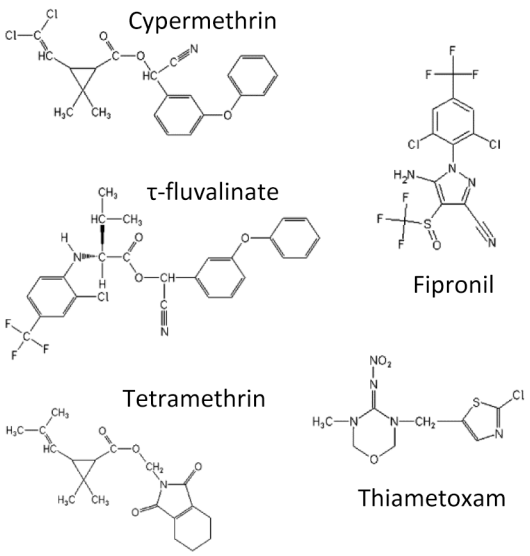

Fig 1. Synthetic insecticides from three classes. Chemical structure of 3 pyrethroids (cypermethrin, tau-fluvalinate, tetramethrin), a phenylpyrazole (fipronil) and a neonicotinoid (thiamethoxam).

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sublethal range was defined as doses producing a mortality level not statistically different from the control 48 hours after exposure (S1 Table). Moreover, for each insecticide, a dose two fold higher than the SLD_{48h} caused a mortality significantly higher than the control 48 hours after exposure (S1 Table). The sublethal range for each insecticide was validated on a minimum of four replicates of 30 bees (up to eight, S1 Table). Selected sublethal doses (SLD_{48h}) were 2.5, 33 and 70 ng/bee for the pyrethroids cypermethrin, tau-fluvalinate and tetramethrin respectively. Selected SLD_{48h} were 0.5 and 3.8 ng/bee for fipronil and thiamethoxam, respectively. In control modalities (acetone only), mortality at 48 h did not exceed 2.5% (see Results). Control mortality was measured from a minimum of six replicates of 30 bees (up to eight). A long-term survival test (up to 5 days after exposure) was performed as well, to quantify mortality over durations longer than 48 h. If any, dead bees were removed daily from cages. Mortality at 48 h and long-term survival (at 120 h) were assessed on different bees from those assayed for locomotion.

Video tracking analysis

Locomotor activity was monitored for 3 minutes using a webcam controlled with VirtualDub (GNU free software, acquisition frequency 1 Hz, http://sourceforge.net/projects/virtualdub/files/). The arena set up allows video tracking one bee at a time, every 5 minutes (3 minutes of effective video tracking and 2 additional minutes to transfer the bee from its cage to the arena and to allow for short time acclimation, and to transfer it back to a cage at the end of the tracking). Video tracking was performed between 2 and 6 pm and bees were allowed to recover from anesthesia during several hours in a ventilated incubator (29°C, 40% humidity, dark). For a single insecticide, control and insecticide-exposed bees were alternatively assayed and the total series duration was thus >200 minutes. Three minutes of video tracking were sufficient to characterize a distance covered at a nearly steady-state speed. The three minute duration also prevented speed adaptation (S3 Fig) that has been shown to arise quickly in some arena systems (e.g. a significant speed decrease ~10 minutes after placing the bee in the arena [29]). The vertical observation arena, inspired from existing arenas [10], measured 30 x 30 x 4 cm (height:

width: depth), was illuminated from above and placed in a dark chamber to avoid any variation due to daylight. The light source consisted of two parallel flicker free LED ramps (length 10 inches, 9 LED each), for a total of 0.72 W, 70 lumens of cold light (StarLED sticks, Starlicht, Germany). Experiments were done at room temperature (22–24°C). For each insecticide, bees were taken alternatively from control and exposed groups (random selection in each case) and introduced into the arena through a hole at the bottom with entomological forceps. Videos were semi-automatically analyzed using Image J (open source, Rasband WS, National Institutes of Health, Bethesda, http://imagej.nih.gov/ij/) with available filters and plugins in order to obtain a series of x,y coordinates for each bee. Individual paths were analyzed with Excel and Origin softwares (OriginLab) and the total distance covered by insecticide-exposed bees was expressed relative to the respective mean value obtained in control bees for each pesticide.

Statistics

Distances are expressed as mean ± S.E.M. The absolute total distance (in meters) covered by individuals during the 3-min time slots was compared among trials using a linear mixed model (LMM) framework. To gain statistical robustness, we handled the five control-*vs*-treated trials (cypermethrin, fipronil, tau-fluvalinate, tetramethrin, thiamethoxam) simultaneously as a part of the same model, followed by post-hoc pairwise comparisons with Bonferroni *p*-value adjustments for multiple testing. In a preliminary step, we assessed the constancy and stability of the experimental design by comparing monitored distances among the five control groups only (simple linear model LM and Tukey multiple pairwise comparisons). In a second step, we introduced into the model the five treated groups and set the correct matching with their respective control group by specifying the trial identity as a random grouping factor (LMM and Dunnett multiple comparisons with control). We verified that the LMM normality and homoscedasticity assumptions were met by graphically inspecting model residuals and QQ-plots [30]. We further statistically confirmed residual normality (Shapiro-Wilk test, $w = 0.98$, $p = 0.15$) and variance homogeneity among all trials (Bartlett test, $K^2 = 1.84$, $df = 4$, $p = 0.76$) and all treatments ($K^2 = 11.17$, $df = 9$, $p = 0.26$). Statistical analyses were performed with the R software for statistical computing [31]. Fisher exact tests were performed with the JMP software (SAS) to compare mortality rates, assuming significant differences for $P<0.01$.

Results

Determination of sublethal doses

Sublethal doses (SLD_{48h}) were determined from mortality assays preceding the locomotion tests. Two criteria were mandatory in our experiments to select experimental SLD_{48h}: i) a dose producing a mortality level not statistically different from the control was considered as a SLD_{48h} and ii) twice the chosen dose (SLD_{48h}) had to produce a mortality level significantly higher than the control. SLD_{48h} for each insecticide are given in S1 Table, with results of the statistical analysis on mortality assays (p-values from exact Fisher tests). SLD_{48h} were 2.5, 33 and 70 ng for the three pyrethroids cypermethrin, tau-fluvalinate and tetramethrin respectively. SLD_{48h} were 3.8 and 0.5 ng for thiamethoxam and fipronil respectively. Mortality levels after insecticide exposure were not corrected for control mortality levels [26], which were low in all series (0–2.5%).

Locomotion in control bees

Locomotor function and deficits produced after exposure to an insecticide were evaluated by video tracking bees placed in a closed vertical arena. Individual honeybees subjected to this