Introduction

Pollinators play a crucial role in maintaining vegetal biodiversity but also participate in improving agricultural production. Therefore, a number of managed honeybee colonies are periodically moved in the vicinity of agricultural fields, not only to increase honey production but to improve crop pollination as well. As a consequence, insecticide exposure of honeybee colonies does not only occur when individuals are foraging, but also happens directly in the hive, as demonstrated by measurements of in-hive pesticide residues [1, 2]. In the last few decades, an increase in honeybee colony mortalities has been reported around the world and focused the attention of a broad scientific community on the potential consequences of pesticide misuse on pollinator survival [3–5]. These studies have been especially focused on two families of insecticides, neonicotinoids and phenylpyrazoles, owing to their use as systemic insecticides in seed treatment [6]. Recently, the sublethal toxicity of neonicotinoids towards honeybees has been demonstrated in real-world environments and led the European Union to restrict the use of three members of this class for two years [7–9]. Similarly, fipronil, a phenylpyrazole highly toxic to bees even at sublethal levels (by impairing memory and synergistically enhancing sensitivity to the pathogen Nosema [10, 11]) has also been banned as an agrochemical product in France and more recently in other countries of the European Union, although it is still widely used elsewhere, like the neonicotinoids [12]. It is worth mentioning that these temporary restrictions apply for seed coating only, whereas other agrochemical formulations are still authorized (Official Journal of the European Union OJ L219/22–15.8.2013 and OJ L139/12–25.5.2013). Besides neonicotinoids and phenylpyrazoles, pyrethroid insecticides constitute a large insecticide family produced through chemical synthesis, with a limited number of compounds (e.g. deltamethrin, cypermethrin, λ-cyalothrin, permethrin) accounting for the majority of sales [13]. The restrictions imposed on neonicotinoids and phenylpyrazoles may lead to an increase in pyrethroid use. Many pyrethroids are also highly toxic towards honeybees [14], and very few studies have compared the sublethal toxicities of pyrethroids, neonicotinoids and phenylpyrazoles in honeybees [15, 16]. These insecticides all target ion channels involved in the function of a variety of tissues (including the nervous and the muscular systems), and it is known that their primary mode of action is to interfere with the normal function of voltage-gated sodium channels (for pyrethroids), nicotinic acetylcholine receptors (for neonicotinoids) and glutamate and GABA receptors (for phenylpyrazoles).

The effects of sublethal doses of insecticides on the neuromuscular system of honeybees are not easy to analyze. Methods for evaluating the ability of bees to fly back to the hive after exposure to a sublethal dose of insecticide (the 'homing flight assay') have been recently developed [7, 17, 18]. Besides the importance of flight for bees, efficient ambulation (walking) inside the hive is required for many tasks, including cell construction and cleaning, larval feeding and social interactions in general [19]. Muscle contraction, allowing physical movements, also produces heat [20] and thus participates in maintaining proper temperature levels around the brood. In feral colonies and in managed hives, the combs, built vertically, add an additional physical challenge by requiring vertical displacements. Experimentally, evaluation of locomotor abilities inside the hive is challenging and requires special observation hives with glass sides. Locomotion assays in laboratory conditions (open-field arena) are easier to set-up and produce a simple standardized and reproducible test to evaluate the effect of sublethal doses of insecticides [21]. Sublethal doses of neonicotinoids can (acetamiprid 0.1 μg/bee) or cannot (thiamethoxam 1 ng/bee) modify walking locomotion [22]. Whereas chronic exposure to thiamethoxam or imidacloprid sublethal concentrations (24h, 10 nM) did not modify the walking behavior, the righting reflex was affected [23]. Imidacloprid sublethal doses reduce waggle dancing 24 h after ingestion [24]. Low doses of phenylpyrazole (fipronil 1 ng/bee) modify

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