut+Fip group exhibited latencies that were not significantly different from bees in the Fip group (Fip: Mean±SE: 2.6s±0.2; Rut+Fip vs. Fip t_{84} =0.32, P=0.74; Fig. 3B).

When analyzing the population through the acquisition curves, we excluded bees in the Imid group since none exhibited a conditioned response (Fig. 3C). We found that bees were affected by the training protocol and the feeding treatment (Fig. 3C; GLMM: Trial: F=61.3, P<0.0001; Treatment: F=21.7, P<0.0001). Importantly, we found a significant interaction between the feeding schedule and training trial (Treatment*Trial), indicating that the rate of acquisition was different between bees exposed to different treatments (GLMM: Trial*Treatment: F=9.9, P<0.0001). This significant effect was associated with the pattern of response of bees exposed to fipronil (determined after comparisons of all GLMM pairs, not shown). In fact, a more detailed analysis showed that bees exposed to fipronil exhibited significantly lower acquisition than bees in the Control group (Fip vs. Control: one-sided t=-1.95, P=0.027; Fig. 3C) with a decrease in the percentage of conditioned PER after the third trial. In contrast, bees fed with rutin even exhibited significantly higher levels of acquisition than bees in the Control group (Rut vs. Control: t=2.2, P=0.027; Fig. 3C). Also, bees prophylactically fed with rutin and then exposed to fipronil (Rut+Fip) exhibited higher acquisition than bees exposed to fipronil (Fip vs. Rut+Fip: one-sided t=3.30, P=0.0007; Fig. 3C). Moreover, bees fed prophylactically with rutin and then exposed to fipronil (Rut+Fip) exhibited levels of acquisition that did not significantly differ from bees fed with rutin (Rut vs. Rut+Fip: t=0.67, P=0.5). In contrast, bees prophylactically fed with rutin and then exposed to imidacloprid (Rut+Imid) exhibited levels of acquisition that were significantly lower than bees fed with rutin alone (Rut+Imid vs. Rut: t=8.37, P<0.0001).

We found that the overall pattern of response after 24h was maintained relative to the last training trial, with bees in the Imid and Fip groups exhibiting the lowest percentages of conditioned response (Fig. 3D). Bees in the Imid and the Fip groups exhibited significantly lower

memory retention than Control bees (Adjusted Wald test for Imid vs. Control: Proportion difference=0.55, P=0.0002; Adjusted Wald test for Fip vs. Control: Proportion difference=0.31, P=0.036) and the odds of remembering were estimated to be 23.3 times higher (95% CI=2.6-208.6) for Control bees relative to Imid bees and 3.5 times higher relative to Fip bees (95% CI=1.1-11.5). In contrast, bees in the Rut group exhibited a level of memory retention that did not significantly differ from Control bees (Adjusted Wald test: Proportion difference=0.0, P=1.0). Interestingly, bees in the Rut+Imid group exhibited levels of memory retention that were not significantly different from Imid bees (Adjusted Wald test: Proportion difference=-0.07, P=0.25) but were significantly lower than Rut bees (Adjusted Wald test: Proportion difference=-0.62, P=0.0002). In the case of the protection against fipronil, the bees in the Rut+Fip group exhibited levels of memory retention that were significantly higher than the bees in the Fip group (Adjusted Wald test: Proportion difference=0.31, P=0.036). The odds of remembering were estimated to be 3.6 times higher (95% CI=1.03-12.8) for Rut+Fip bees relative to Fip bees. Also, the levels of memory retention of Rut+Fip bees did not significantly differ from Rut bees (Adjusted Wald test: Proportion difference=0.007, P=1.0), suggesting a full level of protection. However, retention itself was not generally affected by the administration of the treatments as shown by the comparison of the probability of exhibiting a conditioned PER during the last training trial and during the 24h retention test (Fisher's exact test: Control: P=0.76; Imid: P=1.0; Rut+Imid: P=0.49; Fip: P=1.0; Rut+Fip: P=0.48; Rut: P=0.19).

DISCUSSION

The negative ecological and economic impact of pesticides on pollinators, such as bees, calls for urgent and multidimensional approaches. For decades, evidence has highlighted the role of nutrition in the overall health of bees. Here, we tested whether rutin, a plant secondary metabolite, may protect against sublethal impairments induced by different schedules of oral administration of two neuroactive pesticides. Our results demonstrate that oral administration of both pesticides impairs primarily learning and, under some conditions, memory retention and decision speed. Most importantly, prophylactic administration of rutin, under well-defined doses as well as under *ad libitum* conditions, led to the development of protection against the pesticide effects. However, such protection was not observed in one case, when imidacloprid was provided

ad libitum and led to a total depression of learning acquisition. Interestingly, memory retention was not affected, although a significantly low performance was observed at 24h. In contrast, bees prophylactically fed with rutin exhibited fully normal memory retention after the administration of the insecticide, demonstrating the protection. Thus, our results highlight three key aspects: the sublethal impairments after exposure to imidacloprid and fipronil, the innocuous effect of rutin administration, and the protection provided by prophylactic administration of rutin against the pesticides.

First, the exposure to imidacloprid and fipronil generally resulted in reduced learning performance as inferred from learning scores and acquisition curves (Figures 1A, 2A, 3A), but not to longer response latencies (Figs. 1B, 2B; but see Fig. 3B). Moreover, in most cases bees exposed to the insecticide exhibited lower memory retention (but see below). These effects correspond with well-known impairments derived from multilevel effects by the insecticides and their metabolites. In our case, one of the insecticides, imidacloprid, is known to overstimulate the excitatory cholinergic pathway supporting olfactory learning, whereas fipronil suppresses the inhibitory GABAergic pathway. Eventually, the long-term action of insecticides and their metabolites may lead to neuronal degeneration, presumably as a consequence of mitochondrial instability inducing apoptosis (Peng and Yang, 2016; Wu et al., 2015). These long-term effects may help explain the sustained low performance observed, for example, 28h after the administration of imidacloprid (Fig. 1D). However, low performance during retention tests may result from low learning (impaired acquisition) or impaired storage itself. In our case, the levels of memory retention were generally not significantly different from the performance observed during the last training trial, suggesting a long-term impact of the impairment in acquisition but not necessarily of retention. Nevertheless, the performance of bees administered with imidacloprid or fipronil was always low during the 24h test, which argues for a long-term cognitive impairment. Thus, together our results agree with the impairment observed in previous accounts following the administration of imidacloprid and fipronil; yet suggest a larger variation in the response of the processes associated with memory storage and the speed of information processing. For example, fipronil led to apparent rapid acquisition followed by a decrease in the conditioned responses. Interestingly, the impairment was more severe with the administration of imidacloprid compared to fipronil, an effect probably due to the prevalence of ACh as excitatory transmitter in the olfactory pathway during absolute conditioning (Grünewald, 2012 and

references therein). Since GABA acts as an inhibitory transmitter and contributes to odor discrimination (Wilson and Laurent, 2005), the impact of fipronil might be more significant during differential learning tasks as compared to our absolute conditioning protocol.

As a second aspect, and in contrast to the effects of the neuroactive insecticides, the prophylactic administration of rutin was innocuous. This result is not surprising given the extended presence of rutin in nectar and pollen and the low dosages used here. Importantly, rutin has been found to not act as attractant or deterrent of bees and, unlike alkaloids and other allelochemicals, appears not to be toxic (Detzel and Wink, 1993). In our case, even under *ad libitum* administration of rutin we did not observe any negative effects on performance, although the individual amount ingested could not be quantified due to having multiple bees sharing a feeder. However, separate accounts from our group suggest that bees fed with rutin exhibit enhanced cognitive performance relative to Control bees and the learning curve of bees receiving *ad libitum* rutin was significantly higher. Finally, we highlight the fact that using very low concentrations of rutin allowed the dilution of the flavonoid in water. Rutin, like other flavonoids, is typically dissolved in Dimethyl Sulfoxide (DMSO) during bioassays, which greatly enhances its solubility. In honey bees, acute administration of DMSO has not shown impairments (Guez et al., 2005); yet its impact during extended schedules of exposure is unknown. Nevertheless, DMSO appears to exhibit significant levels of cell toxicity even at low concentrations (Galvao et al., 2014; Kim and Lee, 2021; Milchreit et al., 2016; Verheijen et al., 2019), making it an undesirable solvent for supplements that may require regular administration.

As a third and final aspect, we found that the performance of bees fed prophylactically with rutin, and then exposed to the pesticides, was significantly better than bees administered with the insecticides (Figs. 1A, 2A, 3A). In all cases, the performance of ‘protected’ bees administered with fipronil was undistinguishable from bees fed only with rutin (Figs. 2A, 3A). In the case of imidacloprid-treated bees, we observed full protection in two cases; however, when the bees were allowed *ad libitum* administration of the insecticide, the ‘protection’ was low and partial. Nevertheless, this last scenario occurred when *ad libitum* imidacloprid appeared to fully impair the acquisition of any information (Fig. 3A), thus highlighting the sensitivity of bumble bees even at sublethal levels. These results reflect the subtler, yet significant, impact of fipronil

relative to imidacloprid and the stronger deficiency derived from forcing the *ad libitum* self-administration of imidacloprid for three days (Moffat et al., 2016). This is particularly critical as imidacloprid induces a preference for neonicotinoid-laced sucrose solutions in bumble bees (Kessler et al., 2015). Furthermore, it highlights the variation in effects induced by different pesticides, previously reported even among related neonicotinoids (Moffat et al., 2016). However, direct comparisons are challenging because of the differences in the respective dosages used and the less documented toxicity of fipronil (presented as concentrations and not as dosage/bee).

The overall protection induced by the administration of rutin points to three elements of relevance. First, we observed protection for two neuroactive pesticides featuring distinct toxicodynamic. This implies a protection by rutin involving common targets of activity by imidacloprid and fipronil, such as the stability of mitochondrial function (Nicodemo et al., 2014; Powner et al., 2016) and the activation of detoxification mechanisms (Mao et al., 2011; Mao et al., 2013; Zhang et al., 2012). Importantly, rutin can be metabolized, enhancing its antioxidant and detoxification effect (De Araújo et al., 2013). Moreover, aiming to evaluate a more realistic scenario we relied on commercial formulations of the pesticides; therefore, these results imply a protection against the adjuvants, which probably varied between pesticides and are broadly known to contribute to the observed impairments in bees (Mullin, 2015). Second, the protective effects seem to extend more than 36h after the last dose of rutin. This is reflected by the normal performance of bees during the memory test, when the insecticide-treated bees had not recovered. Lastly, the latency of response of the protected bees tended to be longer than the bees in the rutin group (Fig. 3B), suggesting that rutin may alleviate the accuracy through effects on the speed of the decisions. Thus, together our results suggest that bumble bees can be protected against the sublethal cognitive impairment induced by commercial forms of two major pesticides acting through different mechanisms. Although the mitochondria appear to be a common cornerstone targeted by both the insecticides, in the absence of evidence on mitochondrial structure or function, our results do not allow us to confirm that the protection acts at this level.

Crop productivity is central to global issues associated with food security. Strategies such as the use of neurotoxic pesticides and establishing monocultures play a central role in maintaining productivity by decreasing damage through pests and enhancing crop yield of key products. Thus, it becomes crucial to develop and implement multiple strategies that secure food production while protecting non-target species, such as bees. Our approach relying on a secondary metabolite of plants present in pollen and nectar introduces an alternative that protects the cognition of the bees at realistic levels of pesticide exposure. Our results suggest a direct use for managed species through specific supplementation; however, it also encourages higher plant diversity (for example through flowering species planting programs) to allow and enrich nutrition for wild bees and other pollinators (St Clair et al., 2020). This further calls for evaluations in semi-field and field conditions, particularly addressing the issue of the population decline faced by managed pollinators. Considering the broad range of physiological effects of rutin (Heim et al., 2002; Nkpaa and Onyeso, 2018), one might expect rutin to positively affect other aspects of bee health in addition to protecting the nervous system. This study might also encourage future research into potential pollinator protective effects of other related secondary plant metabolites with established or assumed health effects for humans.

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Competing interests

Part of the results presented here are included in the PCT application W02021046388A1.

Author contributions

AJR conceptualized the project, designed the experiments, collected the data, conducted the statistical analyses, elaborated figures, contributed with discussion and wrote the manuscript. WG conceptualized the project, designed the experiments, collected the bees, and contributed to discussion and writing of the manuscript.

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Figures and Tables

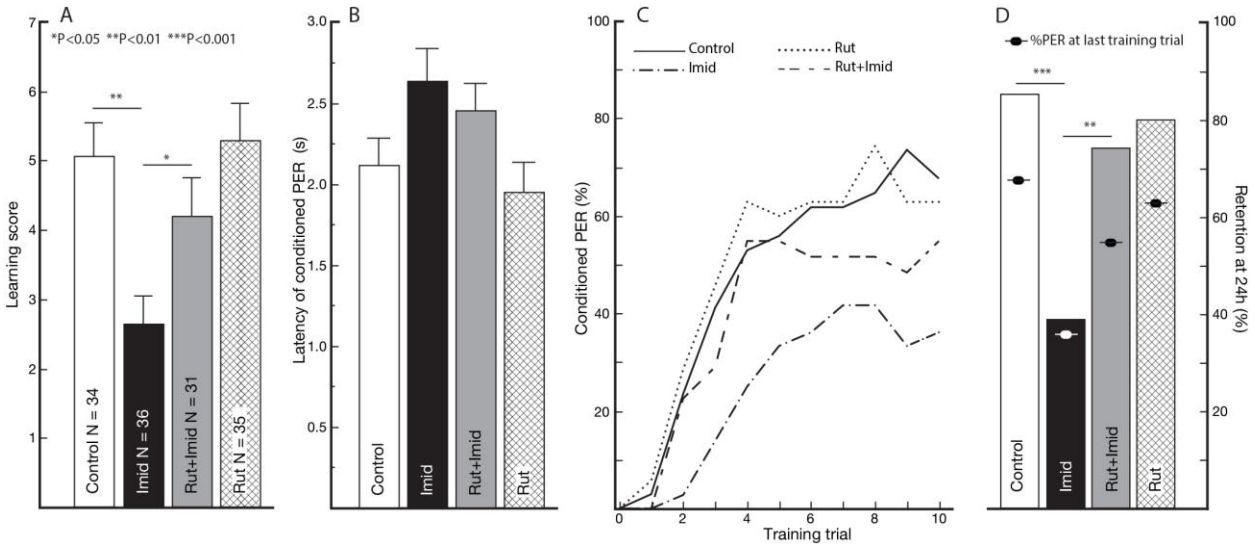

Fig 1. Controlled prophylactic administration of rutin protects learning and memory against acute exposure to imidacloprid. (**A**) Innocuous and protective effect of rutin administration on learning and (**B**) Average latency of response for bees that responded at least twice during training. (**C**) Acquisition curves for bees in all treatments. **(D)** memory retention 28h after exposure to imidacloprid and 40h after the last administration of rutin. (A), (C) and (D) include the same bees. Only statistically significant planned comparisons (see methods) are indicated to facilitate visualization in A and D. Error bars display standard error of the mean.

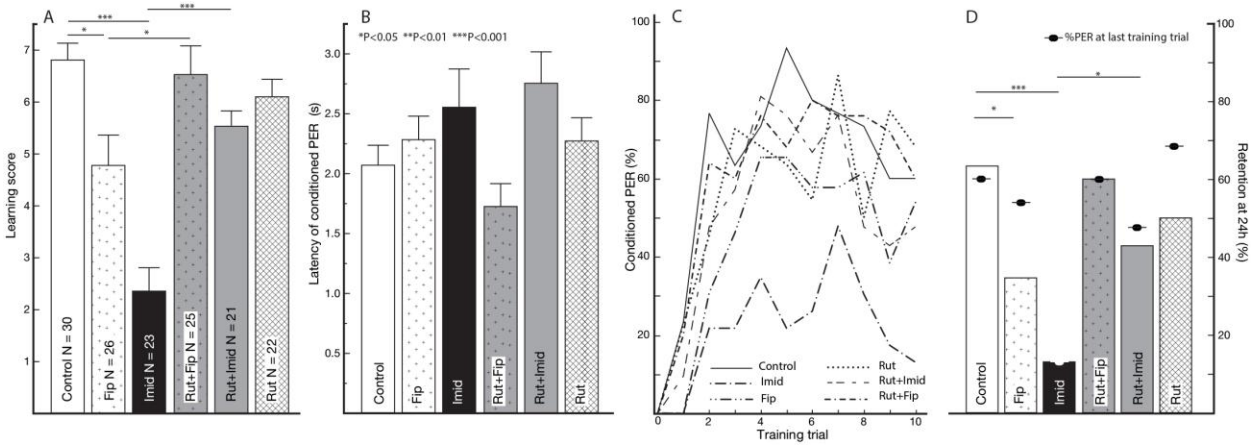

Fig. 2. *Ad libitum* prophylactic administration of rutin protects learning and memory against impairment by acute exposure to imidacloprid and fipronil. (**A**) Innocuous and full protective effect of rutin administration on learning. (**B**) The administration of insecticides did not significantly affect the latency of response. (**C**) Acquisition curves for bees in all treatments. (**D**) Innocuous and protective effect of rutin administration on memory retention 28h after exposure to imidacloprid and 40h after the last administration of rutin. (A), (C) and (D) include the same bees. Only statistically significant planned comparisons (see methods) are indicated to facilitate visualization in A and D. Error bars display standard error of the mean.

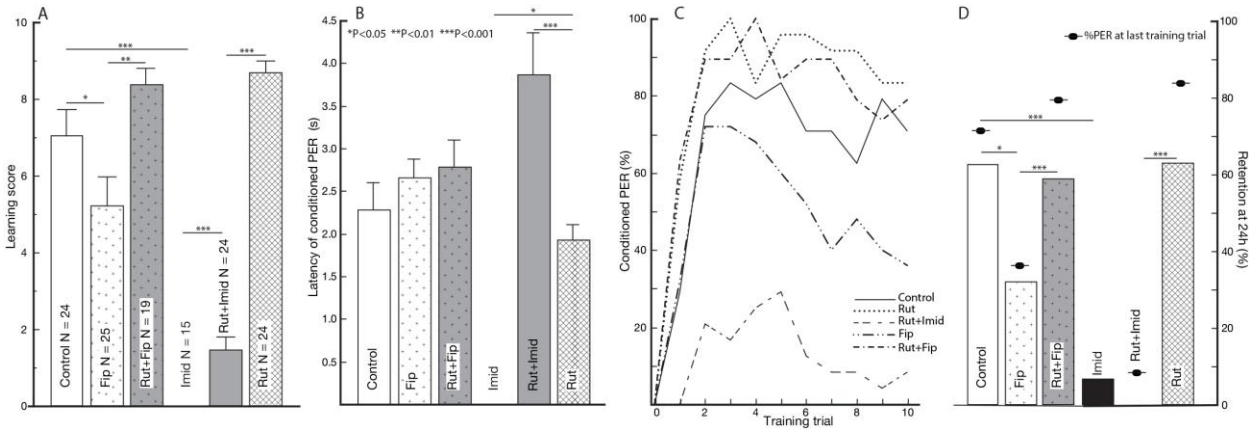

Fig. 3. ***Ad libitum*** **prophylactic administration of rutin protects learning and memory against impairment by chronic exposure to fipronil but not imidacloprid. (A)** Innocuous and protective effect of rutin. **(B)** Effect of feeding treatments on the latency of conditioned response of bees that responded at least twice. (**C**) Acquisition curves for bees in all treatments. **(D)** Effect of rutin administration on memory retention 40h after the last administration of rutin. (A), (C) and (D) include the same bees. Only statistically significant planned comparisons (see methods) are indicated to facilitate visualization. Error bars display standard error of the mean.

Table 1. Experimental design for the evaluation of cognitive protection against commercial forms of imidacloprid and fipronil

Experiment	Schedule	Treatments
1	*Days 1-3*: Bees assigned to one of two treatments: -Rut: 20 µL of 1 µM rut (12ng/bee) (6 doses) -Control: 20 µL of 1M sucrose (6 doses) *Day 4*: *Bees assigned to one of two treatments 2h before training:* -Imid: 20 µL of 5nM (0.03ng/bee) (1 dose) -Control: 20 µL of 1M sucrose (1 dose) *Olfactory conditioning* *Day 5*: Memory retention test	Control Rut Rut+Imid Imid
2	*Days 1-3*: Bees assigned to one of two treatments: -Rut: *Ad libitum* 1µM rut -Control: *Ad libitum* 1M sucrose *Day 4*: *Bees assigned to one of three treatments:* -Imid: 20 µL of 5nM (0.03ng/bee) (1 dose) -Fip: 20 µL of 0.11µM (1ng/bee) (1 dose) -Control: 20 µL of 1M sucrose (1 dose) *Olfactory conditioning* *Day 5*: Memory retention test	Control Rut Rut+Imid Rut+Fip Imid Fip
3	*Days 1-6*: Bees assigned to one of two treatments: -Rut: *Ad libitum* 1µM rut -Control: *Ad libitum* 1M sucrose *Day 4-6*: *Bees assigned to one of three treatments to replace one of two feeders:* -Imid: *Ad libitum* 5nM (1.3ppb) -Fip: *Ad libitum* 0.11µM (48ppb) -Control: *Ad libitum* 1M sucrose Day 7: Olfactory conditioning *Day 8*: Memory retention test	Control Rut Rut+Imid Rut+Fip Imid Fip