ASSESSMENT OF THE TOXIC EFFECT OF PESTICIDES ON HONEY BEE DRONE FERTILITY USING LABORATORY AND SEMIFIELD APPROACHES: A CASE STUDY OF FIPRONIL

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Abstract: Concern about the reproductive toxicity of plant protection products in honey bee reproducers is increasing. Because the reproductive capacity of honey bees is not currently considered during the risk assessment procedure performed during plant protection product registration, it is important to provide methods to assess such potential impairments. To achieve this aim, we used 2 different approaches that involved semifield and laboratory conditions to study the impact of fipronil on drone fertility. For each approach, the drones were reared for 20 d, from emergence to sexual maturity, and exposed to fipronil via a contaminated sugar solution. In both groups, the effects of fipronil were determined by studying life traits and fertility indicators. The results showed that the survival and maturity rates of the drones were better under laboratory conditions than under semifield conditions. Moreover, the drones reared under laboratory conditions produced more seminal fluid. Although these differences could be explained by environmental factors that may vary under semifield conditions, it was found that regardless of the approach used, fipronil did not affect survival rates, maturity rates, or semen volumes, whereas it did affect fertility by inducing a decrease in spermatozoa quantity that was associated with an increase in spermatozoa mortality. These results confirm that fipronil affects drone fertility and support the relevance of each approach for assessing the potential reproductive toxicity of plant protection products in honey bees. Environ Toxicol Chem 2017;36:2345–2351. © 2017 The Authors. Environmental Toxicology and Chemistry published by Wiley Periodicals, Inc. on behalf of SETAC.

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INTRODUCTION

During the last century, the worldwide increase in the use of plant protection products in agriculture has led to concerns regarding the impact of humans on environmental health. To address these concerns, developed nations have implemented strategies to assess the risks posed by plant protection products and have initiated policies to regulate their use. To enhance measures aimed at protecting humans, animals, and the environment, newly developed substances are subjected to a risk assessment procedure that involves biological assays and a consideration of the available scientific data. During this procedure, biological assays must be performed in accordance with guidelines that are defined by intergovernmental organizations, such as the Organisation for Economic Co-operation and Development and the European and Mediterranean Plant Protection Organization, which involved input from 35 and 50 member countries, respectively. In Europe, plant protection products placed on the market, including active substances, softeners, and synergists, are regulated and harmonized by Regulation EC 1107/2009 [1], which replaced Council Directive 91/414/EEC [2]. These directives concern assessments of the risks presented by pesticides to humans (consumers and operators) and the environment (vegetal and animal [vertebrate and invertebrate] species). To comply with regulations, a new substance should not be used if it is carcinogenic, mutagenic, an endocrine disruptor, or toxic to reproduction in humans under realistic conditions proposed for its use [1]. With regard to reproduction, regulatory authorities consider its potential to induce adverse reproductive effects that can potentially result in impairments to fertility in either males or females or the abnormal development of offspring [2].

However, there are gaps in the plant protection product registration dossier regarding assessments of the sublethal effects of plant protection products on the reproductive functions of nontarget species. In mammals, the adverse effects of active substances are assessed using 1) toxicological tests focused on evaluating prenatal toxicity [3]; and 2) a 2-generation study [4,5] in a model species, such as the rat. In these tests, sperm quality and estrus cycle parameters are investigated as fertility indicators. Following analyses of the effects of exposure, the results are extrapolated to humans using toxicokinetic studies, although reactions to toxicants can differ between the 2 species [6]. In ecotoxicological studies, which use nontarget organisms, tests that are focused on reproduction may be required when a risk is suspected. However, the currently available registered tests are less thorough than those performed on mammals, they are mainly focused on the production of offspring by exposed parents, and they were developed for only a few species, such as fish [2,7,8], birds [2,9], crustaceans [2,10], and earthworms [2,11], but not for threatened pollinating insects, which are of substantial ecological and economical interest [12,13].

Assessments of the risks associated with the use of plant protection products in pollinating insects produce even more superficial results. For the honey bee, the regulations merely specify that the use of plant protection products under the
proposed conditions “will result in a negligible exposure of honeybees, or has no unacceptable acute or chronic effects on colony survival and development, taking into account effects on honeybee larvae and honey bee behaviour” [1]. When exposure is likely to occur, the regulatory authorities recommend performing laboratory tests to assess acute oral and contact toxicity in adults [14–16]. If a product is likely to have systemic or insect growth-regulating properties, a larval toxicity test must be performed [17]. Following these tests, if toxicity is demonstrated, a semi field test is performed to assess the effects at colony scale [14,18]. However, these tests do not consider sublethal effects on learning, orientation, locomotion, or physiological functions such as reproduction. Because reproductive functions are highly important for colony growth and sustainability, scientists advise that they should be taken into consideration [19,20]. However, no standardized methods are available to assess the effects of plant protection products on reproduction, whereas an increasing amount of evidence indicates that fertility has been impaired in reproducers and that this has impacted honey bee decline [20–26].

In this context, it may be important to develop tools that can be used to assess the reproductive toxicity of plant protection products in queens and drones, which are the key reproductive individuals in honey bee colonies. Up until now, in honey bee research, entire hives in fields have been contaminated to assess the effects of pesticides on drone fertility and their impact on hives and on the environment. We used fipronil as a relevant active substance to develop 2 novel approaches to assess the effects of plant protection products on drone fertility. Fipronil targets the γ-aminobutyric acid receptor as an antagonist and is used around the world as a pesticide in agricultural and veterinary practices. It has also been shown to have adverse reproductive effects in invertebrates, such as honey bee males [26], in addition to vertebrates [27]. At the cellular level, fipronil is known to impair oxidative phosphorylation in mitochondria [28], which are not only ubiquitous but also essential for spermatozoa functionality.

The first approach uses semi field conditions, which differ from open-field conditions in that access to food is controlled and the bees are isolated from exposure to other environmental pollutants [29]. The second approach improves on the methods used under laboratory conditions that were proposed by Abdelkader et al. [29]. In the present study, we focused on comparisons between the 2 approaches instead of 1) performing an exhaustive characterization of fipronil toxicity, which would require an evaluation of concentration-dependent responses; 2) intracolony variation; and 3) the ages of drones. These comparisons required a large number of repetitions, which were performed at the expense of investigations into the aforementioned subjects. However, it would be very interesting to investigate these in the future. Thus the effects of fipronil on drones were determined by studying drone survival, maturity, semen volume, and fertility parameters such as the number and the mortality rate of spermatozoa in semen. We then compared the relevance of each evaluated approach.

MATERIALS AND METHODS

These experiments, which involved semi field and laboratory conditions, were performed simultaneously in Avignon, France, in June to July 2014 to avoid seasonal influences. The biological material was obtained from experimental honey bee colonies (Apis mellifera L.) that were monitored for their sanitary status and treated with Amitraz in September 2013 to control the Varroa mite population. The drones and workers used were collected from 15 healthy colonies and homogenized before they were introduced to hives and large cages [29] (Figure 1A and B). In both approaches, the workers were used to take care of the drones from emergence to sexual maturity (20 d old; Figure 1C), and the bees were fed with a sugar solution, crushed pollen, and water [29]. Under each rearing condition, the exposed bees were fed steadily with a sugar solution contaminated with fipronil (0.1 μg/L) in a sugar solution. At the end of the experiments, 20-d-old drones were caught (C) to collect semen. Drone life traits related to drone survival, sexual maturity rates, and semen volumes were determined during semen collection (D). The semen quality was then investigated by analyzing fertility indicators, such as spermatozoa quantity, using a counting chamber (E), and spermatozoa mortality rates were determined using propidium iodide staining (F).

Varroa mite population. The drones and workers used were collected from 15 healthy colonies and homogenized before they were introduced to hives and large cages [29] (Figure 1A and B). In both approaches, the workers were used to take care of the drones from emergence to sexual maturity (20 d old; Figure 1C), and the bees were fed with a sugar solution, crushed pollen, and water [29]. Under each rearing condition, the exposed bees were fed steadily with a sugar solution contaminated with fipronil at a low environmental concentration (0.1 μg/L) [30]. Food was changed every day, and the amount that was gathered was recorded. Twenty days after introduction, the surviving drones were caught for semen collection, which was performed after a manual eversion of the endophallus (Figure 1D). The drones that provided the semen samples were considered to have reached sexual maturity. The collected semen was analyzed to assess the impact of fipronil on drone fertility (Figure 1).

Rearing method used under semi field conditions

Under semi field conditions, the drones were reared in a queenless colony and introduced into compartmentalized tunnels that were covered with an insect-proof net (Figure 1A). The colonies were composed of 5000 workers, 1 brood comb, and 5 comb without food. In each colony, 300 drones were cloistered using a queen excluder, as described in previous studies [29]. Eight control colonies (control semi field) and 8 colonies that were exposed to fipronil (fipronil semi field) were used. For 20 d, the colonies were supplied by foragers that collected food provided daily to feeders outside the colony. Fipronil was previously dissolved in 100% dimethyl sulfoxide (DMSO) and diluted in the food at the appropriate concentration to obtain a...
final DMSO concentration of 0.1% (w/v). Thus the food consisted of a sugar solution (50% w/v) containing 0.1% (v/v) DMSO that was or was not contaminated with 0.1 μg L⁻¹ fipronil. A 50% sugar solution was used in the semifield conditions because this was close to the natural conditions under which foragers normally collect nectar. To avoid rapidly overloading of the hive with food, which could result in a decrease in foraging activity because of completely filled combs, we restricted the feeding period. This had no effect on exposure. The sugar solution was available from 8:30 AM to 11:30 AM. After 11:30 AM, it was replaced with crushed pollen and water for the remaining time [26]. Pollen was provided to force the bees to sustain foraging activity, to maintain conditions closer to natural conditions, and to prevent disrupting the normal functions of the colony and the larvae rearing (larvae consume pollen that were previously transformed into beebread).

Rearing method used under laboratory conditions

Under laboratory conditions, drones were reared in large cages that were placed in the dark in a thermostat-controlled chamber set at 33 ± 1°C with 60 ± 10% relative humidity. According to the methods described by Abdelkader et al. [29], the large cages were composed of 1000 emerging workers, 150 drones, 3 wax combs fixed on the top of the cage, a Beeboost® stick (Pherotech) that released a queen mandibular pheromone, and a movable floor [29] (Figure 1B). Filter paper was placed on the floor and replaced every 2 d to maintain good hygiene conditions. At the beginning of the present study, the emergence of bees over a 24-h period enabled to fill 6 cages, which were used as controls (control laboratory), and 5 cages in which the drones were exposed to fipronil (fipronil laboratory). For 20 d, the bees were continuously fed with a sugar solution (70% [w/v] sucrose, 1% [v/v] of the solution of proteins and vitamins [Bee Food], and 0.1% [v/v] DMSO) that either did or did not contain 0.1 μg L⁻¹ fipronil. Crushed pollen and water were provided ad libitum. The syrup that was given to the bees maintained under laboratory conditions was more concentrated than the syrup given to the bees under semifield conditions, to maintain hive conditions as close to normal as possible. The syrup was supplemented with proteins to ensure that the bees maintained a correct level of protein intake [31]. This enabled the bees to achieve better survival rates and a higher degree of cage cleanliness because the bees were unable to leave for their hygienic flights. Some of the previously described methods were improved on. At 10 d after the beginning of the experiment, 500 newborn nurses were added to each cage, and these bees took care of the drones. From the 12th d until the end of the experiment, the floor, which was previously positioned under fixed waxes, was lowered by 3 cm every 2 d to progressively increase the space available to the drones for flying. The mortality rates in the workers and drones were checked every 2 d throughout the experimental period.

Chemicals

Fipronil was purchased from TechLab, and DMSO was purchased from Sigma-Aldrich. The propidium iodide dye was obtained from a LIVE/DEAD® Sperm Viability Kit that was purchased from Molecular Probes (L-7011). The protein and vitamin solution Bee Food was obtained from Apiculture Remuax.

Semen collection and analyses

At the end of the experiments, for each cage or hive, the surviving 20-d-old drones were caught, and semen samples were collected. The semen of drones from the same hive or cage was pooled in the same glass capillary. During semen collection, the maturity rate of the drones obtained from each colony was determined, and the results were used to determine the ability of the surviving drones to provide semen after stimulation of the drones intended to induce eversion of the endophallus. Semen was subsequently immediately collected using a glass capillary tube connected to a syringe filled with Kiev solution (36 g/L trisodium citrate, 3.6 g/L sodium bicarbonate, 0.6 g/L potassium chloride, 5 g/L glucose and 3 g/L sulfuramidine, pH 8.5, osmotic pressure, 460 mOs/mL) [29,32]. To limit discrepancies in semen quality determination, a single operator handled the drones to collect semen. The survival rate, the maturity rate (calculated as the number of drones that provided sperm after stimulation), and the overall semen volume were determined for each cage or hive. To decrease discrepancies resulting from differences in semen volume, semen was pooled to eliminate individual variation. The average semen volume per drone was determined using the number of drones that provided semen and the total volume of semen collected in calibrated glass capillary tubes. Using these endpoints, the average semen volume per drone was calculated for each group. For these life traits, 1 measurement was performed per hive/cage (i.e., n = 8 for control semi and fipronil semi modalities, n = 6 for control laboratory, and n = 5 for fipronil laboratory). Then fertility was assessed by analyzing semen properties. To perform these semen analyses, semen was collected from each hive or cage and then equally divided to obtain fractions, which were diluted in Kiev solution to assess the concentration of and the mortality rate of spermatozoa. The concentration of spermatozoa was studied using a counting cell (Neubauer improved/Petroff®) [26] (Figure 1E). The number of spermatozoa per drone was calculated from the concentration, and the average volume of semen was calculated for the drones in each hive/cage. The spermatozoa mortality rate was determined by staining the cells with propidium iodide (Figure 1F) and then analyzing the results using a fluorimeter (TECAN Infinite® F500 plate reader). This assay was performed using 100 μL of diluted semen in Kiev solution containing 1 × 10⁷ spermatozoa and 60 μM propidium iodide. The level of fluorescence in the dead spermatozoa (FI λex = 535 nm, λem = 617 nm) was measured after the cells were incubated in the dark for 10 min at 34°C. The percentage of spermatozoa that died was determined using fluorescence intensity in a standard range up to 1 × 10⁷ dead spermatozoa, which was obtained by successively freezing and thawing standard sperm samples [26]. To obtain these parameters, several samples were measured for each cage or hive (i.e., n = 24 for control semi and fipronil semi, n = 36 for control laboratory, and n = 30 for fipronil laboratory).

Statistical analyses

For data related to drone life traits (e.g., survival, maturity, and semen volume), the following 2-step statistical analyses were performed: 1) a linear model plus analysis of variance to determine the potential effects of each rearing method and treatment on different parameters; and then 2) a post hoc Wilcoxon test because the small number of data points required a nonparametric test for confirmation. To analyze semen parameters, statistical analyses were performed using a generalized linear mixed model with a random effect on the hive/cage from which the drones were obtained. These statistical analyses were performed using the package lme4 in R software [33].
RESULTS

Food collection and consumption

The amount of food collected in the feeders was measured daily to assess the effect of fipronil on worker feeding behavior. Regardless of the rearing method, fipronil did not affect the amount of syrup and pollen that was gathered by the bees (data not shown), as was previously observed in bees grown under semifield conditions [26]. A comparison of the amount of food that was gathered by workers between bees reared using different methods was not relevant because the feeding methods were different. Bees reared under laboratory conditions did not need to fly to gather food because food was directly supplied in the cage, whereas food was not directly supplied to the bees reared under semifield conditions. When the bees were reared in a cage or in a hive, the amount of food assessed by individuals within a hive/cage was not possible because most of the food that was gathered was stored in comb cells.

Drone life traits

The survival, maturity, and volume of semen collected from 20-d-old drones was compared between bees raised using different rearing and exposure conditions. When these parameters were analyzed, fipronil did not induce any significant effects between the rearing methods used in the present study (Table 1). However, there were differences between bees reared from different methods. Drones had higher survival rates when reared under laboratory conditions than when reared under semifield conditions (mean: 73.7% in control laboratory vs 59.2% in control semifield, \( p = 0.043 \) and mean: 79.7% in fipronil laboratory against 44.1% in fipronil semifield, \( p = 0.002 \)). Similarly, the maturity rate was higher in the laboratory bees than in the semifield bees (mean: 73.2% in control laboratory against 59.2% in control semifield, \( p = 0.003 \) and mean: 72.2% in fipronil laboratory against 61.8% in fipronil semifield, \( p = 0.065 \); Table 1). Moreover, the drones raised under laboratory conditions had a slightly higher semen volume (up to 1 \( \mu L/drone \)).

Semen quality

Spermatozoa quantity and mortality were used as indicators of drone fertility. Fipronil altered drone fertility in the same way regardless of the rearing method. Spermatozoa quality was altered as a result of a decrease in the concentration of spermatozoa in the semen (median: \( 8.3 \times 10^6 \) spermatozoa/\( \mu L \) for fipronil semifield vs \( 9.8 \times 10^6 \) spermatozoa/\( \mu L \) for control semifield, \( p \leq 0.01 \); and \( 7.3 \times 10^6 \) spermatozoa/\( \mu L \) for fipronil laboratory vs \( 8.2 \times 10^6 \) spermatozoa/\( \mu L \) for control laboratory, \( p \leq 0.05 \). Figure 2A) and a decrease in the number of spermatozoa per drone (median: \( 8.0 \times 10^6 \) spermatozoa for fipronil semifield vs \( 8.8 \times 10^6 \) spermatozoa for control semifield, \( p \leq 0.05 \); and \( 6.7 \times 10^6 \) spermatozoa for fipronil laboratory vs \( 8.4 \times 10^6 \) spermatozoa for control laboratory, \( p \leq 0.05 \); Figure 2B). In addition, the insecticide induced an increase in the spermatozoa mortality rate (median: 41.0% for fipronil semifield vs 32.4% for control semifield, \( p \leq 0.01 \); and 32.2% for fipronil laboratory vs 28.0% for control laboratory, \( p \leq 0.05 \); Figure 3).

Despite the similarities observed in the responses of the drones to the stressor, bees raised under the 2 rearing conditions presented differences in fertility parameters in addition to differences in drone mortality and maturity. Thus drones reared under semifield conditions presented higher concentrations of spermatozoa than were observed in the drones reared under laboratory conditions (median: \( 9.8 \times 10^6 \) spermatozoa/\( \mu L \) for control semifield vs \( 8.2 \times 10^6 \) spermatozoa/\( \mu L \) for control laboratory, \( p \leq 0.001 \); and \( 8.3 \times 10^6 \) spermatozoa/\( \mu L \) for fipronil semifield vs \( 7.3 \times 10^6 \) spermatozoa/\( \mu L \) for fipronil laboratory, \( p \leq 0.05 \); Figure 2A). Nevertheless, the number of spermatozoa per drone was not significantly different between bees reared under these 2 different methods (median: \( 8.8 \times 10^6 \) spermatozoa/\( \mu L \) for control semifield vs \( 8.4 \times 10^6 \) spermatozoa/\( \mu L \) for control laboratory and \( 8.0 \times 10^6 \) spermatozoa/\( \mu L \) for fipronil semifield vs \( 6.7 \times 10^6 \) spermatozoa/\( \mu L \) for fipronil laboratory; Figure 2B). Finally, there was no significant difference in the spermatozoa mortality rate between the rearing methods (median: 32.4% for control semifield vs 28.0% for control laboratory and 41.0% for fipronil semifield vs 32.2% for fipronil laboratory; Figure 3).

DISCUSSION

The adverse sublethal effects of plant protection products on nontarget invertebrate species, including the honey bee, are a major concern [34,35], but they remain difficult to assess. In this context, we have developed tools that can be used to take into consideration a portion of the potential hazard posed by plant protection products to honey bee reproduction. These include a laboratory approach, in which conditions are entirely controlled, and an semifield approach, in which conditions are closer to natural ones. The 2 approaches present specific features that should be emphasized. It is important to note that the laboratory method provides some advantages, such as the ability to monitor behavior and mortality on a daily basis (data not shown), and requires fewer bees than the semifield method to collect a

| Table 1. Effects of exposure to fipronil on drone life traits |
|-------------------------------------------------------------|
| **Semiafield conditions** | **Laboratory conditions** |
| **Drone survival rate (%)** | **Drone survival rate (%)** | **Drone survival rate (%)** | **Drone survival rate (%)** |
| Ctrl SF (n = 8) | Fip SF (n = 8) | Ctrl Lab (n = 6) | Fip Lab (n = 5) |
| 50.9 ± 20.1 A | 44.1 ± 15.4 A | 73.7 ± 13.9 B | 79.7 ± 06.5 B |
| 59.2 ± 8.6 A | 61.8 ± 9.0 AB | 73.2 ± 7.2 B | 72.2 ± 6.1 B |
| 69.5 ± 26.7 A | 69.7 ± 31.0 A | 71.5 ± 9.6 A | 72.3 ± 10.8 A |
| 0.91 ± 0.07 A | 0.93 ± 0.07 A | 1.00 ± 0.07 A | 0.94 ± 0.09 A |

*Cloistered drones (20 d old) were exposed or not exposed to Fipronil and then captured to collect semen.

*Data represent mean values ± standard deviations and were obtained from drone populations that were recovered from hives/cages; n indicates the number of repetitions for each modality. Statistical analyses were performed using post hoc Wilcoxon tests. For each parameter, significant differences (\( p \leq 0.05 \)) between modalities are expressed using non-corresponding uppercase letters.

*The drone survival rate (%), sexual maturity rate (%), and semen volume (\( \mu L \)) were measured for each hive/cage.

*The semen volume per drone (\( \mu L \)) was calculated from the overall semen volume and the number of drones that were collected.

Ctrl = control; SF = semifield; Lab = laboratory; Fip = fipronil.
Assessment of pesticide reprotoxicity in the honey bee

condition. Asterisks indicate signiﬁcant differences: * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001. Ctrl = control; Fip = ﬁpronil; Lab = laboratory; SF = semiﬁeld; Spz = spermatozoa.

Figure 2. Effects of exposure to ﬁpronil on the quantity of spermatozoa. To assess spermatozoa quantity, semen was diluted and observed under a phase contrast microscope. (A) Spermatozoa concentrations in semen are expressed per microliter of semen. (B) The number of spermatozoa per drone was calculated from the spermatozoa concentration and the semen volume per drone. Statistical analyses were performed using a linear mixed model with a random effect on hive/cage. n indicates the number of repetitions for each modality. Asterisks indicate significant differences: * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001. Ctrl = control; Fip = ﬁpronil; Lab = laboratory; SF = semiﬁeld; Spz = spermatozoa.

Figure 3. Effects of exposure to ﬁpronil on the mortality rates of spermatozoa. The spermatozoa mortality rate was assessed by staining the cells with propidium iodide and then reading the ﬂuorescence intensity using a ﬂuorimeter. The spermatozoa mortality rate is expressed as a percentage (%) of the total spermatozoa population. Statistical analyses were performed using a linear mixed model with a random effect on hive/cage. n indicates the number of repetitions for each modality. Asterisks indicate signiﬁcant differences: * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001. Ctrl = control; Fip = ﬁpronil; Lab = laboratory; SF = semiﬁeld; Spz = spermatozoa.

similar volume of semen (Table 1). However, whatever the exposure method, food intake and level of exposure cannot be precisely determined because a portion of the collected food was stored in wax before it was consumed. In all cases, the drones were supplied by nurses during the early stages of life before they were able to take themselves to the food stored in wax [36]. The semiﬁeld approach has the advantage of mimicking foraging activity and colony behaviors that are subjected to climatic inﬂuences. Indeed, weather conditions, such as precipitation and extreme temperatures, are additional stress factors that can lead to colony weakening [37]. Given that environmental factors and food have a substantial inﬂuence on drone maintenance [38,39] and fertility [40-42], it is not surprising that the 2 different approaches used in the present study produced slightly different results, similar to what was observed in a previous study [29]. These differences revealed that better survival and maturity rates were achieved when the laboratory method was used (Table 1). Moreover, we could not exclude the fact that the proteins and vitamins provided under laboratory conditions might have inﬂuenced the survival and maturity of the drones. Interestingly, a lower mortality rate, a higher maturity rate, and a higher semen volume collectively explain the similar volumes of semen that were collected per group (~70 μL) even though there were half as many drones in the cages as in the hives (Table 1). Moreover, a lower semen concentration of spermatozoa was observed in the drones reared under laboratory conditions than in drones reared under semiﬁeld conditions (Figure 2A). However, when the number of spermatozoa per drone was taken into account, no difference was observed (Figure 2B). This ﬁnding supports the conclusion that drones reared under laboratory conditions had higher volumes of seminal ﬂuid, which is an essential component of sperm. Among the essential functions it performs in conserving spermatozoa, seminal ﬂuid is required to provide nutrients to spermatozoa [43,44], support cell metabolism [45], and protect against oxidative stress [45,46] and microbial threats [45]. Hence, exposure to environmental stressors, such as biological or chemical agents, can have different effects on drone life traits and fertility depending on the approach used. To better explore this notion, at least with regard for chemical agents, in the present study, we reared drones with different methods using bees that were or were not exposed to a relevant concentration of the insecticide ﬁpronil, which has been previously shown to have effects on drone fertility under semiﬁeld conditions and on the reproductive potential of the queen [26].

Although 2 different approaches were used, exposure to ﬁpronil resulted in similar effects on drone life traits and fertility in both. No effect on mortality, maturity, and semen volume was observed between controls and exposed drones (Table 1). Regarding fertility parameters, the exposed drones presented similarly impaired semen quality, which was attributed to a decrease in the amount of spermatozoa (Figure 2A and B) and an increase in the spermatozoa mortality rate (Figure 3), which were described in a previous study [26]. The effects of proteins and
vitamins supply or the absence of climate variations could have been masked under these laboratory conditions. However, in the present study, no difference was observed in the intensity of the effect of fipronil between the 2 approaches, and this result did not support such a hypothesis. Moreover, the lack of an effect on maturity rate and semen volume, which are 2 parameters previously been associated with the ability to ejaculate, further reinforces the notion that fipronil is cytotoxic to spermatozoa. Thus, despite the differences that were observed and associated with environmental conditions, the observation that fipronil had the same effects on drone fertility under both conditions provides strong support for the reprotoxic nature of this substance and indicates that each approach is relevant for assessing the reprotoxic effects of plant protection products on drones. Thus, the results obtained under laboratory conditions predicted the results obtained under semifield conditions and could potentially predict field conditions. It has frequently been argued that toxicological effects, such as those observed under laboratory conditions, may not predict the effects observed under field conditions either because exposure was overestimated or because compensating factors were introduced or initially present in the field [47–50]. However, if compensating factors are found to be present, the findings should be viewed as more legitimate because they were obtained in the presence of aggravating factors, which can include wind, low temperature, hard worker labor, travel over long distances to achieve efficient foraging, competition with other pollinators, scarce food resources, habitat fragmentation, monoculture farming, and exposure to pathogens and other environmental pollutants. Thus, field or semifield studies that lead to a ban, restriction, or moratorium on the use of a substance or a family of substances may merely con

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Data availability—The dataset is available from: https://figshare.com/s/47d8bece6849d453e47 or from the corresponding author (jean-luc.brunet@inra.fr).

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