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Abstract – Many problems have been reported on honey bees colonies including fertility problems of queens resulted in production failure. Pesticides can be the cause of this failure in connection with the quality of sperm drone. Thus, the aim of this study was to assess the influence of exposure to syrup contaminated with clothianidin at 0.1 µg/L on semen parameters of drones. Results showed a significant decrease of semen volume and sperm concentration and an increase in sperm mortality rate. As for the energetic state, clothianidin increased cell redox potential, the ATP content of spermatozoa as well as the lactate dehydrogenase activity (LDH). It was concluded that exposure to clothianidin during the sexual maturity of drones could affect the semen quality.

Keywords: pesticides, drones, spermatozoa, fertility, metabolic state

1. Introduction

Populations of honey bees are declining worldwide for more than a decade (Grossman 2013). Colony losses are attributed to numerous causes including exposure to pesticides (DeGrandi-Hoffman et al. 2013). Increasingly, there are concerns about the way by which pesticides may weaken and kill bee colonies at sublethal levels (Frazier et al. 2008). In parallel, the increased use of agrochemicals coincided with increased beekeeper complaints on problems associated with queen performance in the hive (Burley et al. 2008). Many beekeepers have noted problems with supersedure after the introduction of a new queen as well as an inability of colonies to naturally renew queen (Sanford, 2001 in (Pettis 2004). It is known that the reproductive live of queen depends, among others, on the success of her mating with drones(Connor 2010). Decreasing sperm quality of drones might play a role in the current problems associated with queen performance(Burley et al. 2008). Queens mated with sperm of low viability would become drone layers faster than well-mated queens(Collins 2000). Pesticides are among factors that have an effect on drone semen quality. Burley et al. (2008) report that a decrease in quality of sperm contained in the spermatheca of queens mated with miticide-exposed drones could lead to queen loss and supersedure. Miticides used in honey bee colonies can also reduce drone survival (Rinderer et al. 1999) and production (De Guzman et al. 1999), body and mucus gland weights (Rinderer et al. 1999) and spermatozoa production (Fell and Tignor 2001). Besides, many studies have reported adverse effects of pesticides on the reproductive system of vertebrates. Bal et al. (2012a) report reduced sperm production in rats exposed to imidacloprid, a neonicotinoid, at 2 mg/kg bw/day, a dose representing about 1/250th of the LD50 per day. Exposure to clothianidin at low doses during critical stages of sexual maturation of male rats has moderate detrimental effects on reproductive system but more severe effects are observed at higher dose levels (Bal et al. 2012b). The reproductive system may be more sensitive to exposure to clothianidin early in the development (prenatal and early postnatal) (Bal et al. 2012b). Moreover, it has been reported that some neonicotinoid pesticides induce oxidative stress in the serum of rat (Mohany et al. 2011), liver (El-Gendy et al. 2010) and testis (Zhang et al. 2011) of mice. Honey bee colonies are often exposed not only to miticides but also to other insecticides through contaminated nectar and pollen stored and subsequently shared among nestmates including drones (DeGrandi-Hoffman et al. 2013) which could affect their role inside the colony. The objective of this study was to determine the effects of clothianidin on the quality of semen from drones daily exposed up to their sexual maturity through contaminated food gathered in field and brought to the hive by foragers. To this end,

analyses were focused on semen volume, concentration, mortality rate, redox potential, energetic state (ATP) of spermatozoaand lactate dehydrogenase (LDH) activity.

2. Materials and Methods

Experiments were performed from May to July 2013 (two repetitions) in Avignon (France) with *Apis mellifera* L. colonies carefully monitored for their health status. To stimulate drone production, drone combs were introduced in 10 strong colonies (35,000-45,000 bees/colony; 1 comb/colony) 25 days before the beginning of experiments. These colonies were treated conventionally with Amitraz in September 2012 to control varroa population. The day before emergence, brood frames were placed into an incubator in the dark at 34°C and 70% relative humidity. Queenless and droneless nuclei composed of 5000 honey bees, one brood frame, and four empty frames with no food storage were placed in an outdoor tunnel (32 x 8 m) covered with an insect proof mesh divided 4 compartments. Two nuclei were placed in each compartment (8 x 8 m). To avoid overexposure to the sun, umbrellas were placed above each nucleus and food feeder. No food was supplied inside the nuclei. Three hundred emerging drones were introduced in each droneless nucleus and enabled for free movement in the colony.

2.1. Exposure to clothianidin

The honey bee nuclei were introduced in the tunnel three days before drone introduction to train bees to visit a feeder placed at equal distance from the nuclei. This feeder consisted of a yellow cup containing sugar syrup (50% sucrose solution, w/v) on which a piece of cork and filter paper were placed on the surface to enable the honey bees to land. Treated nuclei received sugar syrup with a final concentration of 50% sucrose, 0.1% DMSO (v/v) and 0.1 µg/L clothianidin. Great care was taken to avoid the direct contact of the sunlight with the feeder. Controls received sugar syrup containing 50% sucrose and 0.1% DMSO. Honey bees were fed or exposed to clothianidin from 9:00 to 12:00 a.m. each day for a period of 20 days. Outside this period, honey bees received water and pollen *ad libitum*.
The amounts of syrup and pollen foraged were measured daily in each compartment. Thus, during their sexual maturity, drones were chronically fed with a food gathered to the hive by foragers, as in natural conditions. In total, 22 queenless nuclei were used in the experiment, 10 for treatment and 12 for control (4 and 6 for the first experiment in 2013, 6 and 6 for the second experiment in 2013).

2.2. Drone survival rate, maturity rate and semen collection

Drones were collected after 20 days. The survival rate was recorded. Semen was gathered by a manual eversion of the drone endophallus. 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). The average volume of semen was determined. During semen collection, the maturity rate of drones from each colony was assessed by the ability to provide sperm after stimulation. The average semen volume per drone was also determined. A part of the fresh semen from each sample was used for the determination of the spermatozoa concentration, mortality rate and metabolic activity (as assessed by measuring the reducing potential, adenosine triphosphate (ATP) content) and another part of fresh semen from each sample was transferred into a microcentrifuge tube, diluted two-fold in Kiev solution and centrifuged at 4°C for 20 min at 16,000 *g*. The supernatant (diluted seminal plasma) was kept for LDH assay.

2.3. Spermatozoa concentration

The semen was diluted in the Kiev solution ($1/1500^{th}$) and spermatozoa were counted under a phase contrast microscope by using a Neubauer improved Type Petroff cell with 0.02 mm of depth. Five repetitions were done for each sample.

2.4. Sperm mortality

Sperm mortality was assessed with the conventional dead-cell stain propidium iodide (PI) from the Live/Dead® Sperm viability kit (Life Technologie, France). This test was performed in 96-black-well microplate. According to the recommendations of the manufacturer, propidium iodure was diluted in DMSO at a final concentration of 60 µM. Five µl of PI solution were added to each well containing

1.10^7 spermatozoa. The microplate was incubated at 37°C for 5 min before measuring the fluorescence intensity.

2.5. Sperm reduction potential

Sperm reduction potential was estimated by the capacity of spermatozoids to reduce resazurin into resorufin, which is fluorescent (λ_{exc} = 569 nm, λ_{em} = 586 nm) and absorbs at 470 nm (Erb and Ehlers 1950; Shiloh et al. 1997). The test was performed in quadruplicate with a Prestoblue® kit (Invitrogen). Ninety µL of diluted semen samples and 10 µL of Prestoblue® were put in a 96 white-well microplate and kept in darkness for 10 min before measuring the absorbance at 570 nm according to the recommendations of the manufacturer.

2.6. ATP content

ATP content was determined with ATPlite® kit (PerkinElmer, Courtaboeuf, France) in the same wells as the viability test. 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 (PerkinElmer®) were added to the 100 µl of diluted semen in each well and the microplate was covered and gently shaken for 5 min with an orbital shaker at 700 rpm. Then, 50 µl of substrate solution was added, the microplate was covered and shaken for 5 min at 700 rpm and then was kept in the darkness for 10 min before measuring the luminescence intensity (LI).

2.7. Lactate deshydrogenase

The principle of the measure of LDH activity is based on the reduction of NAD^+ to $NADH/H^+$ during the conversion of lactate to pyruvate by LDH. The assay medium contained diluted semen and 50 mMtriethanolamine buffer, 5 mM EDTA, 0.2 mM reduced β-Nicotinamide adenine dinucleotide (β-NADH) and 2 mM sodium Pyruvate.LDH activity was spectrophotometrically followed at 340 nm for 5 min.

2.8. Statistical analysis

Mann-Whitney U test was used to determine differences between groups. Results are presented as mean ± S.E. Values were considered statistically significant if $P< 0.05$. The SPSS/PC program (Version 16.0) was used for the statistical analysis.

3. Results and discussion

The nicotinic acetylcholine receptors (nAChRs) of the nervous system of insects are the primary and selective targets for Neonicotinoid insecticides including clothianidin (Tomizawa and Casida 2003). As some nAChR subunits are expressed in other organs, any neonicotinoid may have multiple effects outside the central nervous system (Bal et al. 2012b). To our knowledge, no studies have been carried out on the toxicity of Neonicotinoid on drones exposed during the sexual maturity stage at which sperm has completed migration from the testes to the seminal vesicles and when mucus glands are fully developed (Rousseau et al. 2015). In this context, drones were exposed to clothianidin via food brought back to the hive by foragers during the period from emergence to drone sexual maturity. Hence, drones directly consuming the syrup with clothianidin at 0.1 µg/L upon the return of foragers. The apparent effects show no significant effects on the foraging behavior of workers during the 20 days of exposure either for contaminated syrup (Figure 1A) or the pollen (Figure 1B).One recent study showed that limited sublethal effects on the tested behavioral functions of winter bees were observed following a chronic exposure to clothianidin at 15 lg/kg(Alkassab and Kirchner 2016). Although many factors are different under field conditions compared to semi-fields conditions, ours results play an important role in assessing the exposure risk.

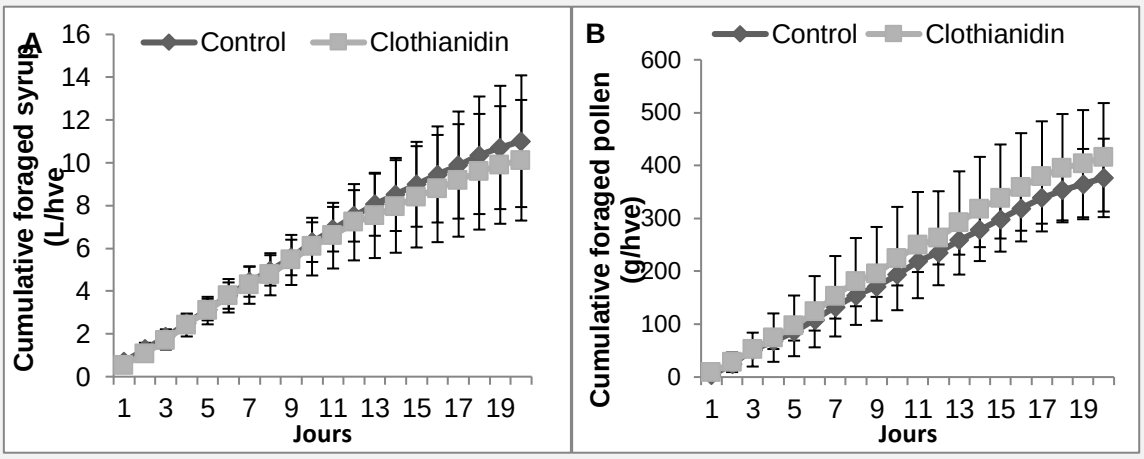

Figure 1. Effect of chronic clothianidin exposure on the cumulative foraged syrup (**A**) and pollen (**B**). The data represent the mean ± the standard deviation of the daily cumulative foraged quantity. For each treatment, the data correspond to the set of values from 2 experiments conducted in 2013 (n =6 for control and n= 5 for clothianidin). Vertical bars represent standard error of the mean

A total of 46.04 ± 25.33% of survived drones was recorded in exposed group (33.88 ± 19.6% for control) (Figure 2). The percentage of mature drones recorder in our study was higher in exposed group (60.97 ± 4.33%) compared to control (56.35 ± 8.56%) (Figure 3A) but statically these results are not significant.

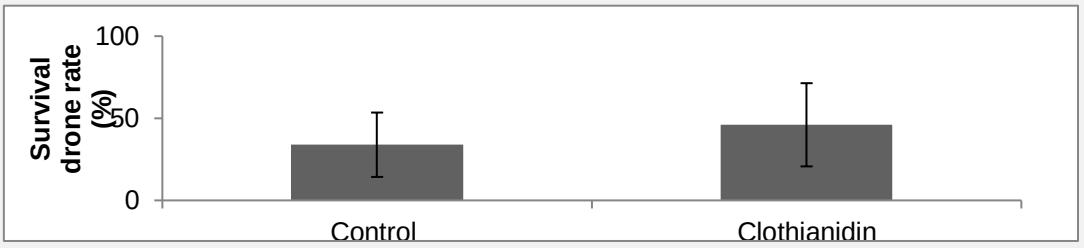

Figure 2. Effects of chronic clothianidin exposure on drone survival rate. The data represent the mean values ± standard deviations obtained from drone populations recovered from hives in 2 experiments monitored in 2013 (n=12 for control and n=10 for clothianidin). Vertical bars represent standard error of the mean

The quantity and quality of semen parameters such as ejaculate volume, sperm concentration and sperm mortality are used as biological endpoints to evaluate drone fitness and spermatozoal competitiveness (Gençer and Kahya 2011).In this context, the quality of semen was studied in greater detail by analyzing sperm count and sperm mortality. The mean semen volume per drone as shown in Figure 3B was lower in drones exposed to clothianidin (median of 0.7 µl/drone vs 0.82 in controls, *P<0.05*).

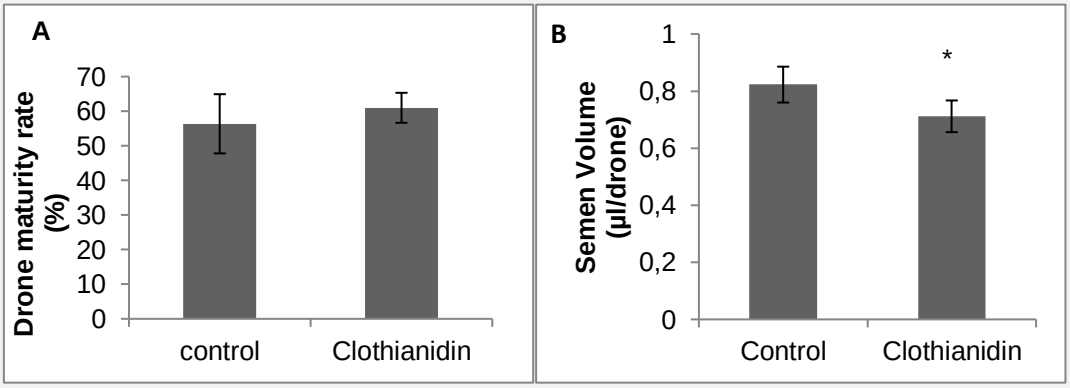

Figure 3. Effects of chronic clothianidin exposure on drone maturity rate (A) and on the semen volume (B). The data represent the mean values ± standard deviations obtained from drone populations recovered from hives in 2 experiments monitored in 2013 (n=12 for control and n=10 for clothianidin) Vertical bars represent standard error of the mean. *Comparison of control with clothianidin-treated group (P < 0.05)

The exposure to clothianidin induced a decrease in total spermatozoa concentration (median of 8.20 × 10^6 spermatozoa vs 10.88 × 10^6 spermatozoa in controls, *P< 0.001*, Figure 4A) and an increased spermatozoa mortality rate (median of 39.92% vs. 47.57% in controls, *P< 0.001*, Figure 4B). In animals, a chronic exposure to clothianidin causes a decrease of spermatozoa count and the appearance of deformed spermatozoa (Watts 2011). Bal et al. (2012b) found also that exposure to clothianidin during 90 days decreases significantly epididymal sperm concentration in male rats. Unlike Straub et al. (2016) who found that exposure drones to clothianidin before emergence did not affect the sperm quantity, our results are in agreement with (Kairo et al. 2016) who find that exposure to Fipronil induced a decrease in total spermatozoa concentration and increase in sperm mortality. This suggests that reproductive effects of clothianidin are not limited to vertebrates and the number of species that could be affected seems much underestimated.

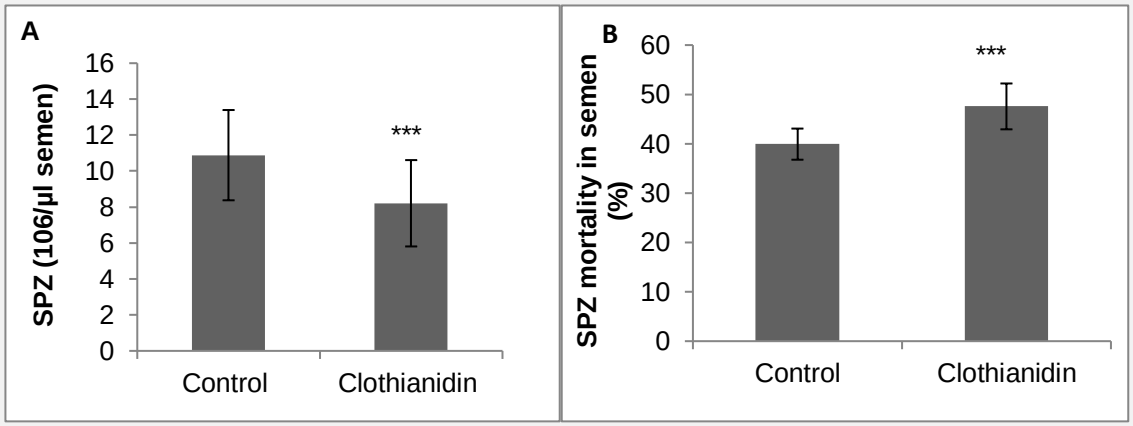

Figure 4. Effects of chronic clothianidin exposure on spermatozoid (SPZ) number (A) and semen mortality rate (B). The data represent the mean values ± standard deviations obtained from drone populations recovered from hives in 2 experiments monitored in 2013 (n=12 for control and n=10 for clothianidin). Vertical bars represent standard error of the mean. *** Comparison of control with clothianidin-treated group (P < 0.001)

At the cellular level, reducing potential corresponds to the ability of cells to metabolically reduce compounds or to passively reduce oxidants resulting from metabolic activity or ionizing rays. Reducing potential was increased by exposure to clothianidin (1.03 absorbance units (AU)) compared to control (0.85 AU, *P*< 0.001, Figure 5A). The cellular ATP content in spermatozoa produced by mitochondria was also increased (median of 2048.48 luminescence intensity (LI) vs. 1457.28 LI in controls, *P*<0.05, Figure 5B).

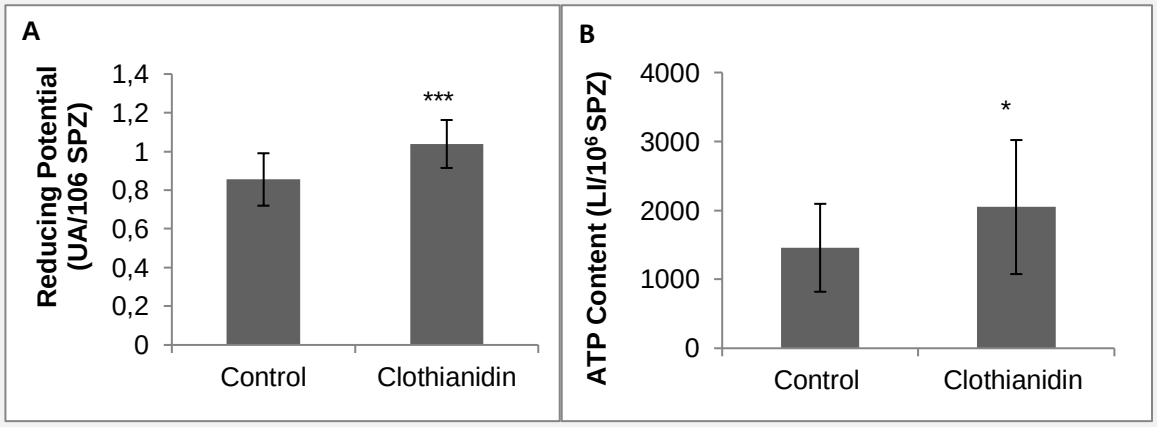

Figure 5. Effects of chronic clothianidin exposure on Reducing potential (A) of semen expressed in absorbance units (AU) and on ATP content expressed in luminescence intensity (LI). The data represent the mean values ± standard deviations obtained from drone populations recovered from hives in 2 experiments monitored in 2013 (n=12 for control and n=10 for clothianidin). Vertical bars represent standard error of the mean. * and *** Comparison of control with clothianidin-treated group (P < 0.05) and (P < 0.001) respectively

The redox potential is important for control of metabolism. Redox potentials are used to infer the direction and free energy cost of reactions involving electron transfer, one of the most ubiquitous and important types of biochemical reactions (Milo and Phillips 2016). According to Trachootham et al. (2008), oxidative stress can either enhance cell survival or promote cell death, depending on the magnitude and duration of the stress, the genetic background and redox states of the cells. The same authors reported that redox regulation of the key factors affecting cell death/survival is often bifurcated (*i.e.*, the same protein can either be activated or inhibited by redox alteration). Though ATP is often claimed to be the energy currency of the cell, in fact, for the energetic balance of the cell the carriers of reducing power are themselves no less important (Milo and Phillips 2016).
Sperm energy metabolism can be also characterized by the activities of marker enzymes reflecting the capacities of various metabolic pathways such as lactate dehydrogenase (LDH) (Al-Lawati and Bienefeld 2009).The LDH activity in seminal plasma of drones exposed to clothianidin was also significantly higher (1.54 ± 0.41mAU/min/10^6spz) compared to control (0.97 ± 0.16mAU/min/10^6 spz, P< 0,05, Figure 6).

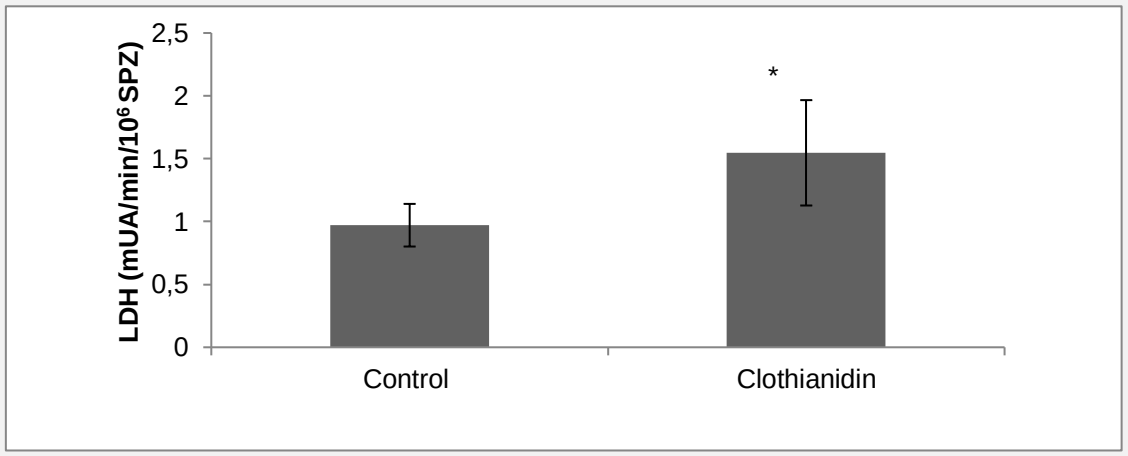

Figure 6. Effects of chronic clothianidin exposure on LDH activity expressed in milli absorbance unities (mUA). The data represent the mean values ± standard deviations obtained from drone populations recovered from hives in 2 experiments monitored in 2013 (n=12 for control and n=10 for clothianidin). Vertical bars represent standard error of the mean. *Comparison of control with clothianidin-treated group (P < 0.05)

LDH is essential for metabolic processes which provide energy for survival, motility, capacitation and fertility of spermatozoa (Sirat et al. 1996). The seminal fluid LDH can be used as good indicator of sperm viability(Stamatiadis et al. 1984). It has thus been proposed that increased LDH levels, as shown in treated group in our study, can be used as a good indicator of less of integrity of the plasma membranes(Dube et al. 1982). Moreover, extracellular activity LDH and lactate increases under the condition of oxidative stress, since the cell integrity can be disrupted during the lipid peroxidation process (Jovanovic et al. 2010). Although spermatozoa exhibit antioxidant defenses, an overload of these defenses can lead to cell death, explaining the high mortality rate of spermatozoa in drones exposed to clothianidin.

4. Conclusion

In conclusion, this study is the first report investigating the effect of exposure to clothianidin, via contaminated food brought to the hive by foragers, on the quality of honey bee drone semen. Clothianidin had negative effects and elicit a decrease of semen volume and sperm concentration, and an increase of sperm mortality rate and other sperm parameters. These results reveal for the first time the toxicity of neonicotinoid insecticides to reproduction in honey bee drone exposed during their sexual maturity. The results were obtained from new investigation methods, and therefore introduce a new perspective on the studies on queen performances and colony development.

Acknowledgments

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