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RESEARCH ARTICLE

The flavonoid kaempferol protects the fruit fly *Drosophila melanogaster* against the motor impairment produced by exposure to the insecticide fipronil

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ABSTRACT

Exposure to pesticides across species has been associated with cognitive and motor impairments. As the problem impacts ecosystem stability, food production and public health, it is urgent to develop multifactorial solutions, from regulatory legislation to pharmacological alternatives that ameliorate the impairments. Fipronil, a commonly used insecticide, acts as a $GABA_A$ receptor ($GABA_AR$) antagonist and induces motor impairments in vertebrates and invertebrates. Here, we hypothesized that kaempferol, a secondary metabolite derived from plants, acting as an allosteric modulator of $GABA_ARs$, would protect against the negative effects induced by the administration of fipronil in adults of the fruit fly *Drosophila melanogaster*. We further evaluated our hypothesis via co-administration of flumazenil, a competitive antagonist on the $GABA_AR$, and through *in silico* analyses. We administered kaempferol prophylactically at three concentrations (10, 30 and 50 µmol l^{-1}) and evaluated its protective effects against motor impairments induced by fipronil. We then used a single dose of kaempferol (50 µmol l^{-1}) to evaluate its protective effect while administering flumazenil. We found that oral administration of fipronil impaired motor control and walking ability. In contrast, kaempferol was innocuous and protected flies from developing the motor-impaired phenotype, whereas the co-administration of flumazenil counteracted these protective effects. These results are supported by the binding of the ligands with the receptor. Together, our results suggest that kaempferol exerts a protective effect against fipronil via positive allosteric modulation of $GABA_ARs$, probably within brain areas such as the central complex and the mushroom bodies. These findings further support current attempts to use metabolites derived from plants as protectors against impairments produced by pesticides.

KEY WORDS: Motor control, Central complex, Mushroom bodies, GABA, Dopamine, Flumazenil

INTRODUCTION

For almost a century, the subtle, yet significant effects of exposure to sublethal levels of pesticides have been of concern because of their impacts ranging from food security to pollinator declines and public health. A renowned example is the association between neuroactive insecticides and the decline of bee populations (Goulson et al., 2015; Holder et al., 2018; Sánchez-Bayo and Wyckhuys, 2019). Neonicotinoids, insecticides acting as agonists of acetylcholine receptors (AChRs), are broadly used for pest control because of their presumed low affinity for human AChRs. However, the exposure to neonicotinoids impairs learning and memory, motor control and sensory sensitivity of beneficial species such as honey bees, even at very low dosages (Charreton et al., 2015; Decourtye et al., 2004; Li et al., 2019; Williamson et al., 2014). Similarly, in humans, exposure to pesticides is associated with a higher risk of developing neurocognitive diseases, such as Parkinson's disease (Elbaz et al., 2009; Godinho et al., 2016; Narayan et al., 2017; Ratner et al., 2014). These concerns have led to regulations and bans that must be supported by further understanding of the mechanisms of action of the pesticides and by strategies to protect animals, including humans, before and after exposure.

Among pesticides, fipronil indiscriminately affects invertebrates and vertebrates through the blockage of inhibitory pathways. Fipronil acts as a non-competitive antagonist of the $GABA_A$ receptor ($GABA_AR$), blocking inward currents of chloride ions (Ikeda et al., 2004; Zhao et al., 2003). Hence, not surprisingly, exposure to fipronil leads to neuronal hyperexcitation, loss of motor control and a decrease in memory retention (Bharatiya et al., 2020; Godinho et al., 2016; Park et al., 2016; Stehr et al., 2006; Suzuki et al., 2021; Zaluski et al., 2015). Moreover, exposure to fipronil leads to reduced dopamine and tyrosine hydroxylase levels, thus deteriorating motor activity (Bharatiya et al., 2020; Park et al., 2016), characterizing the so-called 'parkinsonian phenotype' in rats (Park et al., 2016). In fact, in humans, this impact on the GABAergic and dopaminergic pathways partially explains the association between the onset of parkinsonian traits such as tremors, dyskinesia and in some cases seizures following exposure to fipronil (Mohamed et al., 2004).

Over recent decades, flavonoids, secondary metabolites derived from plants (Jan and Abbas, 2018), have been recognized as inducers of physiological protection (Jung and Kim, 2018; Maher, 2017), antioxidants (Williams et al., 2004), anti-inflammatory agents (Hämäläinen et al., 2007), modulators of GABAergic activity (Benkherouf et al., 2019; Eghorn et al., 2014; Hall et al., 2014) and inducers of detoxification against xenobiotics (Bernklau et al., 2019; Liao et al., 2017). For example, in honey bees, prophylactic administration of quercetin and *p*-coumaric acid increases longevity in individuals exposed to low concentrations of imidacloprid (Wong et al., 2018), propiconazole and chlorantraniliprole (Liao et al., 2020). Also, in bumble bees, the prophylactic administration of rutin induces protection against the cognitive impairment produced by oral administration of

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imidacloprid and fipronil (Riveros and Gronenberg, 2022). However, the potential effect of flavonoids in the regulation of GABAergic pathways supporting motor control has not been addressed. Can prophylactic administration of a flavonoid protect against impairments to motor control produced by exposure to fipronil?

Here, our goal was threefold. First, we aimed to test the impairment produced by the administration of sublethal doses of fipronil on motor control in the fruit fly *Drosophila melanogaster*. We used the fruit fly because it has been a central model in the understanding of behavioral ecotoxicology and in the development of new drugs (Nagoshi, 2018; Tasman et al., 2021a,b). In *D. melanogaster*, exposure to pesticides negatively impacts reproduction (Tasman et al., 2021b), immunity (Daisley et al., 2017), memory (Tasman et al., 2021a) and locomotion (Chaudhuri et al., 2020), and induces neuronal death (Martelli et al., 2020). Moreover, *D. melanogaster* has been used to understand the effects of exposure to pesticides such as paraquat and rotenone in connection with the development of Parkinson's disease (Bastías-Candia et al., 2019; Coulom and Birman, 2004; Maher, 2019; Nagoshi, 2018; Pallanck and Whitworth, 2005; Park et al., 2012).

Second, we aimed to test the protective effect against fipronil of the prophylactic administration of the flavonol kaempferol, primarily recognized by its antiviral (Mitrocotsa et al., 2000), anti-inflammatory (Hämäläinen et al., 2007) and antioxidant (Grünz et al., 2012) effects. However, several accounts suggest an impact on the nervous system because of its role as anxiolytic (Grundmann et al., 2009; Grünz et al., 2012) and inducer of neuroprotective effects (Filomeni et al., 2012). Remarkably, kaempferol might act as a positive allosteric modulator of the $GABA_AR$ (Liu et al., 2018), as suggested by the abolishment of its protective effects following the co-administration of flumazenil (Grundmann et al., 2009), a competitive antagonist of the benzodiazepine site (Whitman and Amrein, 1995). Thus, we hypothesized that the allosteric modulation of the GABAergic pathway by kaempferol enhances the release of GABA, thus counteracting the impairment produced by fipronil.

As a third and final goal, we evaluated whether any protective effect produced by kaempferol was dose dependent and whether it would be absent in the presence of flumazenil. As a part of our third goal, we analyzed *in silico* the effect of administration of kaempferol, flumazenil and fipronil by focusing on the interaction between kaempferol, flumazenil and fipronil with the $GABA_AR$ in flies and humans.

MATERIALS AND METHODS

Subject maintenance

We commercially acquired wild-type fruit flies (*Drosophila melanogaster* Meigen 1830) from a local provider (Universidad Nacional de Colombia, Bogotá, Colombia) and reproduced them and maintained them under laboratory conditions (25°C, 12 h:12 h light:dark cycle). Flies were fed using a diet modified from the recipe proposed by the Bloomington *Drosophila* Stock Center (Indiana University at Bloomington). The diet is composed of agar (1.6 g), sucrose (5 g), corn starch (6 g), yeast (2 g) and water (to a final volume of 100 ml). Diet was supplemented with propionic acid (0.3% v/v) and replaced once a week if needed to avoid mold growth. For all experiments, we arbitrarily selected females (most likely not virgins as they were collected 3 days after eclosion; see below), aiming to decrease a potential confounding effect of sex.

Selection of concentrations of fipronil, kaempferol and flumazenil

The information available on doses of fipronil is scarce and whereas concentrations are reported, individual dosages are not available. Thus, we utilized our empirically determined dose of 1 ng per individual based on our separate accounts in honey bees and bumble bees indicating sublethal cognitive impairment (Riveros and Gronenberg, 2022; L. M. Garcia, V. Caicedo-Garzón and A.J.R., unpublished). In contrast, the concentrations of kaempferol were selected based on the protective and antioxidant effect demonstrated in a model of Parkinson's disease in *D. melanogaster* (Rahul et al., 2020). All solutions of kaempferol (CAS: 520-18-3; Sigma, St Louis, MO, USA) were obtained through dilutions of a sonicated (90% of maximum intensity) stock (170 µmol l^{-1}) and varied depending on the experiment between 10 and 50 µmol l^{-1}. Finally, as information on *in vivo* concentrations of flumazenil in *D. melanogaster* and other insects is scarce, we decided to adjust the concentration from the dose that reverses the anesthetic effect of midazolam, a benzodiazepine, and has no behavioral effects on the invertebrate *Daphnia pulex* (Dong et al., 2013).

Experiment 1: dose-dependent effect of kaempferol against fipronil

Three days after hatching, we collected females and individually kept them for 24 h of starvation in the wells of culture plates (2 ml volume per well). Following this period of starvation, we randomly assigned each fly to one of four feeding treatments (Table 1): sucrose water (Control: 146 mmol l^{-1} sucrose solution), 10 µmol l^{-1} kaempferol (K10), 30 µmol l^{-1} kaempferol (K30) or 50 µmol l^{-1} kaempferol (K50). After 22 h, we randomly assigned each fly to one of two treatments: 146 mmol l^{-1} sucrose water or 2.06 µmol l^{-1} fipronil (Fip; Astuto 200 scv; ∼0.9 ng of pesticide per fly). Thus, each fly belonged to one of eight treatments depending upon their feeding schedule: Control, Fip, K10, K30, K50, K10/Fip, K30/Fip, K50/Fip. In all cases, solutions were prepared by adding 1 µl of the main component (e.g. 1 µl of 30 µmol l^{-1} kaempferol) to a sucrose solution (final concentration of sucrose: 146 mmol l^{-1}). Lastly, we conducted evaluation of motor activity 4 h after the administration of fipronil (see below).

Table 1. Experimental design for the evaluation of cognitive protection against a commercial form of fipronil

Experiment	Schedule	Administration of kaempferol	Exposure to insecticide	Insecticide
1	Day 1: starvation	Prophylactic	Acute	Fip
	Day 2: Control, K10, K30, K50	1 dose of K10, K30 or K50	4 h before training	Fip
	Day 3: Fip, motor evaluation			
2	Day 1: starvation	Prophylactic	Acute	Fip
	Day 2: Control, K50, Flum, K50/Flum	1 dose of K50 or K50/Flum	4 h before training	Fip
	Day 3: Fip, motor evaluation			

Control: 1 µl 146 mmol l^{-1} sucrose; Fip, 1 µl 2.06 µmol l^{-1} fipronil; K10, 1 µl 10 µmol l^{-1} kaempferol; K30, 1 µl 30 µmol l^{-1} kaempferol; K50, 1 µl 50 µmol l^{-1} kaempferol; Flum, 1 µl 20 µmol l^{-1} flumazenil; K50/Flum, 1 µl 50 µmol l^{-1} kaempferol+1 µl 20 µmol l^{-1} flumazenil.

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Experiment 2: effect of co-administration of flumazenil on kaempferol protection against fipronil

Following our results from the first experiment, we tested whether the effect of kaempferol was due to its action as a positive allosteric modulator of $GABA_A$Rs. We selected the K50 treatment because its administration led to protection against the deleterious effects of fipronil in most behavioral assays.

Thus, 3 days after hatching, we collected females and individually kept them for 24 h of starvation in 24 well culture plates. Following starvation, we randomly assigned each fly to one of four feeding treatments: sucrose water (Control: 146 mmol l^{-1} sucrose solution), 50 µmol l^{-1} kaempferol (K50), 20 µmol l^{-1} flumazenil (Flum; Diazenil) or 50 µmol l^{-1} kaempferol+20 µmol l^{-1} flumazenil (K50/Flum). After 22 h, we randomly assigned each fly to one of two treatments: 146 mmol l^{-1} sucrose water or 2.06 µmol l^{-1} fipronil (Fip; Astuto 200 scv; ∼0.9 ng of pesticide per fly). Thus, each fly belonged to one of eight treatments depending upon their feeding schedules across the three phases: Control, K50, Flum, K50/Flum, Fip, Flum/Fip, K50/Fip, K50/Flum/Fip. In all cases, solutions were prepared by adding 1 µl of the main component (e.g. 1 µl of 50 µmol l^{-1} kaempferol) to a sucrose solution (final concentration of sucrose: 146 mmol l^{-1}). Lastly, we conducted the evaluation of motor activity 4 h after the administration of fipronil.

Motor evaluation

Open-field tests

We evaluated the tendency of the flies to walk and remain on the border of a container by recording their individual trajectories within the cell for 1 min (30 frame s^{-1}, iPhone 6, Apple Inc.). The distance and trajectory of each fly inside the cell were analyzed using the plugin *Mtrack* and the Scholl's analysis from *Fiji* (sampling rate: 5 Hz; Schindelin et al., 2012). We defined the 'edge' as a region 0.3 cm from the wall (one body length) and counted the total number of intersections outside this range (Fig. 1A). We counted the number of flies that completed at least one lap inside the edge region (n_{onelap}). Then, the border preference index (BPI) was defined as $[(n_{onelap}/n_{total})\times100]$. The time spent walking was recorded manually from the videos using a chronometer. All treatments were represented during each run of the test.

Negative geotaxis test

We transferred each fly to an individual lane of a 3D printed arena and allowed them to acclimate for 5 min before conducting the test (Fig. 1B). The arena consisted of 15 lanes (each lane 7.5×0.4×0.2 cm) covered with three microscope slides. Once flies were in the arena, we taped it to displace the flies to the bottom. We recorded the flies using a camera (30 frames s^{-1}, iPhone 6) and measured the walking distance after 10 s using *Fiji* (Schindelin et al., 2012). We further calculated the percentage of flies below a threshold of 5 cm. All treatments were represented during each run of the test and each fly was recorded once.

Experiment 3: bioinformatic analysis of the interaction between ligands and the $GABA_A$R

Homology modeling

We aligned the *D. melanogaster* $GABA_A$R ($DGABA_A$R) amino acid sequence obtained from NCBI (NP_523991.2) against the PDB database to find the most suitable template (PDB 5TIN). We generated 3D homology modeling of $DGABA_A$R using the program MODELLER 9.3 (Webb and Sali, 2016). We then scored 10 models using MODELLER's DOPE scoring function and selected the lowest for further analysis. Finally, we selected the best model after validation of the alignment using the stereochemical quality (Ramachandran plot) and by comparing the *Z*-score of the modeled $GABA_A$R with the *Z*-scores of the experimental structures of proteins of the same size.

Molecular dockings

We conducted molecular dockings of the interaction between kaempferol, flumazenil, fipronil and fipronil sulfone, the principal metabolite of fipronil, which is also toxic (Suzuki et al., 2021), with the allosteric site of $GABA_A$R from *D. melanogaster* and humans using the open-source software *Chimera* UCSF (v.1.15 for Mac; Pettersen et al., 2004). We performed the analyses using *Autodock vina* (v.1.1.2 for Mac; Trott and Schroer, 2010). The binding site of flumazenil and kaempferol was located between the residues Ser205 and Tyr58 in *D. melanogaster*. The binding site for fipronil and fipronil sulfone both in *D. melanogaster* and humans in $GABA_A$ was located around the centroid of the residues −2′ and 2 from the five chains.

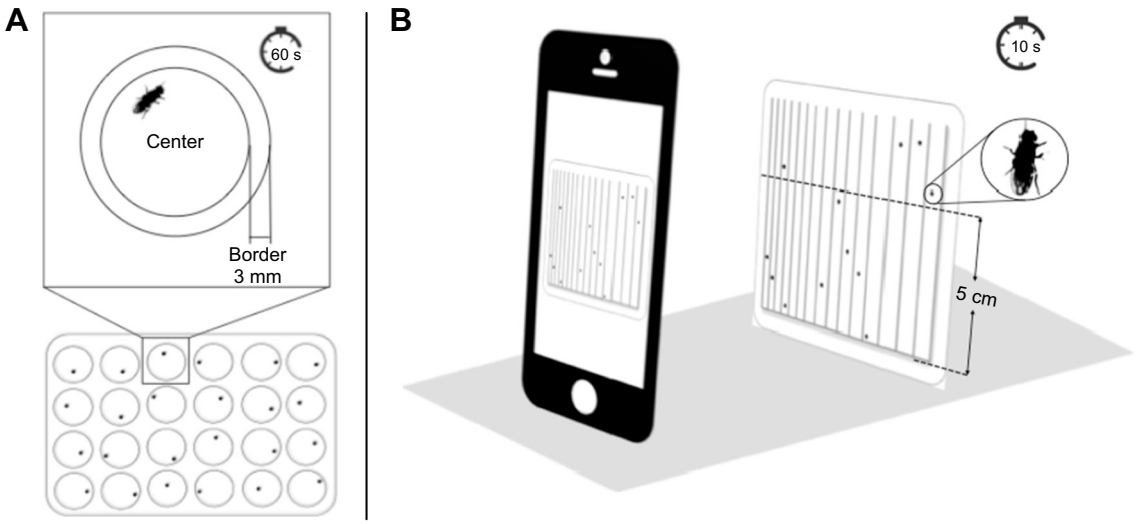

Fig. 1. Experimental setup for motor evaluation. (A) The behavior of individual flies inside the well was recorded for 60 s. We calculated the distance traveled, border preference index (BPI) and number of intersections. (B) Each fly was transferred to an individual lane and, after 10 s, the total distance traveled and percentage of flies below 5 cm were calculated.

Data analyses

We analyzed the normality and homoscedasticity parameters of continuous variables (distance, time, velocity) using a Kolmogorov–Smirnov and Levene test, respectively (Massey, 1951; Glass, 1966). Data exhibiting a normal distribution and homoscedasticity were analyzed using an ANOVA followed by multiple *t*-test planned comparisons (Student, 1908). Of interest were the following comparisons (depending on the experiment, see above): Control versus Fip to evaluate the effect of the insecticide, Control versus K (10, 30, 50) to evaluate the innocuousness of kaempferol, K (10, 30, 50) versus K (10, 30, 50)/Fip, to test whether there was full protection against Fip, and Fip versus K (10, 30, 50)/Fip, Fip versus K50/Flum/Fip to test whether there was any significant protection. If the data did not exhibit a normal distribution, we compared the treatments using a Kruskal–Wallis test (Hoffman, 2019) followed by the Wilcoxon signed-rank test (for planned comparisons, see above; Woolson, 2008). Nominal variables (e.g. yes/no climbed above threshold or inside the edge region) were analyzed using a chi-square test (Tallarida and Murray, 1987). We identified and excluded outliers after a single run of the Mahalanobis distance test (Rousseeuw and van Zomeren, 1990). Analyses of behavioral results were performed in JMP v.14.2 (SAS Institute). In all cases, error associated with multiple comparisons was corrected with the false discovery rate (FDR) method (Benjamini and Hochberg, 1995) and the corrected *P*-values (*q*-values) are presented.

RESULTS

Experiment 1: dose-dependent effect of kaempferol against fipronil

Open field-test

We collected, maintained and evaluated 206 flies. Some flies were excluded following an outlier analysis (see above). Thus, for our final analysis, we included 194 flies distributed across eight treatments: Control (N=26), K10 (N=23), K30 (N=28), K50 (N=19), Fip (N=23), K10/Fip (N=26), K30/Fip (N=27) and K50/Fip (N=22). Fig. 2 shows the results of the open field test for experiment 1, as trajectories, number of intersections, BPI, distance traveled, time walking and average speed.

We found that the trajectory patterns differed among treatments in terms of BPI and the number of intersections (Kruskal–Wallis test: χ^2_3=34.21, $P\leq$0.0001). Flies fed with fipronil (Fip group) changed their trajectory patterns relative to Control flies (Fig. 2A–C). For instance, Control flies followed circular trajectories, whereas Fip flies typically exhibited trajectories often distanced from the wall. Thus, the trajectories of Fip flies had more intersections than those of Control flies (mean±s.e.m. number of intersections: Control 1.6±0.47, Fip 6.5±1.05; Wilcoxon test: Z=−4.292, P=0.039; Fig. 2B) and shorter BPI ($\chi^2_{1,47}$=19.67, P=0.012; Fig. 2C). Additionally, we found that the administration of fipronil and kaempferol affected the distance traveled (ANOVA: $F_{7,186}$=5.46, P<0.0001), time (Kruskal–Wallis test: χ^2_3=57.18, P<0.0001) and speed (Kruskal–Wallis test: χ^2_3=21.57, P=0.003). For instance, flies in the Fip group spent a significantly shorter time walking and, when moving, traveled significantly shorter distances than flies fed with sucrose solution (Fip versus Control: mean±s.e.m. time: Control 36.92±2.7 s, Fip 15.13±3.21 s; Wilcoxon test: Z=−4.77, P=0.0005; Fig. 2E; mean±s.e.m. distance: Control 20.45±1.4 cm, Fip 12.10±1.11 cm; t_{182}=−4.92, P=0.0005; Fig. 2D). Interestingly, Fip flies were significantly faster than Control flies (Control 0.58±0.03 cm s^{-1}, Fip 1.02±1.02 cm s^{-1}; Wilcoxon test: Z=2.57, P=0.025; Fig. 2F).

In contrast, administration of kaempferol was innocuous to the flies at all concentrations. Flies across all concentrations of kaempferol exhibited trajectory patterns that did not differ from those of Control flies (mean±s.e.m. number of intersections: K10 2.78±0.65; Wilcoxon test: Z=−0.50, P=0.61; K30 2.73±0.7; Wilcoxon test: Z=−1.15, P=0.31; K50 2.5±0.6; Wilcoxon test: Z=−1.17, P=0.31; Fig. 2A,B; BPI: K10: $\chi^2_{1,51}$=0.38, P=0.56; K30: $\chi^2_{1,56}$=0.487, P=0.60; K50: $\chi^2_{1,47}$=0.02, P=0.89; Fig. 2A,C). Further, flies in the K10, K30 and K50 groups exhibited a performance in terms of distance, time and speed of trajectories that did not differ from that of Control flies (mean±s.e.m. distance: Control 20.45±1.4 cm, K10 19.2±1.04 cm; t_{182}: 0.71, P=0.096; K30 18.37±1.45 cm; t_{182}: 1.65, P=0.14; K50 18.33±0.67 cm; t_{182}: 1.19, P=0.24; Fig. 2D; mean±s.e.m. time: Control 36.92±2.7 s, K10 36.17±2.21 s; Wilcoxon test: Z=0.06, P=0.952; K30 37.32±2.57 s; Wilcoxon test: Z=0.06, P=0.952; K50 37.75±2.34 s; Wilcoxon test: Z=−0.66, P=0.64; Fig. 2E; mean±s.e.m. speed: Control 0.58±0.03 cm s^{-1}, K10 0.65±0.68 cm s^{-1}; Wilcoxon test: Z=0.27, P=0.23; K30 0.50±0.03 cm s^{-1}; Wilcoxon test: Z=2.13, P=0.062; K50 0.51±0.04 cm s^{-1}; Wilcoxon test: Z=2.33, P=0.05; Fig. 2F).

Most remarkably, the prophylactic administration of kaempferol led to an overall dose-dependent performance improvement in flies exposed to fipronil. Flies in the K50/Fip treatment followed trajectories that did not differ from those of Control flies in the total number of intersections or BPI (mean±s.e.m. number of intersections: K50 2.5±0.6, K50/Fip 4.32±1.35; Wilcoxon test: Z=0.7221, P=0.522; BPI: $\chi^2_{1,41}$=1.420, P=0.17; Fig. 2A–C), but significantly differed from those of Fip flies (mean±s.e.m. number of intersections K50/Fip 4.32±1.35; Wilcoxon test: Z=0.72, P=0.045; BPI: $\chi^2_{1,41}$=1.42, P=0.007; Fig. 2A–C), demonstrating full protection. However, this protection was not observed when we prophylactically administered the lower concentrations of kaempferol in the K10/Fip or the K30/Fip flies (Fip versus 10/30K/Fip: mean±s.e.m. number of intersections: K10/Fip 5.42 ±1.03; Wilcoxon test: Z=−0.867, P=0.31; K30/Fip 7.90±1.29; Wilcoxon test: Z=0.722, P=0.31; Fig. 2A,B; BPI: K10/Fip: $\chi^2_{1,49}$=11.31, P=0.08; K30/Fip: $\chi^2_{1,55}$=8.20, P=0.13; Fig. 2A,C). Importantly, flies in the K10/Fip, K30/Fip and K50/Fip groups had walking distances that did not significantly differ from those of Fip flies (mean±s.e.m. distance: K10/Fip 14.61±1.28 cm; t_{182}=−148, P=0.12; K30/Fip 15.06±1.27 cm; t_{182}=−0.85, P=0.22; K50/Fip 13.86±1.29 cm; t_{182}=−1.0, P=0.2; Fig. 2D), but significantly differed from those of Control flies (K10/Fip: t_{182}=3.56, P=0.02; K30/Fip: t_{182}=4.10, P=0.02; K50/Fip: t_{182}=3.84, P=0.02; Fig. 2D). Further, flies that were fed with kaempferol spent a significantly longer time walking than flies in the Fip group (mean±s.e.m. time: Fip 7.37±0.62 s, K10/Fip 24.58±3.09 s; Wilcoxon test: Z=−2.0, P=0.004; K30/Fip 23.04±1.68 s; Wilcoxon test: Z=−3.0, P=0.003; K50/Fip 26.95±2.66 s; Wilcoxon test: Z=−3.32, P=0.002; Fig. 2E), but a significantly shorter time than their respective controls (K10/Fip: Wilcoxon test: Z=2.77, P=0.012; K30/Fip: Wilcoxon test: Z=3.96, P=0.002; K50/Fip: Wilcoxon test: Z=2.44, P=0.027; Fig. 2E). We further observed protection when evaluating the speed of K30/Fip and K50/Fip flies (30/50K/Fip versus Fip: mean±s.e.m. speed K30/Fip 0.75±0.11 cm s^{-1}; Wilcoxon test: Z=2.10, P=0.049; K50/Fip 0.68±0.15 cm s^{-1}; Wilcoxon test: Z=2.67, P=0.025; Fig. 2F). Additionally, protected flies walked at speeds that did not differ significantly from those of Controls (K10/Fip: Wilcoxon test: Z=−0.70, P=0.232; K30/Fip: Wilcoxon test: Z=−0.57, P=0.062; K50/Fip: Wilcoxon test: Z=0.55, P=0.232; Fig. 2F). These results suggest that K30 and K50 are the most protective concentrations, while K10 seems to lead to an intermediate level of protection.

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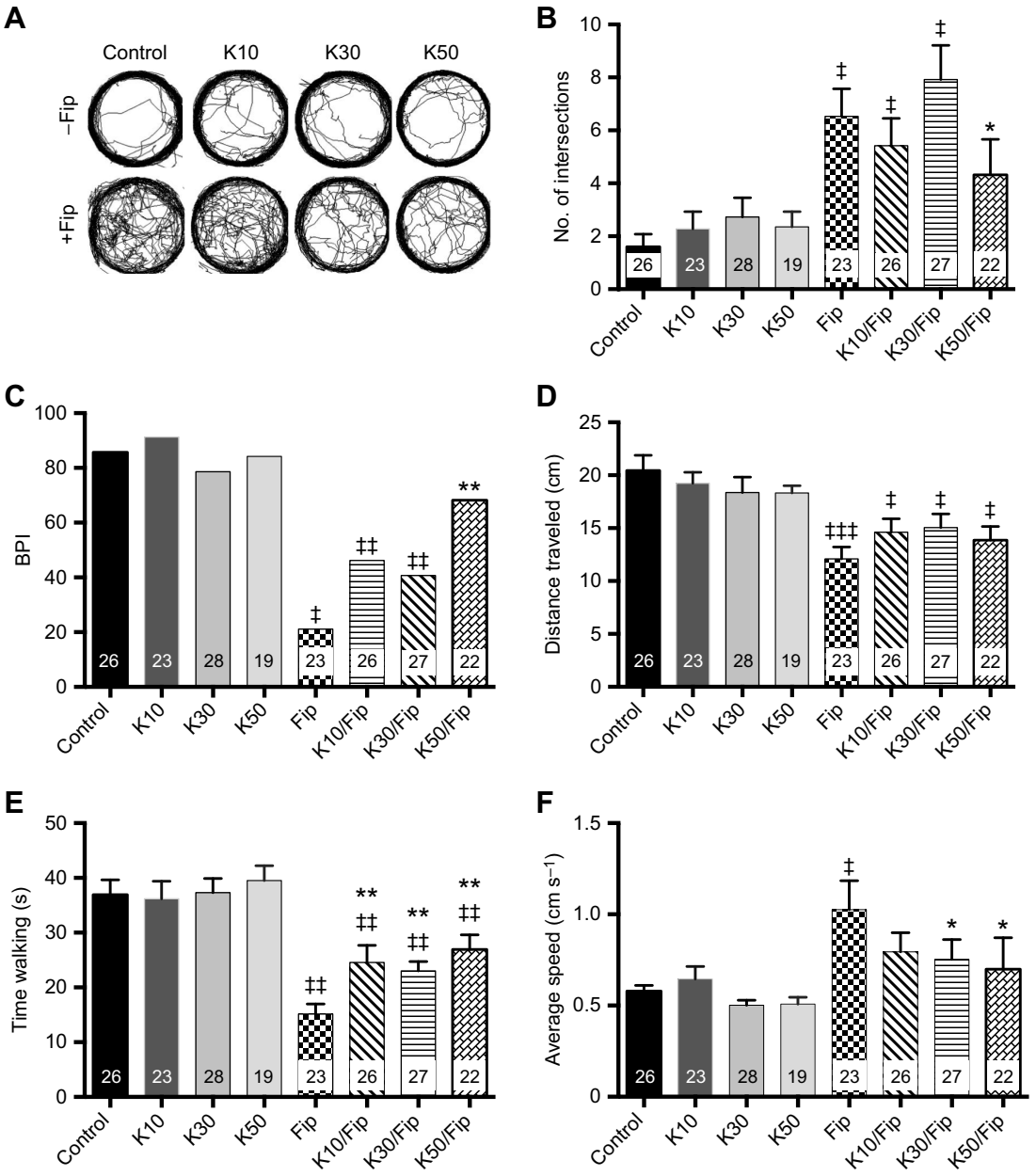

Fig. 2. Effects of kaempferol and fipronil on walking control. (A) Overlapping trajectories within the circular cell for flies in the four feeding treatments [control and 10, 30 or 50 μmol l^{-1} kaempferol (K10, K30, K50)] with or without fipronil treatment (Fip). (B) Number of intersections of trajectories with an imaginary circular region one body length away from the border. (C) BPI. (D) Distance traveled. (E) Time walking. (F) Average speed. Data are means±s.e.m. **P*<0.05, ***P*<0.01 compared with flies in the Fip group; ‡*P*<0.05, ‡‡*P*<0.01, ‡‡‡*P*<0.001 compared with flies in the Control group. *P*-values are reported after false discovery rate (FDR) correction. Only significant planned comparisons are shown (see Materials and Methods). Sample sizes (*N*) are given within the bars.

Negative geotaxis test

We evaluated geotaxis in a total of 192 flies distributed across eight treatments: Control (*N*=25), Fip (*N*=25), K10 (*N*=23), K30 (*N*=25), K50 (*N*=19), K10/Fip (*N*=22), K30/Fip (*N*=28), K50/Fip (*N*=25). We found that feeding flies with fipronil or kaempferol had an effect on the distance climbed (Kruskal–Wallis test: χ^2_3=48.70, *P*<0.0001). Flies in the Fip group climbed shorter distances compared with flies in the Control group (mean±s.e.m. distance: Control 5.43±0.40 cm, Fip 1.26±0.25 cm; Wilcoxon test: *Z*=−5.292, *P*=0.003; Fig. 3A). Also, 78.9% of flies in the Fip group remained below the 5 cm threshold while only 14.3% of the flies in the Control group remained below 5 cm ($\chi^2_{1,50}$=4.348, *P*=0.003; Fig. 3B). Prophylactic treatment with K10 and K50 increased the distance traveled but flies in the K30/Fip treatment did not differ from those in the Fip group (mean±s.e.m. distance: K10/Fip 3.23±0.54 cm; Wilcoxon test: *Z*=2.59, *P*=0.01; K30/Fip 2.88±0.52 cm; Wilcoxon test: *Z*=1.67, *P*=0.08; K50/Fip 3.22±0.44 cm; Wilcoxon test: *Z*=3.44, *P*=0.003; Fig. 3A). Moreover, all groups receiving kaempferol showed a decrease in the percentage of flies below the 5 cm threshold compared with the Fip group (K10/Fip: $\chi^2_{1,46}$=9.83, *P*=0.003; K30/Fip: $\chi^2_{1,53}$=9.68, *P*=0.003; K50/Fip: $\chi^2_{1,49}$=7.12, *P*=0.01; Fig. 3B). Notably, only the K50/Fip group behaved similar to its control in distance (mean±s.e.m. distance: K10/Fip 3.23±0.54 cm; Wilcoxon test: *Z*=−2.53, *P*=0.012; K30/Fip 2.88±0.52 cm;

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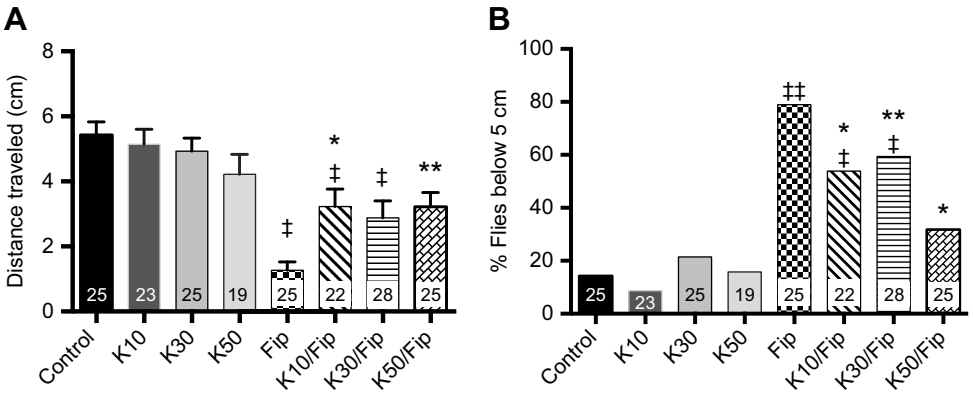

Fig. 3. Effects of kaempferol on the negative geotactic response after exposure to fipronil. (A) Distance traveled. (B) Percentage of flies below the 5 cm threshold. Data are means±s.e.m. *P<0.05, **P<0.01 compared with flies in the Fip group; ‡P<0.05, ‡‡P<0.01 compared with flies in the Control group. P-values are reported after FDR correction. Sample sizes (N) are given within the bars.

Wilcoxon test: Z=−2.59, P=0.012; K50/Fip 3.22±0.43 cm; Wilcoxon test: Z=−1.24, P=0.15; Fig. 3A) and the percentage of flies below 5 cm (K10/Fip: $\chi^2_{1,46}$=6.88, P=0.02; K30/Fip: $\chi^2_{1,53}$=8.40, P=0.02; K50/Fip: $\chi^2_{1,49}$=10.8, P=0.09; Fig. 3B), suggesting partial protection by kaempferol at the lowest concentrations. Importantly, flies exposed to kaempferol did not significantly differ in distance traveled or percentage of flies below the 5 cm threshold from those in the Control group (mean±s.e.m. distance: K10 5.14±0.40 cm; Wilcoxon test: Z=−0.730, P=0.47; K30 4.93±0.40 cm; Z=−1.24, P=0.24; K50 4.22±0.62 cm; Z=−1.44, P=0.19; percentage of flies below 5 cm: K10: $\chi^2_{1,49}$=0.16, P=0.69; K30: $\chi^2_{1,50}$=0.37, P=0.60; K50: $\chi^2_{1,44}$=2.76, P=0.12; Fig. 3).

Experiment 2: effect of co-administration of flumazenil on kaempferol protection against fipronil

Open field

We collected, maintained and evaluated 266 flies. Some flies were excluded following an outlier analysis (see above). Thus, for our final analysis, we included 225 flies distributed across eight treatments: Control (N=31), K50 (N=33), Flum (N=31), K50/Flum (N=28), Fip (N=24), K50/Fip (N=30), K50/Flum/Fip (N=20), Flum/Fip (N=28). Fig. 4 shows the results of the open field test for experiment 2, as trajectories, number of intersections, BPI, distance traveled, time walking and average speed.

We found that the administered compounds affected the distance traveled (ANOVA: $F_{7,217}$=19.11, P<0.0001), time spent walking (Kruskal–Wallis test: χ^2_3=120.4, P<0.0001), speed (Kruskal–Wallis test: χ^2_3=55.50, P<0.0001) and trajectory patterns (intersections: Kruskal–Wallis test: χ^2_3=24, P=0.001). We found that the co-administration of flumazenil to the flies counteracted the protective effect of administration of kaempferol (K50/Fip versus K50/Flum/Fip) in terms of the number of intersections (mean±s.e.m. number of intersections: K50Flum/Fip 4.15±0.89, K50/Fip 1.80±0.34; Wilcoxon test: Z=1.95, P=0.04; Fig. 4A,B), walking time (mean±s.e.m. time: K50Flum/Fip 8.65±0.98 s, K50/Fip 15.67±1.58 s; Wilcoxon test: Z=3.12, P=0.002; Fig. 4E) and speed (mean±s.e.m. speed K50Flum/Fip 0.90±0.05 cm s^{-1}, K50/Fip 0.88±0.004 cm s^{-1}; Wilcoxon test: Z=2.68, P=0.02; Fig. 4F). Interestingly, the BPI and the total distance walked by flies in the K50/Flum/Fip group were not significantly different from those of flies in the K50/Fip group (BPI: $\chi^2_{1,50}$=3, P=0.08; Fig. 4D; mean±s.e.m. distance: K50/Flum/Fip 8.77±0.96 cm, K50/Fip 12.11±1.12 cm; Wilcoxon test: Z=−1.73, P=0.08; Fig. 4E). Thus, flies receiving co-treatment with flumazenil seemed to emulate the performance of flies receiving fipronil alone (mean±s.e.m. number of intersections: Fip 4.04±0.77; Wilcoxon test: Z=0.097, P=0.55; Fig. 4A,B; BPI: $\chi^2_{1,44}$=1.13, P=0.33; Fig. 4A,C; mean±s.e.m. distance: Fip 8.38±0.74 cm; Wilcoxon test: Z=0.80, P=0.43; Fig. 4D; mean±s.e.m. time: Fip 7.37±0.62 s; Wilcoxon test: Z=1.40, P=0.19; Fig. 4E; mean±s.e.m. speed: Fip 1.15±0.07 cm s^{-1}; Wilcoxon test: Z=−0.91, P=0.22; Fig. 4F).

Co-treatment with flumazenil led to a shorter time walking and a lower speed but not to differences in trajectory patterns or distance compared with flies in the Control group (mean±s.e.m. number of intersections: Control 1.065±0.29, K50/Flum 1.71±0.40; Wilcoxon test: Z=−1.18, P=0.38; Fig. 4A,B; BPI: $\chi^2_{1,60}$=0.35, P=0.55; Fig. 4C; mean±s.e.m. distance: Control 20.03±1.31 cm, K50/Flum 17.58±1.33 cm; Wilcoxon test: Z=1.34, P=0.40; Fig. 4D; mean±s.e.m. time: Control 30.06±1.90 s, K50/Flum 22.96±2.14 s; Z=2.06, P=0.02; Fig. 4E; mean±s.e.m. speed: Control 0.69±0.03 cm s^{-1}, K50/Flum 0.83±0.04 cm s^{-1}; Wilcoxon test: Z=2.22, P=0.03; Fig. 4F). Importantly, flumazenil alone did not reverse the effects of fipronil; in fact, flies in the Flum/Fip group did not perform differently from flies in the Fip group (mean±s.e.m. number of intersections: Flum/Fip 3.36±0.76; Z=−1.35, P=0.15; Fig. 4A,B; BPI: $\chi^2_{1,52}$=1.82, P=0.12; Fig. 4A,C; mean±s.e.m. distance: Flum/Fip9.38±0.66 cm; Z=0.80, P=0.29; mean±s.e.m. time: Flum/Fip 8.96±0.71 s; Z=1.40, P=0.1; Fig. 4D; mean±s.e.m. speed: Flum/Fip 1.14±0.08 cm s^{-1}; Z=−0.91, P=0.40; Fig. 4F). Additionally, flies in the Flum group behaved similar to flies in the Control group, except for time walking and speed (mean±s.e.m. number of intersections: Control 1.06±0.29, Flum 1.13±0.31; Z=−1.68, P=0.35; Fig. 4A,B; BPI: $\chi^2_{1,63}$=2.91, P=0.12; Fig. 4A,C; mean±s.e.m. distance: Control 20.03±1.31 cm, Flum 18.10±1.24 cm; Z=0.23, P=0.40; Fig. 4D; mean±s.e.m. time: Control 30.06±1.90 s, Flum 23.45±1.98 s; Z=2.52, P=0.02; Fig. 4E; mean±s.e.m. speed: Control 0.69±0.03 cm s^{-1}, Flum 0.84±0.04 cm s^{-1}; Z=−2.65, P=0.02; Fig. 4F).

Negative geotaxis test

We evaluated geotaxis in a total of 247 flies distributed across eight treatments: Control (N=28), K50 (N=37), Flum (N=32), K50/Flum (N=28), Fip (N=31), K50/Fip (N=30), K50/Flum/Fip (N=30), Flum/Fip (N=31). We found that these compounds affected the distance climbed (Kruskal–Wallis test: χ^2_3=38.48, P<0.0001). For instance, the co-administration of flumazenil and kaempferol significantly reduced the distance climbed and increased the percentage of flies under the 5 cm threshold compared with that in the K50/Fip group (mean±s.e.m. distance: K50/Fip 3.42±0.48 cm, K50/Flum/Fip 2.08±0.42 cm; Z=−2.08, P=0.04; Fig. 5A; percentage of flies below 5 cm: $\chi^2_{1,58}$=8.73, P=0.03; Fig. 5B). Moreover, flies in the K50/Flum/Fip group did not differ from those in the Fip group in terms of distance or percentage of flies below the 5 cm threshold

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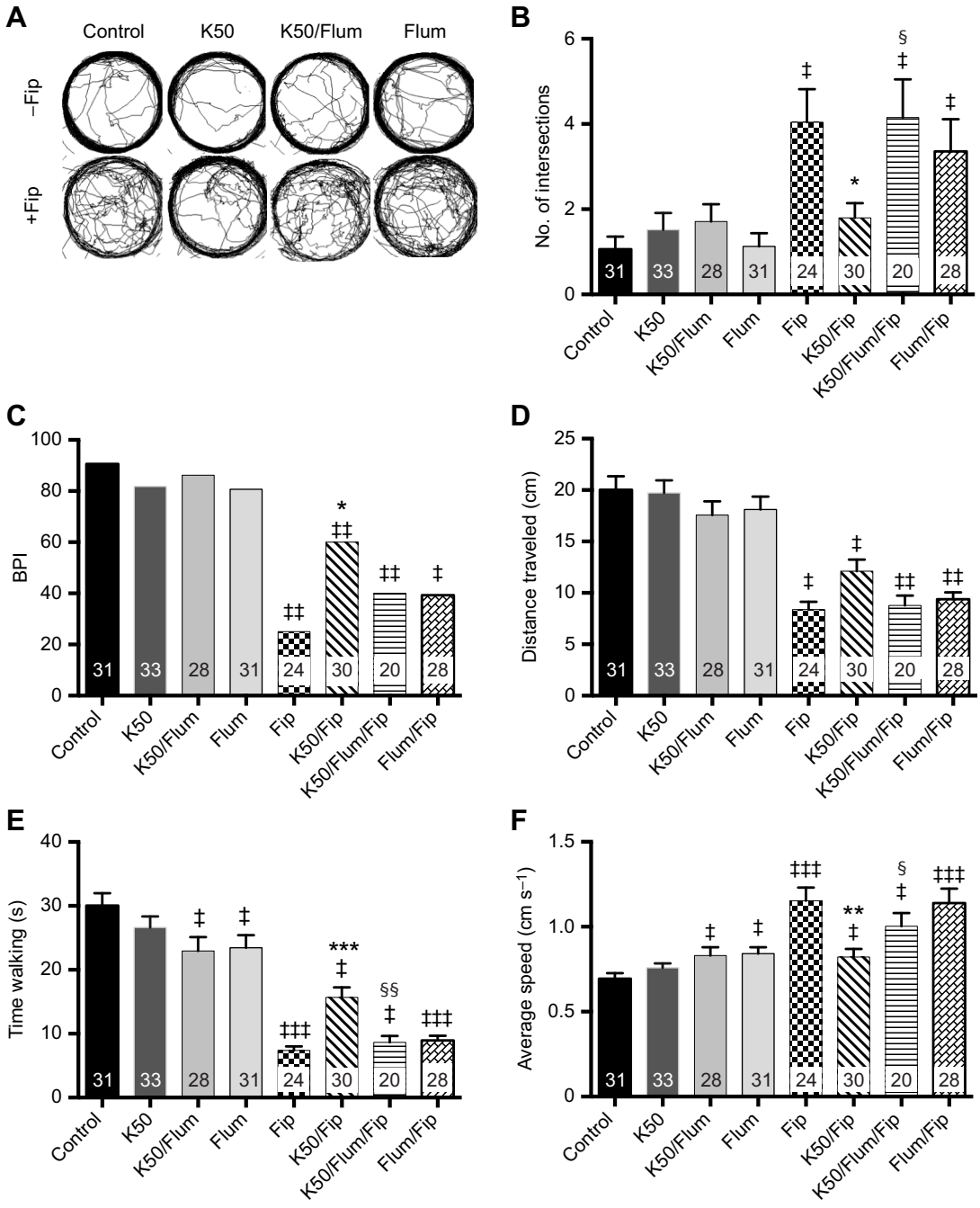

Fig. 4. Effects of co-administration of flumazenil, kaempferol and fipronil on walking control. (A) Overlapping trajectories within the circular cell for flies in the four treatment groups [control, 50 μmol l^{-1} kaempferol (K50), 20 μmol l^{-1} flumazenil (Flum) and 50 μmol l^{-1} kaempferol+20 μmol l^{-1} flumazenil (K50/Flum)], with or without fipronil treatment. (B) Number of intersections of trajectories with an imaginary circular region one body length away from the border. (C) BPI. (D) Distance traveled. (E) Time walking. (F) Average speed. Data are means±s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.001 compared with flies in the Fip group; §*P*<0.05, §§*P*<0.001 relative to flies in the K50/Fip group; and ‡*P*<0.05, ‡‡*P*<0.01, ‡‡‡*P*<0.001 compared with flies in the Control group. *P*-values are reported after FDR correction. Only significant planned comparisons are shown (see Materials and Methods). Sample sizes (*N*) are given within the bars.

(mean±s.e.m. distance: K50/Flum/Fip 2.077±0.22 cm, Fip 2.14±0.42 cm; Z=0.62, P=0.36; percentage of flies below 5 cm: $\chi^2_{1,62}$=0.1, P=0.40; Fig. 5A,B). Importantly, administration of flumazenil alone did not have any effect against fipronil (Flum/Fip versus Fip: mean±s.e.m. distance: Flum/Fip 2.47±0.45 cm; Z=0.62, P=0.42; Fig. 5A; percentage of flies below the 5 cm threshold: $\chi^2_{1,62}$=0.10, P=0.47; Fig. 5B). Finally, the performance of flies in the Flum group did not significantly differ from that of flies in the Control group (mean±s.e.m. number of intersections: Flum 1.13±0.31, Control 1.06±0.29; Z=−0.05, P=0.959; Fig. 5A; BPI: $\chi^2_{1,60}$=0.58, P=0.50; Fig. 5B).

Experiment 3: bioinformatic analysis of the interaction between ligands and the $GABA_AR$

Homology modeling

We found that 88.3% of residues from the modeled $DGABA_AR$ were in the most favorable area, 10.8% were in the allowed regions, 0.5% were in the generously allowed regions and just 0.4% were in

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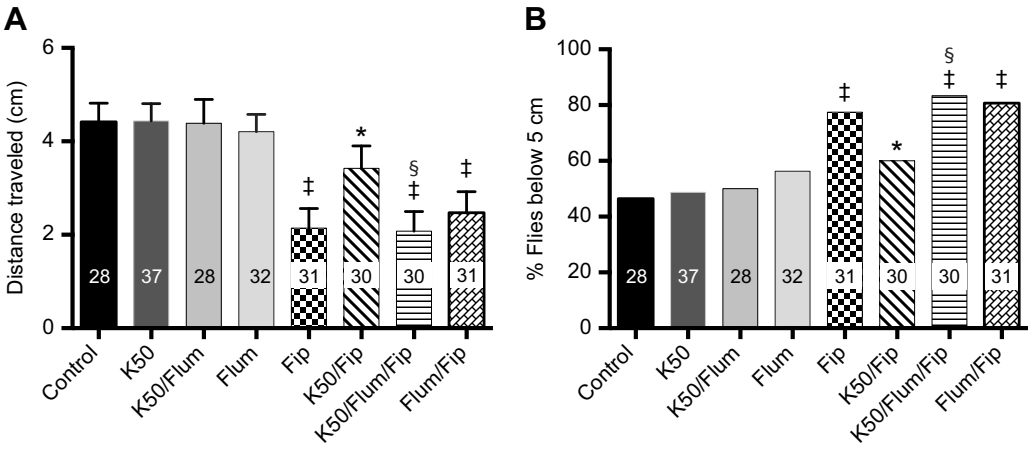

Fig. 5. Effects of co-administration of flumazenil and kaempferol on the negative geotactic response after exposure to fipronil. (A) Distance traveled. (B) Percentage of flies below the 5 cm threshold. Data are means±s.e.m. **P*<0.05 relative to flies in the Fip group; §*P*<0.05 relative to flies in the K50/Fip group; ‡*P*<0.05 relative to the corresponding control. *P*-values are reported after FDR correction. Sample sizes (*N*) are given within the bars.

the disallowed region (Ramachandran plot; Fig. 6A). The *Z*-scores of the modeled DGABA$_A$R were similar to those obtained by NMA or X-ray data of protein of similar size (Fig. 6B). We considered that the model was of good quality as the *Z*-scores relied on the range of similar proteins even though the percentage of residues in the most favorable area in the Ramachandran plot was below 90%. However, we took special care in subsequent steps for docking preparation.

Molecular dockings

We docked kaempferol and flumazenil to the allosteric site, and fipronil and fipronil sulfone to the antagonist site on DGABA$_A$R (Fig. 7A,B). We found that flumazenil and kaempferol bond to the DGABA$_A$R in the canonical allosteric site in the extracellular domain. Flumazenil, the competitive antagonist of benzodiazepines at the GABA$_A$R (Whitman and Amrein, 1995), binds to the DGABA$_A$R through the interaction between the Y254, L249, Y90, Y109, F206 and S205 residues with a free energy (ΔG) of −8.5 kcal mol^{-1} (Fig. 7C). Kaempferol forms hydrogen bonds with the S205 and S176 residues with the OH-group of the B ring and with the oxygen from the C ring (ΔG=−8.3 kcal mol^{-1}; Fig. 7D). We corroborated that fipronil and its principal metabolite fipronil sulfone binds to the transmembrane domain (TMD) of the five chains of the receptor (Zheng et al., 2014). Fipronil and fipronil sulfone bind with the trifluoromethyl group directly to the intracellular domain and interact with the 2′ Ala, 6′ Thr and 9′ Leu residues from the five chains (Fipronil: ΔG=−8.1 kcal mol^{-1}, fipronil sulfone: ΔG=−7.9 kcal mol^{-1}; Fig. 7E,F).

Interestingly, we found that kaempferol binds to the allosteric site in human GABA$_A$R and corroborated that fipronil binds to the TMD of human GABA$_A$R (Ci et al., 2007). Also, we found that fipronil sulfone binds to the GABA$_A$R. Kaempferol forms hydrogen bonds with the S205 residue with oxygen from the A and C rings, and interacts with Y160, Y58, F100, R144 and F77 from the binding pocket (ΔG=−8.7 kcal mol^{-1}; Fig. 8A). Fipronil and fipronil sulfone bind to the TMD via the trifluoromethyl group directly to the intracellular domain through interaction with L259, T256, A252

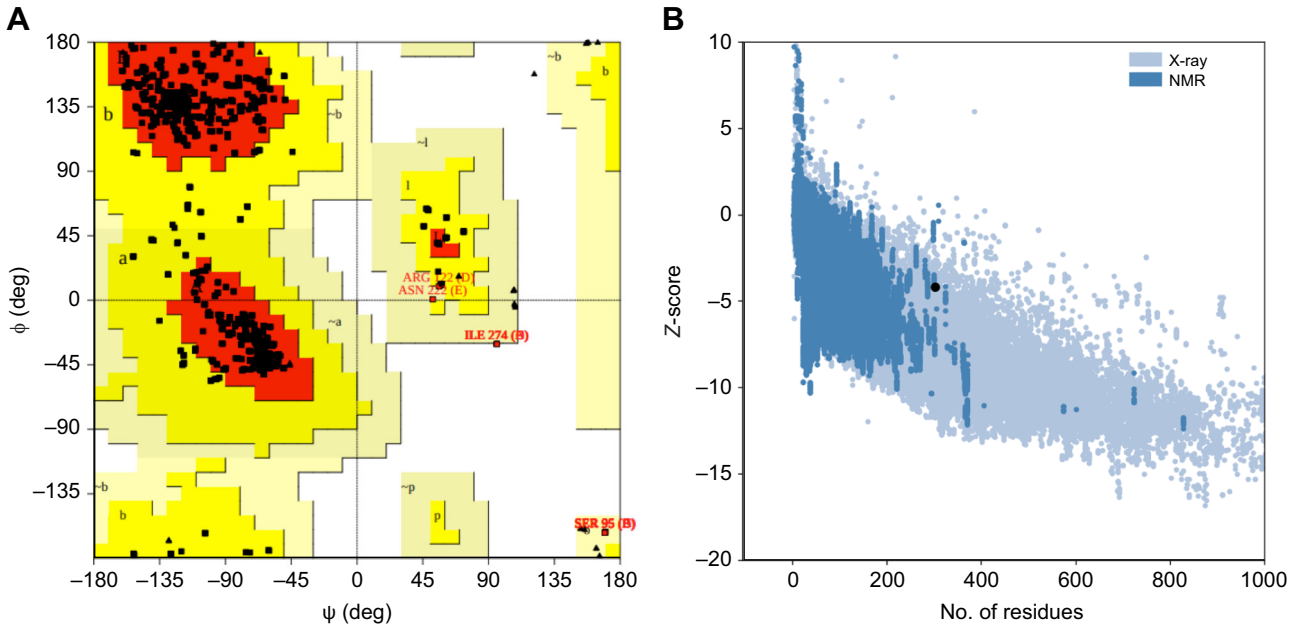

Fig. 6. Structure validation of the modeled GABA$_A$Rs of *Drosophila melanogaster*. (A) Ramachandran plot showing 88.3% of residues from the modeled *D. melanogaster* GABA$_A$ receptors (DGABA$_A$R) were in the most favorable area, 10.8% were in the allowed regions, 0.5% were in the generously allowed regions and just 0.4% were in the disallowed region. (B) Estimation of the *Z*-score of chain A of DGABA$_A$R compared with experimental data. The calculated value is shown as a black dot in the plot.

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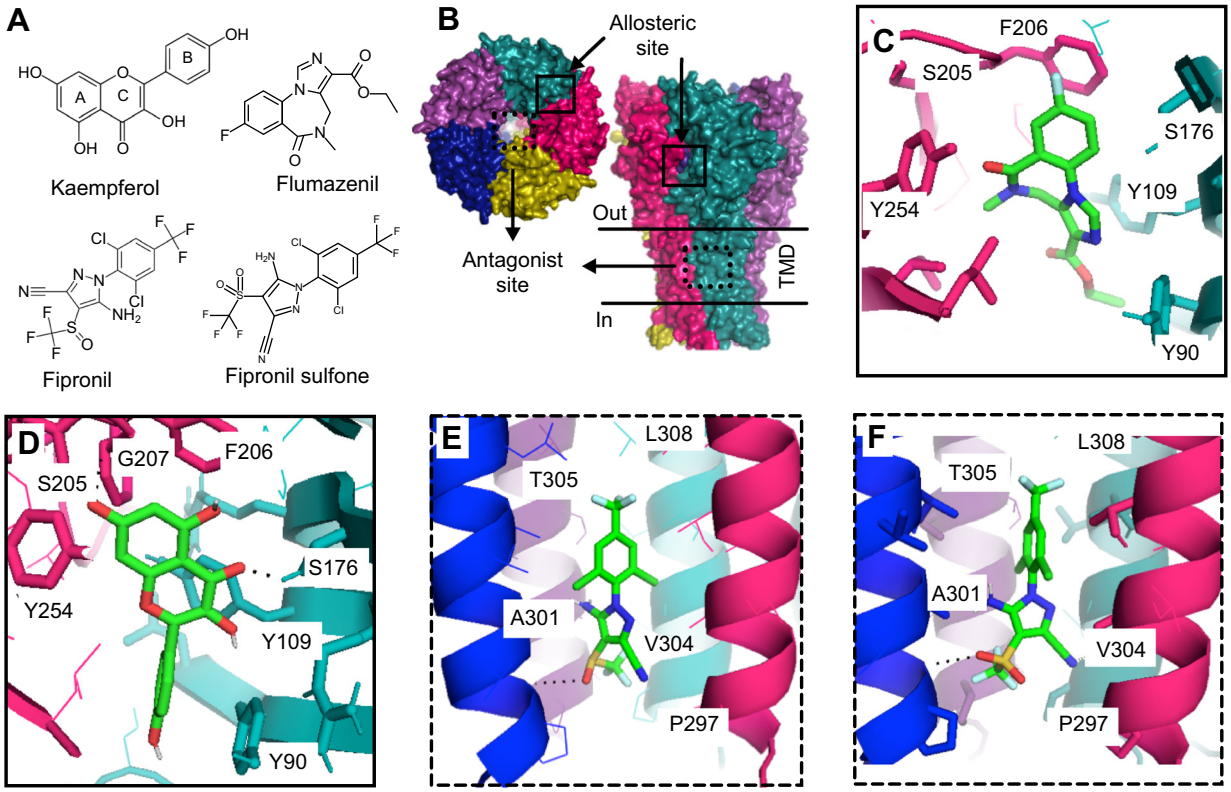

Fig. 7. Allosteric and antagonist binding site on the DGABA$_A$R. (A) Structural formulae of flumazenil, kaempferol, fipronil and fipronil sulfone. (B) Top and side view of the allosteric and antagonist binding site in the DGABA$_A$R. TMD, transmembrane domain. (C,D) Detailed architecture of the allosteric binding pocket in complex with (C) flumazenil and (D) kaempferol. (E,F) Detailed architecture of the antagonist binding pocket in complex with (E) fipronil and (F) fipronil sulfone. Black dotted lines indicate hydrogen bonds.

and P253 residues (fipronil: ΔG=−8.4 kcal mol^{-1}, fipronil sulfone: ΔG=−8.0 kcal mol^{-1}; Fig. 8B,C).

DISCUSSION

Recent decades have seen a rise in concerns over the negative effects of exposure to sublethal doses of pesticides in invertebrates and vertebrates. Current approaches include restrictions and bans, yet the continuous development of new pesticides calls for the development of strategies that counteract the deleterious effects in animals. Fipronil, a commonly used insecticide, acts as a GABA$_A$ antagonist, promoting neuronal hyperexcitation, dopamine depletion and motor loss (Stehr et al., 2006; Zhao et al., 2003). Here, we evaluated the protective effects of the flavonoid kaempferol against motor impairment induced by the administration of fipronil. We used kaempferol because its neuroprotective properties have been studied in neurodegenerative diseases and because of its action as a potential regulator of the GABAergic system. Our results confirm the detrimental effects of exposure to fipronil on motor activity. The impairments resemble parkinsonian features, characterized by fast walking, shorter walking activity and shorter distances climbed. In contrast, we found that kaempferol was innocuous and conferred protection to the flies against the impairing effects of fipronil. Importantly, the use of co-administration of flumazenil and the bioinformatic

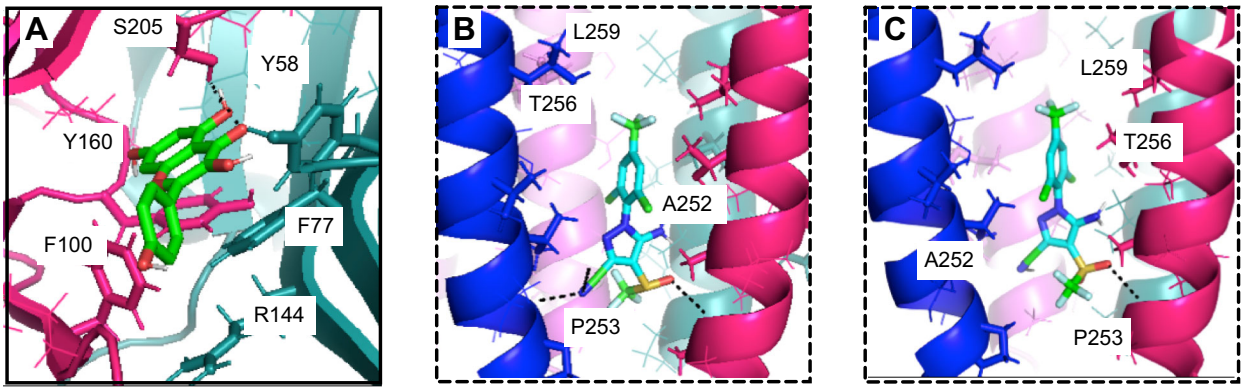

Fig. 8. Detailed binding of kaempferol, fipronil and fipronil sulfone to human GABA$_A$Rs. (A) Allosteric binding pocket in complex with kaempferol. (B,C) Antagonist site in the TMD in complex with (B) fipronil and (C) fipronil sulfone.

analyses enabled us to infer interactions between ligands and $GABA_AR$ that help explain the observed protection. Thus, together, our results highlight the motor impairment produced by fipronil, the innocuous effect of kaempferol and the prophylactic protection induced by its administration.

First, we observed that flies exposed to fipronil were faster but spent a shorter time walking, a trait resembling the involuntary hastening and propulsion-like gait in those with Parkinson's disease (PD; Hohler and de Leon, 2011; Koller, 1984; Pallis, 1971), albeit this effect decreased over time (Hohler and de Leon, 2011). Interestingly, whereas the faster pace in people with PD contrasts with a freezing phenotype (Nonnekes et al., 2019), the higher speed in the flies exposed to fipronil reflected bursts of fast walking episodes followed by longer periods of immobility. These higher speeds may be caused by the release of the inhibitory action imposed by the GABAergic pathways within the central complex (CC; a major area within the brain associated with motor control), controlling limb coordination and therefore speed (Martin et al., 1999). The irregular oscillation between periods of immobility and walking was perhaps initiated by impairment of the regulatory control of walk termination supported by the mushroom bodies (MBs; Martin et al., 1998; Poeck et al., 2008). This argument is further supported by the recent discovery of a tract connecting the central complex and the MBs (Li et al., 2020).

A second key feature of the Fip flies was erratic and shorter trajectories distanced from the border, probably explained by an impairment of visual orientation. The exposure to fipronil is associated with decreases in dopamine levels produced indirectly by the impairment of GABAergic pathways (Bharatiya et al., 2020; Błaszczyk, 2016; Kottler et al., 2019). This is consistent with the cognitive impairments in PD patients exhibiting visuo-spatial orientation and attentional deficits that predate motor symptoms and, in some cases, worsen it (Azulay et al., 1999; Davidsdottir et al., 2005). In fruit flies as in other animals, coordinated execution of walking and climbing depends on the integration of proprioceptive and exteroceptive (mostly visual) information (Bausenwein et al., 1994; Strauß and Heisenberg, 1990; Weir and Dickinson, 2015). We hypothesize that fipronil induced changes in the GABAergic system and an indirect reduction in dopamine levels, causing impairment of optomotor responses. Although $GABA_ARs$ are widely distributed in the fruit fly brain (Enell et al., 2007), the impairment of optomotor responses may result from damage to at least two structures: the CC and the MBs. CC mutants lose their ability to orient in Buridan's paradigm (Strauss and Heisenberg, 1993). Also, orientation memory relies on GABAergic neurons in the ellipsoid body, a sub-region within the CC (Neuser et al., 2008). Further, in fruit flies, border preference is related to spatial orientation, visual processing and dopamine signaling (Kottler et al., 2019; Soibam et al., 2012; White et al., 2010). However, the neuronal circuit is not yet fully understood. Although border preference has been primarily associated with the MBs (Besson and Martin, 2005), a role of the CC cannot be disregarded because of its coordination of movements based on visual inputs (Martin et al., 1999).

In flies, the hallmark for protection in models of motor impairment focuses on a better performance in the climbing assay (Nagoshi, 2018) rather than cognitive features such as attention deficit and visual impairments. Here, we found that kaempferol had protective effects on the detrimental impacts of fipronil in walking features associated with coordination and visual orientation. The protective effect of kaempferol may be explained by its direct action on $GABA_ARs$, affecting primarily but not exclusively the visual processing and motor circuits within the CC and the MBs. Kaempferol improves cognitive function via $GABA_AR$ modulation (Grundmann et al., 2009); however, the importance of the modulation in improving motor functions is unknown. Interestingly, we found that the co-administration of flumazenil, a competitive antagonist of the allosteric site in $GABA_ARs$ (Whitman and Amrein, 1995), reversed the protective effects of kaempferol against fipronil. The counteraction was evident through three features of the phenotype: lower walking activity, trajectory patterns separated from the border and shorter distances climbed. This is consistent with our hypothesis that kaempferol acts as a positive allosteric modulator (PAM) of the $GABA_ARs$. The effect of PAMs is dependent on ligand concentration, mostly exhibiting a biphasic response (Sigel and Baur, 1988). This supports our results of a dose-dependent protective effect of kaempferol against fipronil.

That kaempferol has a protective effect against fipronil via the GABAergic system may be explained by its action through at least three mechanisms: enhancement of $GABA_A$ transmission, blocking the binding of fipronil and regulation of antioxidant activity. First, kaempferol allosterically enhances $GABA_AR$ transmission, which prevents hyperexcitation in the system and halts the oxidative stress induced by fipronil and later neuronal loss. This agrees with previous accounts showing that a balance between excitation and inhibition is essential to a normal transmission in the brain. Second, it is possible that the binding of kaempferol in the allosteric site inhibits the binding of fipronil and thus prevents the blockade of chloride influx. For example, PAMs inhibit the binding of picrotoxin, a $GABA_AR$ antagonist that binds in the same site as fipronil (Leeb-Lundberg et al., 1981). Also, benzodiazepines (recognized PAMs) have been used to treat fipronil intoxication in humans (Mohamed et al., 2004). Third, in PD models, protective effects of kaempferol are primarily attributable to its antioxidant activity (Li and Pu, 2011; Rahul et al., 2020). We observed that the protective effects were reverted by flumazenil; therefore, we suggest that the antioxidant activity is not the primary mechanism of protection against fipronil, although it cannot be ruled out as an indirect course of protection. Hence, kaempferol may maintain the integrity of neuronal circuits within the CC and the MBs via $GABA_AR$ enhancement, allowing an improvement in motor performance and, probably, visual orientation in flies.

Thus, we conclude that acute exposure to fipronil impairs motor control in the fruit fly *D. melanogaster* while kaempferol protects against motor impairment induced by fipronil in a dose-dependent fashion. The protective effects of kaempferol are presumably the result of its positive allosteric activity on $GABA_ARs$. Hence, our results highlight the potential of kaempferol as a prophylactic nutraceutical to protect against motor impairment produced by exposure to fipronil, a pesticide broadly used around the globe.

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Competing interests

Part of the results presented here are included in Patent Cooperation Treaty (PCT) application W02021046388A1.

Author contributions

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