behavior (impaired olfactory learning and decreased sucrose sensitivity) without any effect on locomotion [10], while locomotion is affected by sublethal doses of one of the least toxic pyrethroids (tau-fluvalinate at a doses causing 10% mortality, LD10 [25]). Although these studies suggest that the sublethal effects of most insecticides are molecule-specific and cannot simply be extrapolated directly from the LD_{50} values, none of these molecules have been tested together in parallel tests in the same experimental conditions. Owing to the prevalence of pesticides in the vicinity of hives but also inside the hive's matrices, locomotor deficits may affect multiple developmental stages of bees, from foragers (after contact with treated plants) to larvae and recently emerged, young bees (through contact with contaminated matrices). However, the current testing protocols only impose toxicological tests on either young larvae or unselected adults (samples shall be collected without regard to the age of the bees). While the exploration of specific toxicological effects on newly emerged bees are not yet required, this early stage of imaginal development may show a different sensitivity compared to older adults [26, 27].

The aim of this study was therefore to compare the deleterious effects of sublethal doses of several pyrethroids, one neonicotinoid and one phenylpyrazole on the locomotion of honeybees in their first day of adult life. This study shows for the first time that an acute contact exposure to sublethal doses of pyrethroid or neonicotinoid insecticides induces serious locomotor deficits in young bees that can be quantified several hours after exposure.

Materials and Methods

Honeybees

Newly emerged bees (*Apis mellifera*) were obtained during the spring season from two hives (with sister queens) maintained in the experimental apiary of the *Abeilles & Environnement* Research Department on the Avignon INRA PACA campus. Colonies received a treatment against *Varroa* in October (Apivar™, active ingredient amitraze) and were healthy, without any obvious symptoms of disease. Thiamethoxam experiments were performed on colony 1 (summer 2013), all other experiments were performed on colony 2 (summer 2014). To collect bees, frames of developing brood were gently brushed to get rid of adult bees and placed into an incubator (30°C, high humidity) overnight in order to harvest newly emerged bees the next morning (upon emergence, bees were fed on food stored in combs).

Exposure to insecticides

Technical-grade insecticides (the active ingredients) were purchased from EhrenStorfer GmbH (cypermethrin, tetramethrin, tau-fluvalinate, fipronil and thiamethoxam 96, 98, 94, 97 and 98% pure, respectively). Molecules (whose molecular structure are given in Fig 1) were dissolved in acetone and final concentrations were obtained by successive dilutions in amber glass vials thoroughly vortexed at each step. Exposure to insecticides was performed between 9 and 10 am. Honeybees were anaesthetized with CO_2 (batches of bees were exposed to a controlled volume of CO_2 (final concentration 50%) for 30 seconds in an anesthesia induction chamber). They were placed on ice while 1 μl solution was applied to the dorsal part of the thorax with a Hamilton syringe mounted in a repeating dispenser. Full acetone evaporation was allowed and bees were placed in standard plastic cages (10.5 cm x 7.5 cm x 11.5 cm, modified from [28]) and provided with water and sugar paste (Apifonda, Ickowicz–sucrose 85%, glucose 5%, fructose 3%, water) in a ventilated incubator (29°C, 40% humidity, dark). Mortality tests were performed for all tested insecticides prior to the locomotion assay in order to determine each insecticide's sublethal dose (SLD). A minimum of two replicates of 30 bees was used at each dose (S1 Table), which is twice the number of bees required in registration tests [26, 27]. The

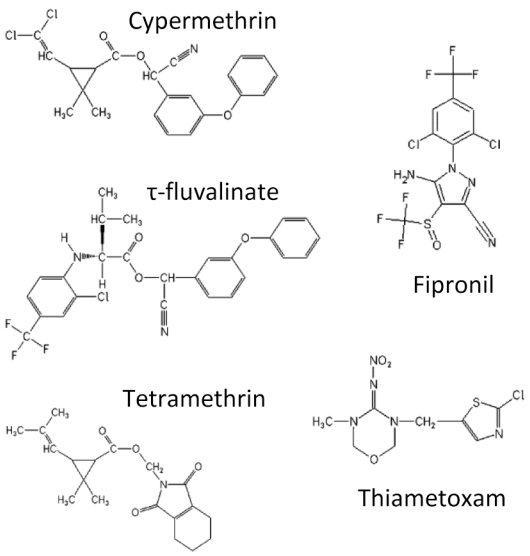

Fig 1. Synthetic insecticides from three classes. Chemical structure of 3 pyrethroids (cypermethrin, tau-fluvalinate, tetramethrin), a phenylpyrazole (fipronil) and a neonicotinoid (thiamethoxam).

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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: