Fig). Given the experimental stability of control groups, we could readily assess the five treatments as a part of a single LMM model. The distance covered by individuals was significantly lower in all treated groups compared to control, except for the fipronil trial (S2 Table, S2 Fig). In bees exposed to an SLD_{48h} of cypermethrin, the mean covered distance was significantly decreased by 71 ± 9% relative to control bees (Fig 3, S1 Fig, S2 Table). Tau-fluvalinate was also very potent at the SLD_{48h}, and the covered distance was significantly diminished by 58 ± 10% (Fig 3, S1 Fig, S2 Table), while tetramethrin (70 ng/bee) significantly decreased the distance covered by 48 ± 7% (Fig 3, S1 Fig, S2 Table). At the chosen SLD_{48h}, all 3 pyrethroids appear qualitatively equally potent. Exposure to an SLD_{48h} of the neonicotinoid thiamethoxam (3.8 ng/bee) resulted in a similar significant decrease by 58 ± 8% of the distance covered (Fig 3, S1 Fig, S2 Table). Exposure to an SLD_{48h} of fipronil (0.5 ng/bee) did not produce any significant effect on locomotion (Fig 3, S1 Fig, S2 Table).

Mortality several days after exposure to fipronil in the absence of early locomotor deficits

In parallel with locomotion assays, long-term survival was measured after exposure to the insecticide SLD_{48h}, to explore whether the locomotor deficits that we have measured could induce any mortality several days after exposure in laboratory conditions. The current legislation imposes that acute contact mortality tests have to be routinely performed for 48h [26]. However, if mortality increases by more than 10% between 24 and 48 h, the assay should be extended up to 96 h. Here, for all pyrethroids and the neonicotinoid tested, mortality rates were stable between 24 and 48 h. We found that the SLD_{48h} was sublethal at 120 h as well, indicating that the early locomotor deficit observed does not compromise survival five days after exposure, at least in a controlled laboratory environment. At 120 h, the SLD_{48h} of cypermethrin, tetramethrin, tau-fluvalinate or thiamethoxam did not induce mortality more than their respective controls (Fisher exact tests, $P>0.14$). Interestingly, whereas fipronil was the only modality in which no locomotor deficits were detected, with a mortality rate stable between 24 and 48 h (1 and 2% respectively, $P = 0.6515$), the SLD_{48h} of this insecticide started to produce an increased mortality at 72 h (14%, $P<0.0001$ as compared to 48 h) and a high mortality rate at 120 h after exposure (78%, n = 180 bees) as compared to control bees (1.5%, n = 180 bees, $P<0.0001$). It is noteworthy to mention that the survival of control bees was stable between 48h and 120h (99.5% and 98.5% respectively, n = 180, $P = 0.6229$).

Discussion

For pollinators, sublethal effects of insecticides increase toxicological risks and thus should be taken more into account in the methods of risk evaluation [32]. For the first time, we analyzed the sublethal effects of pesticides from three major insecticide classes on young bees (day 1 after emergence) in a standardized honeybee walking locomotion test. Emphasis has been put on pyrethroids that were much less studied than the two other insecticide classes despite 1) their high toxicity towards insects, 2) their pervasive use in agriculture and 3) their prevalence in hives. Specific experimentally-determined sublethal doses were selected for each insecticide (see Methods). In our study, assuming a mean individual bee weight of 0.1 g [1], the SLD_{48h} of pyrethroids were 25 ppb for cypermethrin, 330 ppb for tau-fluvalinate and 700 ppb for tetramethrin. By comparison, quantitatively similar values of 13 pyrethroid residues have been detected in North American hives [1]. For instance, in foundation wax, cypermethrin and fluvalinate were found in 23.8 and 100% of samples at maximal levels of 131 and 10120 ppb respectively (average levels 51.6 and 2006 ppb respectively). Knowing that multiple pyrethroids can be found in the same hive, preimaginal bees and newly emerged bees were thus potentially

exposed to cumulated doses [1] that are compatible with SLD_{48h} used in the present study. However, the gaps in the current knowledge on pesticides toxicokinetics (e.g. on the transfer rate of pesticides from hive matrices to the body of a young bee) precludes comparing the level of exposure resulting from contact with contaminated waxes and the level of exposure resulting from a laboratory procedure in which a droplet of contaminated solution is applied on the thorax. Currently, a model that would link these two modes of exposure is unfortunately lacking for a risk evaluation to be accurately performed. For fipronil, our SLD_{48h} was 5 ppb. By comparison, 1.4% of wax samples contained a maximum of 35.9 ppb of fipronil, 0.3% of pollen samples contained a maximum of 28.5 ppb [1]. For thiamethoxam, our SLD_{48h} was 38 ppb. By comparison, 0.3% of pollen samples contained a maximum of 53.3 ppb of thiamethoxam and it was not detected in wax [1].

An SLD_{48h} of all the insecticides tested (except fipronil) triggered serious locomotor deficits. According to available $LD_{50\ at\ 48h}$ values for cypermethrin, that vary from 25 to 121 ng/bee, [33, 34], an SLD_{48h} of cypermethrin 10 to 48 fold lower than the LD_{50} seriously impairs locomotion. The SLD_{48h} for tau-fluvalinate is between 75 and 600 fold lower than published LD_{50} values (2.5 μg—20 μg/bee, [34, 35]). In the case of tetramethrin, the effective SLD_{48h} was only two times lower than the available LD_{50} [36]. Very different maximal ratios between LD_{50} and SLD_{48h} values for the three tested pyrethroids (2, 48 and 600 for tetramethrin, cypermethrin and tau-fluvalinate, respectively) suggest that within a chemical family, deleterious effects of individual insecticides have to be evaluated separately. A similar locomotor deficit was observed with a SLD_{48h} of tau-fluvalinate and a 13 fold lower SLD_{48h} of cypermethrin. This result is consistent with a 20 fold lower LD_{50} obtained with cypermethrin than with tau-fluvalinate [34]. Tau-fluvalinate is commonly used against the bee parasite *Varroa destructor*. The locomotor deficits observed here after exposure to low doses of tau-fluvalinate surely challenge the widespread concept that it can safely be used in hives.

Since honeybees' skeletal muscles do not express functional voltage-gated Na^+ channels, locomotion deficits cannot be explained by a direct action of pyrethroids on such channels in muscle [37, 38]. Locomotor deficits can then be potentially attributed to non-mutually exclusive explanations. First, the locomotor deficits observed in our study could be a consequence of pyrethroid effects on sodium channels located in the central nervous system (in brain and other ganglia) impairing information processing and motor command [39]. Alternatively, impaired electrical activity of sensory neurons housed in the antennae, that are more pyrethroid-sensitive than central neurons, may affect sensory perception and thus impede locomotion [39, 40]. Structural differences between tau-fluvalinate, cypermethrin and tetramethrin in the acidic and alcohol moieties (Fig 1) may produce the different sets of interactions within the channel pore revealed by molecular modeling [41–43], thus giving molecular support for drug-specific modifications in the honeybee sodium channel kinetic parameters [39]. The cloning and expression of the honeybee voltage-dependent sodium channel, $AmNa_v1$ [43] together with the analysis of the changes induced by the different pyrethroids using numerical simulation bring a set of important tools that will be useful to fully characterize and understand the binding differences between these pyrethroids and hence their differential toxicity. Deficits could also be related to pyrethroid potency on secondary targets such as voltage-gated calcium channels [44, 45] that are broadly distributed in honeybee tissues [46, 47]. Calcium channels underlie action potentials in muscles of the honeybee or other insects, [37, 38, 48, 49]. Effects on muscle calcium channels would thus not only impair locomotion but thermoregulation or hemolymph circulation as well. A direct effect on bee muscle cells has actually been shown *in vitro* [37]. Our recent cloning and expression of *Apis mellifera* calcium channels ($AmCa_v$) will allow for more systematic testing of pyrethroids [46, 47].