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

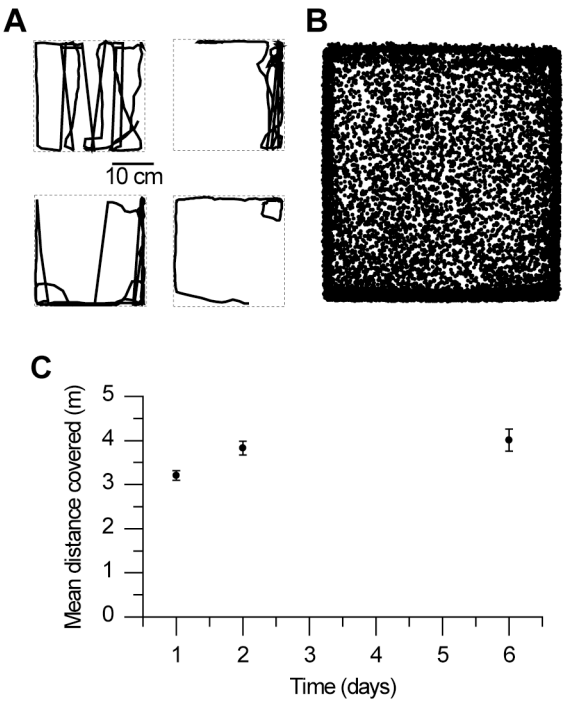

Fig 2. Video tracking of bees using a vertical arena. A, Examples of paths followed during 3 minutes by four individual young bees (day 1 after emergence). **B,** Superimposed paths followed by eighty individual bees. Overall, arena sides were more frequently visited. **C,** Locomotor ability measured at day 1, 2 and 6 after emergence (bees kept in an incubator). Mean distance (± S.E.M) covered by bees slightly increased from 3.2 to 3.8 meters between day 1 and 2 ($p<0.01$, n = 138 and 63 respectively) and did not significantly further increase as shown at 6 days after emergence (n = 38).

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assay displayed variable trajectories, as illustrated for four individuals (first day after emergence) monitored for 3 minutes at a frequency of 1 Hz (Fig 2A). During 3 minutes, each bee can explore only a fraction of the arena. However, overall, bees visited all parts of the arena, although sides were visited more often, possibly owing to a positive thigmotaxis phenomenon (Fig 2B, superimposed trajectories of 80 control bees). The total distance covered was chosen as a proxy for the bee's locomotor ability. In laboratory conditions, a bee's locomotor ability only slightly increased with age as shown by distances measured from bees kept in a cage for 6 days (Fig 2C). Mean distance covered by bees increased from 3.2 ± 1.0 to 3.8 ± 1.6 m between day 1 and 2 (Mann-Whitney U = 3119, $n_1 = 138$, $n_2 = 63$, $p<0.01$) and did not significantly further increase at day 6 after emergence (4.0 ± 2.5 m, n = 38). Bees already showed good locomotion skills at day one after emergence, making this age suitable for the following locomotion assays on insecticide-treated bees.

Locomotion in bees exposed to a SLD_{48h}

Average distances covered by young bees (day 1 after emergence) were measured after exposure to an SLD_{48h} of one of the three pyrethroids: cypermethrin (2.5 ng/bee), tau-fluvalinate (33 ng/bee) and tetramethrin (70 ng/bee). For ease of comparison, distances covered by exposed bees were standardized to the average distance covered by corresponding control bees, set at 1 (Fig 3, relative control distances in black). S1 Fig also reports individual actual distances in meters (S1 Fig). All the five control groups delivered statistically identical locomotion properties, with no significant distance variation in any pairwise combination of trial (S2 Table, S2