Effects of Some Insecticides on the Viability and the ATP Synthesis of Honeybee Drone's Spermatozoid *in vitro* Exposed

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ABSTRACT

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Honeybee (*Apis mellifera*) reproduction is polyandrous: the queen obtains millions of spermatozoid by mating with several drones outside the colony. Fertility problems of honeybee queens are reported where failure of the production and quality of sperm drones are suspected. Several factors can affect sperm quality drones include pesticides. The aim of this study is to determinate the *in vitro* effect of fipronil, ethiprole, imidacloprid, thiamethoxam, cypermethrin, and coumaphos at different concentrations from 0.1 to 100 µM on the viability and the energetic state, through ATP content, of spermatozoids of honeybee drones. Exposure during 24 h showed that all the active ingredients used in this test increase the ATP levels. Four of them i.e. fipronil, ethiprole, imidacloprid, and thiamethoxam reduced significantly the viability of spermatozoids. Hence, pesticides could affect the drone's spermatozoids which may have negative impact on semen quality and further queen fecundity.

Keywords: *Apis mellifera*, ATP, drone, pesticides, spermatozoid, viability

Insect pollination is a key factor in sexual reproduction in angiosperms. Eighty percent of crops around the world are dependent on pollination activity due to insects and foremost bees. As pollinator, the honeybee *Apis mellifera* plays an ecological and economic key role. The pollination effectiveness of bees in agricultural landscapes is due to their large number, anatomy and behavior of foraging (5).

Bees are among eusocial insects with a cooperative brood care, overlapping generations and specialization reproduction limited to a few individuals. The latter distinction is very evident where the queen is the only reproductive and laying female in the colony. Queen has an essential role in the hive; not only renewing the population but emitting pheromones that will punctuate the activity of the colony (9). However, in recent years, many beekeepers report a decrease in queens' longevity and quality. Van Engelsdorp *et*

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al. (22) found that the famine, the varroa mite and the colony collapse disorder were important factors suspected of colony losses (28, 24 and 9%, respectively). The main problem perceived to beekeepers was "poor queens" (31% loss of colonies). The queen is the sole reproductive female in the colony (laying around 1,000 eggs per day) and any stress factor that affects its reproductive output can compromise the colony fate. The poor performance of the queens is considered in recent studies as one of the main reasons for the losses suffered by the colonies. A colony can be affected by many factors that significantly reduce the reproductive potential of the queen. Knowing that its reproductive life also depends on the mating success with drones (9), sperm from drones insufficient in quality and quantity could be a possible cause of queen's performance decrease (6).

Multiple causes can also reduce male fertility and fecundity in the animal kingdom such as pesticides. Many studies have shown that human exposure to environmental pollutants, especially pesticides can decrease fertility (1, 11, 14). These effects cover also most vertebrates as rats (24) and insects (19) and also in drones following chronic exposure to coumaphos, fluvalinate or thymol (6).

The aim of this study is to test *in vitro* the effect of six active ingredients used as insecticides on the drone's spermatozoa exposed during 24 h. These molecules are chosen because honeybee colonies could be exposed to them in intentional and unintentional ways in order to protect the colony against honey bee pathogens such as coumaphos which is used against varroa or through pesticides used in field on crops attractive to honey bee and brought back to hives by foragers such as fipronil, ethiprole, imidacloprid, thiamethoxam, cypermethrin. The effect of pesticides was evaluated by determining the viability and the energy status through Adenosine 5'-TriPhosphate (ATP) content in drone spermatozoa.

MATERIALS AND METHODS

Semen collection.

Experiments were carried out from May to July 2013 in *Laboratoire de Toxicologie Environnementale*, INRA, Avignon (France), with *A. mellifera* colonies carefully monitored for their health status. Semen (Fig. 1) was collected from mature drones captured in front of about 10 hives between 12 h and 16 h. Semen was collected by a manual eversion of the endophallus (Fig. 2). Briefly, drones were stimulated to ejaculate by pressing on the thorax, which usually resulted in eversion of the endophallus. Semen was collected from the tip of the endophallus with a glass capillary 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, 3 g/l sulfanilamide, pH 8.5, osmotic pressure = 486 mOs/ml).

Fig. 1. Drone' semen (8).

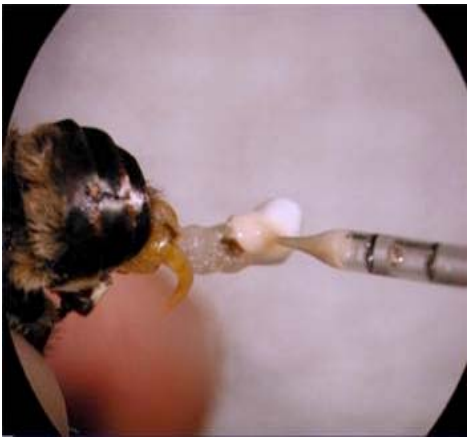

Fig. 2. Collection of the semen (8).

***In vitro* exposure of spermatozoid to insecticides.**

To evaluate effect of insecticides on the viability of spermatozoa, all tests were carried out *in vitro* and performed in triplicate in 96-well microplates. Each well contains 3 million of spermatozoa. Six active ingredients, used as insecticides, were tested in this trial. They belonged to different families such as phenylpyrazoles (fipronil and ethiprole), neonicotinoids (thiamethoxam and imidaclopride), synthetic pyrethroids (cypermethrin) and organophosphorus (coumaphos) (Table 1). Based on data on the estimation of environmental exposure levels, it can be estimated that *in vitro* concentration in the range of 10-100 µM may be considered representative of pesticide levels in cells (21). Therefore, nominal concentrations within and under this range have been used throughout the study.

Five concentrations were tested (0.1, 1, 10, 50, and 100 µM for fipronil, imidacloprid, ethiprole, thiamethoxam and cypermethrin and 1, 10, 25, 50, and 100 µM for coumaphos). These molecules were diluted in DMSO (dimethylsulfoxide) with the exception of cypermethrin and coumaphos which were diluted in acetone. The final concentration of both DMSO and acetone was 1% per well. The plates were incubated for 24 h at 20°C and 5% CO_2. This test was repeated three times during the beekeeping season. Well control contains only solvent without active ingredient.

Sperm count.

To determine the number of spermatozoa in each well, the semen was diluted (1:200) in the Kiev solution and spermatozoa were counted (15 µl of diluted semen) under a phase contrast microscope (× 200) using a Malassez cytometer.

Table 1. Characteristics of active ingredients used in the bioassay

Active ingredient	Purity (%)	Insecticide family	Common trademark name	Oral LD50 (µg/bee) (20)	Agricultural uses
Imidacloprid	99	Neonicotinoid	Gaucho®, Confidor®	0.0037	Cereals, rice, potatoes, vegetables
Thiamethoxam	98	Neonicotinoie	Cruiser®, Actara®	0.005	Leaf vegetables, citrus, tobacco, soybeans
Fipronil	96- 99	Phenylpyrazol	Regent®	0.00417	Coating maize seeds, cotton, beans, sorghum. non-agricultural uses to fight fleas, termites
Ethiprole	97	Phenylpyrazol	Curbix®, Kirappu®	No data available	Rice, citrus, cotton, ornamentals...
Cypermethrin	94	Synthetic Pyrethrinoide	Demon® WP®, Rai®	0.035	Fruits and vegetables, Industrial and domestic use
Coumaphos	95	Organophosphorus	Asuntol®	3.6	Used against varroa

Sperm viability.

Sperm viability was assessed with the conventional live-cell stain Sybr14 from the Live/Dead® Sperm viability kit (Lifetechnologie, France). This test was performed in 96-black-well microplate. As recommended by manufacturer, Sybr14 was diluted in DMSO at a final concentration of 60 µM. Five µl of Syber 14 solution were added to each well after the duration exposure of 24 h. The microplate was incubated at 37°C for 5 min before measuring the fluorescence intensity.

ATP content in spermatoizoid *in vitro* exposed to insecticides.

The ATP content was determined with ATPlite® kit (PerkinElmer, France). This kit contains mammalian cell lysis solution and substrate solution (Fig. 3). The ATPLite® assay system is based on the production of light caused by the reaction of ATP with added luciferase and D-luciferin. The emitted light is proportional to the ATP concentration within certain limits. As recommended by the supplier, 50 µl of a mammalian cell lysis solution were added to diluted semen in each well and the microplate was covered and gently shaked for 5 min with an orbital shaker at 700 rpm. Then, 50 µl of substrate solution was added, the microplate was covered and shaked for 5 min at 700 rpm and then kept in darkness for 10 min before measuring the luminescence intensity.

Statistical analysis.

The effects of pesticides on sperm were evaluated by ANOVA test using SPSS / PC software program (version 16.0).

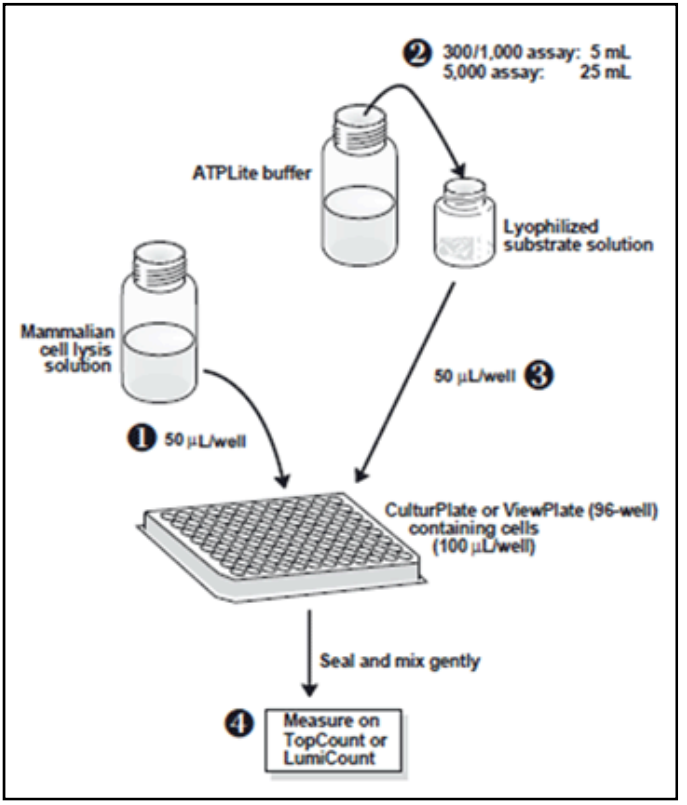

Fig. 3. Protocol of ATP measure (as recommended by manufacturer PerkinElmer®).

RESULTS

Effect of insecticides on spermatozoa viability.

Under our experimental conditions, the concentration of 1% DMSO or acetone does not affect sperm viability. However, exposure to different concentrations of fipronil reduced sperm viability in comparison with the control. This reduction was significant at the concentrations of 0.1, 100, and 50 µM ($P < 0.01$; $P < 0.05$ and $P < 0.001$, respectively). Sperm viability exposed to 1 and 10 µM concentrations was also reduced but the difference was not significant as compared to the control (Fig. 4aA).

Regarding ethiprole (Fig. 4aB), thiamethoxam (Fig. 4bC) and imidacloprid (Fig. 4bD), sperm viability was significantly reduced compared to the control for all concentrations ($P < 0.001$). However, we did not notice any difference in the viability of sperm exposed to cypermethrin (Fig. 4cE) and coumaphos (Fig. 4cF) compared to the control.

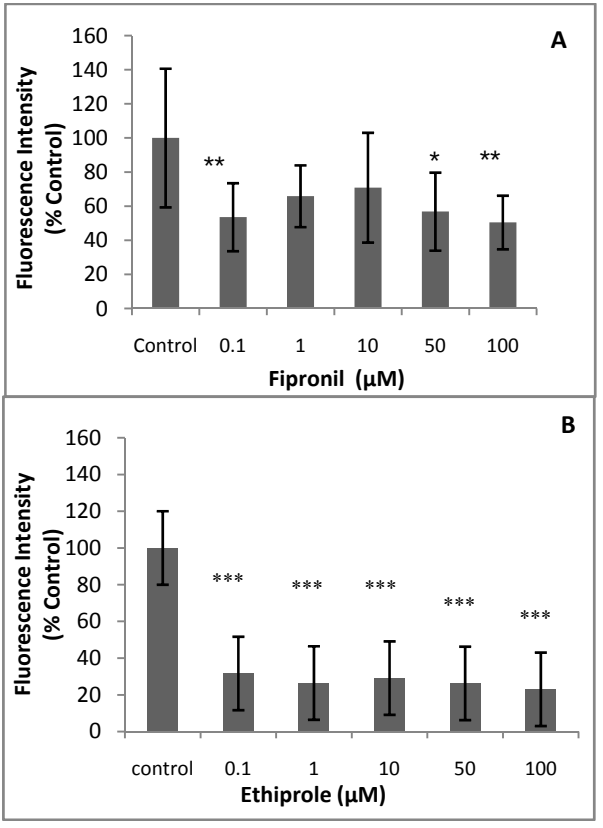

Fig. 4a. Effect of pesticides on viability of spermatozoids *in vitro* exposed during 24 h to fipronil (A) and ethiprole (B). Values were expressed as percentage of control.
*, **, *** indicated significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

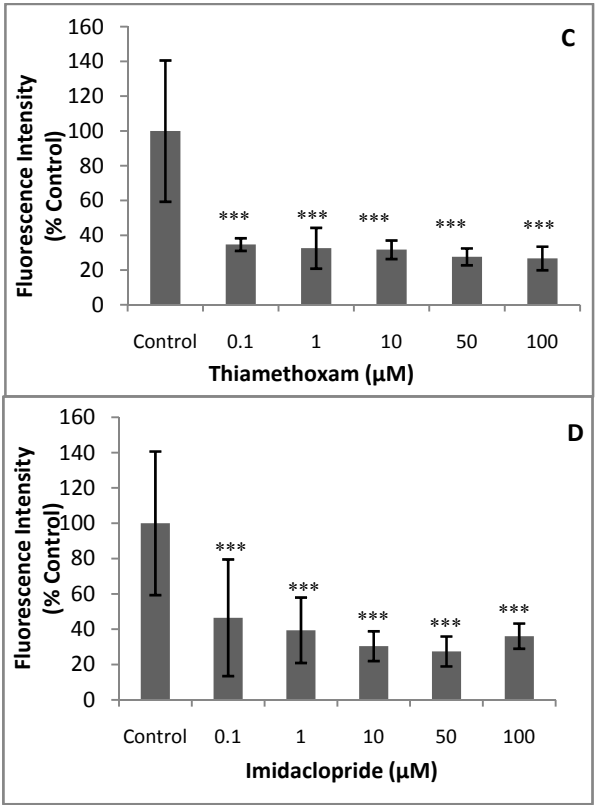

Fig. 4b. Effect of pesticides on viability of spermatozoids *in vitro* exposed during 24 h to thiamethoxam (C) and imidacloprid (D). Values were expressed as percentage of control.
*, **, *** indicated significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

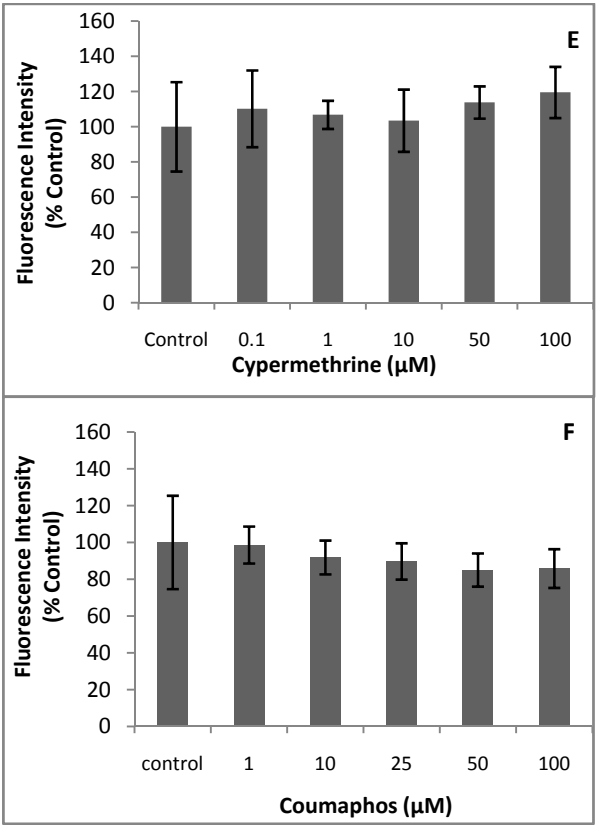

Fig. 4c. Effect of pesticides on viability of spermatozoids *in vitro* exposed during 24 h to cypermethrin (E) and coumaphos (F). Values were expressed as percentage of control.
*, **, *** indicated significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

Effect of insecticides on ATP content in spermatozoid.

The presence of fipronil induced an increase in the rate of ATP (Fig. 5aA). The ATP level was significantly higher of 27% compared to the control at the dose of 10 µM and 50% at doses of 50 and 100 µM ($P < 0.05$, $P < 0.001$ and $P < 0.001$, respectively).

The ATP levels in sperm exposed to ethiprole increased with the exposure dose (Fig. 5aB). Indeed, it is higher of 61% at a dose 0.1 µM ($P < 0.05$) and 78, 81 and 86% more than the control at doses 1, 10 and 50 µM ($P < 0.001$), respectively.

The exposure to thiamethoxam also induced an increase in ATP levels with the increasing of the dose (Fig. 5bC). This increase was significant only at doses of 10 and 100 µM ($P < 0.05$).

For imidacloprid tested at the lowest dose (0.1 µM), the ATP level is significantly higher of 91% compared to the control ($P < 0.001$). This parameter was significantly 73% higher at a dose 1 µM ($P < 0.01$) and 50% at 10, 50, and 100 µM, respectively, compared to control ($P < 0.05$) (Fig. 5bD).

Exposure to cypermethrin (Fig. 5cE) and coumaphos (Fig. 5cF) also induced a slight increase in ATP levels at all doses and it is significant only at the dose 1 µM for cypermethrin ($P < 0.01$).

DISCUSSION

Several experimental studies on human cells *in vitro* have shown that some pesticides (endosulfan, rotenone) may induce oxidative stress resulting in disturbances in process survival and proliferation cellular control (15).

Sperm viability and functional biomarkers has been widely used in toxicity tests of sperm in mammals (16). Sperm viability is a key factor in assessing the quality of drone's sperm. In fact, after the nuptial flight, the number of sperm stored in the spermatheca is on average 6 million. This stock will be used to fertilize the eggs that come from the ovaries of the queen throughout her life since she makes only one nuptial flight. The decrease in the number of viable sperm will result in a reduction in the number of fertilized eggs developing to workers in the post embryonic development. These workers represent the largest caste number, they perform most of the tasks of the colony and on which depends the general condition of the colony. In our study, fipronil, ethiprole imidacloprid and thiamethoxam, decreased significantly sperm viability. This decrease was estimated to 47% compared to the control at the lowest dose (0.1 µM) and reached 70% for fipronil used at 10 µM. For imidacloprid, the viability decreased by 50% with the lowest dose. This increases varied in a dose-dependent manner. In the same way, for thiametoxam and ethiprole, viability decreases with the dose (66% at 0.1µM, 74% at 100 µM and 69% at 77% at 100 µM, respectively). It was shown that alachlor for example, an herbicide belonging to chloroacetamides family, decreases the viability of human spermatozoa (12). Fipronil also inhibits the production, the viability and the motility of human spermatozoa (7). In rats, it has been shown that some active ingredients like dimethoate (organophosphorous) and deltamethrin (pyrethroid) decrease the viability of sperm (18). Chronic exposure to diazinon induced alterations in the quality and integrity of human sperm DNA (10). The exposure of human spermatozoa to 10 µM of cypermethrin for 6 h induced DNA

damage (23). However, in our study, molecules belonging to organophosphorous (coumaphos) and pyrethroid (cypermethrin) families did not elicit any effect on sperm viability. In the other way, after 24 h of exposure to neonicotinoids and phenylperazoles molecules, viability of sperm was reduced.

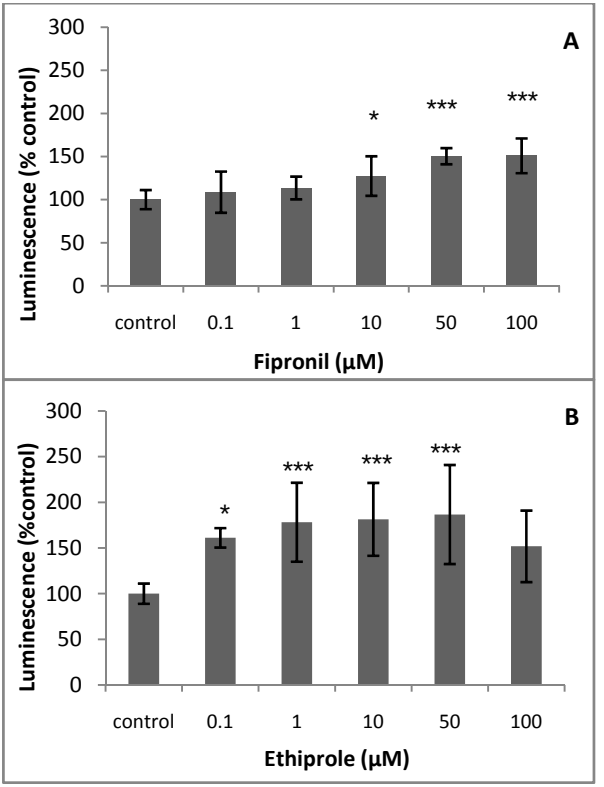

Fig. 5a. Effect of pesticides on the ATP of spermatozoids exposed *in vitro* during 24 h to fipronil (A) and ethiprole (B). Values were expressed as percentage of control.
*, **, *** indicated significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

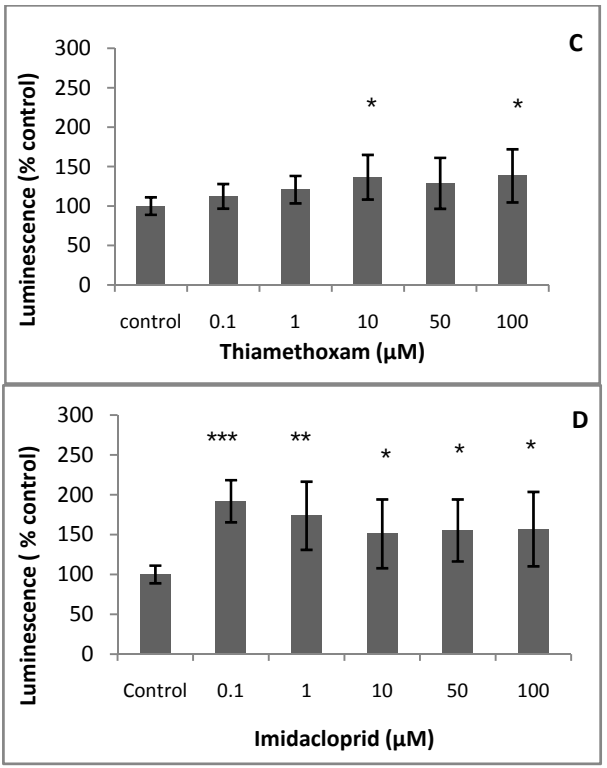

Fig. 5b. Effect of pesticides on the ATP of spermatozoids exposed *in vitro* during 24 h to thiamethoxam (C) and imidacloprid (D). Values were expressed as percentage of control.
*, **, *** indicated significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

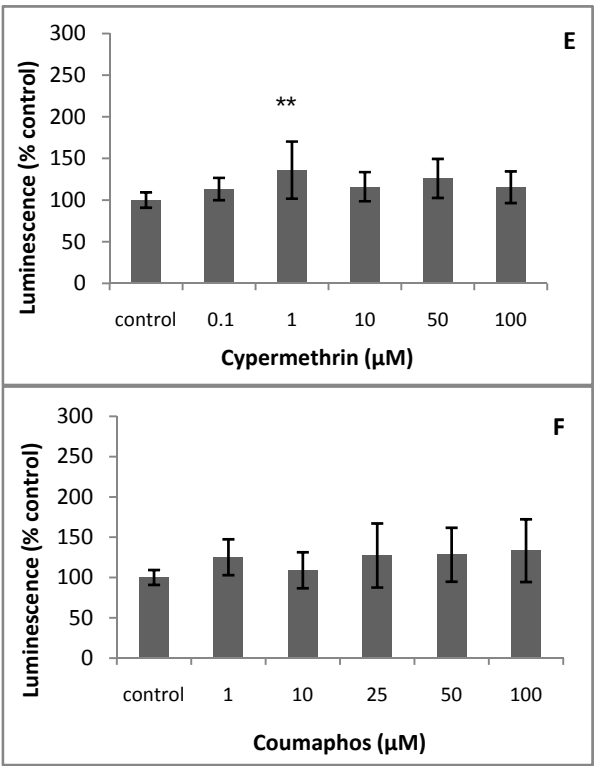

Fig. 5c. Effect of pesticides on the ATP of spermatozoids exposed *in vitro* during 24 h to cypermethrin (E) and coumaphos (F). Values were expressed as percentage of control.
*, **, *** indicated significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

In general, sperm requires ATP mainly for mobility but also in the cellular processes including the hyperactivation, capacitation to fertilize the egg and the acrosome reaction. The results obtained in the study showed that ATP levels increase with the concentration in sperm exposed whatever the molecule tested. In the cell, the synthesis of ATP is determined by the bioavailability of energy substances (carbohydrates, lipids, and proteins), the activity of catabolic pathways and the functional integrity of the mitochondria. Mitochondria, the headquarters of the ATP synthesis, contain several key systems of energy metabolism. This organelle is also the target of several xenobiotic known to disrupt its operation. Pesticides such as rotenone and paraquat, environmental pollutants such as lead and cyanide or some antivirals such as reverse transcriptase inhibitors exert their cytotoxicity by disrupting the functioning of the mitochondria (4, 13, 17). Another study also showed an increase in ATP levels in cerebellar granule cells in the process of apoptosis where ATP results from both oxidative phosphorylation and anaerobic glycolysis (3). In some diseases, for example, ATP may increase with energy needs (2). Thus, ATP increasing in sperm exposed to pesticides could be harmful to the future of the cells. Therefore, we can hypothesize that the increase of ATP observed reflects their responses to oxidative stress caused by xenobiotics.

In conclusion, this study is a first attempt to relate the effect of some analytical standards insecticides on sperm from drones exposed *in vitro* for 24 h. All molecules (fipronil, ethiprole, imidacloprid, thiamethoxam, cypermethrin and coumaphos) appear to affect the quality of the drone sperm. This study was particularly difficult since the drones are only present for a short period of the year, during the spring, and the average volume of semen from a drone is 1 µl and we need important volumes to realize the test.

Recent evidence provided by our work allows reconsidering certain aspects of the toxicological profile of these molecules. At the end of our experiments, we suggest further research on the effect of pesticides on quality drones sperm and semen that play a crucial role in the performance of the queen and later the development of the honey bee colony.

RESUME

Ben Abdelkader F., Barbouche N., Belzunces L. et Brunet J.L. 2015. Effets de quelques insecticides sur la viabilité et la synthèse de l'ATP des spermatozoïdes des mâles d'abeille mellifère exposés *in vitro*. Tunisian Journal of Plant Protection 10: 79-93.

La reproduction chez la reine de l'abeille domestique (*Apis mellifera*) se fait par copulation avec plusieurs faux-bourdons (polyandrique) lors d'un seul vol nuptial qui se fait à l'extérieur de la colonie. Des problèmes de fertilité des reines abeilles ont été rapportés et qui étaient liés, entre autres, à une défaillance de la production et de la qualité du sperme des faux-bourdons. Plusieurs facteurs peuvent affecter la qualité du sperme des faux-bourdons tels que les pesticides. L'objectif de cette étude est de déterminer l'effet *in vitro* de quelques insecticides tels que le fipronil, l'éthiprole, l'imadaclopride, le thiaméthoxam, la cypermethrine et le coumaphos appliqués à différentes concentrations allant de 0,1 à 100 µM sur la viabilité et le statut énergétique des spermatozoïdes des faux-bourdons. L'exposition pendant 24 h a montré que les molécules testées ont augmenté le taux d'ATP au niveau des spermatozoïdes. Quatre d'entre elles à savoir le fipronil, l'ethiprole, l'imidaclopride et le thiamethoxam ont diminué la viabilité des spermatozoïdes d'une manière significative. Par conséquent, les pesticides

peuvent avoir un effet négatif sur les spermatozoïdes des faux-bourdons; ce qui pourrait avoir des répercussions sur la qualité du sperme des mâles et par conséquent, la fécondité des reines.

Mots clés: *Apis mellifera*, ATP, faux-bourdons, pesticides, spermatozoïde, viabilité

ملخص

بن عبد القادر، فاتن ونعيمة بربوش ولوك بلزنز وجون لوك بروني. 2015. تأثير بعض المبيدات الحشرية في المختبر على نمو الحيوانات المنوية وعلى إنتاج الادينوسين ثلاثي الفسفات (ATP) عند ذكر النحل.
Tunisian Journal of Plant Protection 10: 79-93.

تقوم ملكة النحل بعملية الإلقاح مرة واحدة في حياتها مع عشرات الذكور خارج الخلية وذلك في نطاق عملية التكاثر. غير انه تم مؤخرا تسجيل مشاكل في خصوبة الملكات ويشتبه في أن تدني إنتاج ونوعية الحيوانات المنوية لذكر النحل لها دور في ذلك. يمكن لعدة عوامل أن تؤثر على نوعية الحيوانات المنوية كالمبيدات مثلا. الهدف من هذه الدراسة هو تحديد تأثير "الفيبرونيل" و"الاتبرول" و"الامداكلوبريد" و"التيامتوكسم" و"السيبارمترين" و" الكومافوس" بتركزات تتراوح من 0.1 إلى 100 ميليمول في المختبر على الحيوانات المنوية لذكر النحل. أظهر التعرض للمبيدات لمدة 24 ساعة زيادة في نسبة الادينوسين ثلاثي الفسفات (ATP). وأدت أربعة من بين المبيدات وهي "الفيبرونيل" و"الاتبرول" و"الامداكلوبريد" و"التيامتوكسم" إلى انخفاض كبير في نسبة الحيوانات المنوية الحية. لذلك، يمكن للمبيدات أن تؤثر علي الحيوانات المنوية مما قد يكون له اثر سلبي على نوعية السائل المنوي وخصوبة الملكة.

كلمات مفتاحيه: حيوان منوي، ذكر النحل، مبيدات، قابلية الحياة، *Apis mellifera* ، ATP

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