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ORIGINAL RESEARCH ARTICLE

In vitro effects of imidacloprid on honey bee sperm: evaluation using computer-aided sperm analysis (CASA)

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Pesticides are considered as the most important factors of pollinators' decline. Imidacloprid (IMD), belonging to Neonicotinoids class, is widely used as a powerful insecticide but generating important negative effects on honey bees. The current study investigated the direct effect of IMD on bee spermatozoa. The experimental design consisted of *in vitro* co-incubation of bee gametes with three different concentrations of IMD 1, 10, and 25 μM for 15 min. Conventional microscopic and Computer-aided sperm analyses (CASA) were concomitantly used with a dual goal: to evaluate objectively the effects of IMD on sperm parameters on the one hand and to report computer kinematic parameters in the control group, without IMD treatment, on the other hand. The results revealed apparent negative effects in a dose-dependent manner with motility collapsing completely at 25 μM of IMD. The current results highlighted the direct impact of IMD on bee spermatozoa and revealing the potential subsequent effects on bee reproduction. This study reported also drone computer sperm parameters that could serve as a reference in the studied region. Bee sperm velocities showed the lowest values ever reported in the different animal species. The CASA system appeared as objective and a sensitive method to detect subtle toxic effects on bee sperm, and this opens real perspectives particularly in studying existing correlations between CASA parameters and fertility outputs of different environmentally toxic molecules.

Keywords: drone, spermatozoa, pesticides, *in vitro*, CASA system

Introduction

Brittain et al. (2010) Pesticides have been considered as one of the most important drivers of pollinators' decline (Brittain et al., 2010; Faucon et al., 2005; Mullin et al., 2010). They are widespread chemicals mainly used in pest control, applied as a seed dressing or foliar spray, and taken up by plants with different levels of systemic distribution, depending on the used molecule (Sur & Stork, 2003). The drastic effects of pesticides, particularly insecticides, include not only the mortality of non-target organisms but also interferences with normal behavior and functions (Costa et al., 2014; Decourtye et al., 2004).

Neonicotinoids are widely used as insecticides (Elbert et al., 2008) providing powerful impacts on agriculture yields, but generating important toxic effects in insects and mammals (Matsuda et al., 2001; Thany, 2010). Particularly in bee, they are considered as the major cause of bee colony collapse disorder (Henderson et al., 2007; Nahar & Ohtani, 2015). Imidacloprid (IMD), belonging to neonicotinoids class is extensively used particularly on Pomaceae, Drupaceae, citrus, horticultural plants, and seed dressing in corn, sugar beet, sunflower and potato (Medrzycki et al., 2003; Simon-Delso et al., 2015). IMD is effective against a wide range of arthropods, including aphids, scale insects, whiteflies, some heteroptera, coleoptera, and lepidoptera species (Elbert et al., 1991). IMD produces toxicity by binding to nicotinic acetylcholine (Ach) receptors on neurons postsynaptic membranes (Buckingham et al., 1997; Tomlin, 1997) causing paralysis and damaging different normal behavior (Matsuda et al., 2005; Tomizawa & Yamamoto, 1992).

Particularly in honey bees, several studies have demonstrated that IMD and its metabolites exert a broad suite of sublethal neural effects, including brain cell death (Wu et al., 2014) and motor function impairment (Lambin et al., 2001; Williamson et al., 2014). IMD decreases food uptake (Ramirez-Romero et al., 2005), reduces foraging behavior (Mommaerts et al., 2010), diminishes hive entrance activity (Decourtye et al., 2004), failing predator avoidance (Tan et al., 2014), homing failure (Nahar & Ohtani, 2015), impairs learning performance (Mengoni Goñalons & Farina, 2015), memory (Williamson et al., 2013), and immunity (Di Prisco et al., 2013) reduces fecundity and the overall colony fitness (Van dame et al., 1995). Particularly, the most alarming finding is the impairment of reproductive physiology

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(Kairo et al., 2016) in drones and bee queens (Ben Abdelkader & Barbouche, 2015; Williams et al., 2015).

The impact of pesticides on bee drones has been documented concerning reproductive safety (Ramazzini, 2009) and the impacts on the male reproductive system (Zenzes & Bielecki, 2004). *In vivo* studies in mammals have shown that insecticides impair male reproductive function, increase the incidence of sperm abnormalities, reduce testicular weights and alter epididymal sperm count and motility (Mani et al., 2002; Pati & Bhunya, 1989).

In honey bees, neonicotinoids act at very low concentrations reducing fertility outputs (Baylay et al., 2012) and altering ovary size (Williams et al., 2015). Similarly, phénylpyrazoles decrease spermatozoa concentration and viability which indirectly impair queen reproduction (Kairo et al., 2016).

In this respect, it is reported that IMD induces *in vivo* sublethal effects on sperm production (Straub et al., 2016), ATP content, and gametes viability (Ben Abdelkader & Barbouche, 2015; Ciereszko et al., 2017). Kairo et al. (2016) showed that drone exposure to IMD impairs queen reproduction by a significant alteration of spermatozoa stored in the spermatheca.

However, to the best of our knowledge, no previous studies reported a direct impact of IMD on honey bee sperm using *in vitro* models. Moreover, to date, sperm quality was exhaustively analyzed by the traditional microscopic analysis, known as a subjective method. In the current study, Computer-aided sperm analysis (CASA) was used, on the one hand, to evaluate objectively the effects of IMD on bee sperm and on the other hand to report normal computer parameters measured in the control group without IMD treatment.

Materials and methods

Semen collection

Experiments were carried out from May to July 2016, in Bejaia University (36°43′N, 5°04′W) (Algeria). *Apis mellifera intermissa* colonies were carefully monitored for their health status. During the mating season, semen was collected from 324 mature drones captured in front of four hives between 12 and 16 h. Semen collection from seminal vesicles was performed as reported previously (Phiancharoen et al., 2004). Seminal vesicles were separated and immediately transferred into a small glass vessel containing a 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) and pressed with a fine pair of needles to release semen, which is further diluted in 300 μl of Kiev solution.

In vitro exposure of spermatozoa to IMD

To study the direct impact of IMD on sperm motility, the experiment design consisted of a co-incubation during 15 min of 3 μl of pure semen diluted in 300 μl of Kiev solution; three IMD concentrations were tested: 1, 10, and 25 μM. IMD was previously diluted in DMSO (dimethylsulfoxide) at a concentration of 1%. The experiment was repeated 27 times during the beekeeping season. Control treatment consisted of spermatozoa in a Kiev solution without IMD.

Analysis of sperm motility

Traditional analysis of sperm motility

Motility percentage and motility index

A 10 μl of each sample was taken from the semen suspended in Kiev solution and dropped on the center of the Makler chamber (depth 10 mm, Sefie Medical Instruments). Motility was observed under phase contrast at 10× magnification. Motility percentage was measured by considering all motile spermatozoa, independently of the quality of the gametes movement. Then, motility index was scored on the basis of the following scales: 4 when >50% of the sperm exhibited circular and progressive movement, 3 when <20% exhibited circular and progressive movement but more than 50% were vibrating; 2 when there was no circular or progressive movement but >50% were vibrating; 1 when <50% of the sperm were vibrating; and 0 when there was no sperm movement (Locke & Peng, 1993). All motility measurements were taken 1 min after filling Makler chamber on 37 °C heated plate.

Computer sperm motility analysis (CASA)

The overall sperm motility, as well as, the kinematic parameters of all analyzed spermatozoa were assessed by Sperm Class Analyzer® (SCA) Version 5.4 (microptic S.L. Viladomat 321, 6e4 08029 e Barcelona, Spain). The system is provided with Basler A312fc digital camera mounted on a Nikon Eclipse E200 microscope (Nikon, Tokyo, Japan) with a 10 negative phase-contrast lens.

Sperm movement patterns were observed in 10 μ l of diluted semen using Makler chamber. The filled chamber was placed on 37 °C heated plate and allowed to settle 1 min. Three microscopic fields were analyzed in each sample with a phase-contrast objective lens (10 × 0.25).

Motility parameters measured by CASA, as described by Mortimer (1997, 2000), included: straight-line velocity (VSL, um/s): the average path velocity of the sperm head along a straight line from its first to its last position; average path velocity (VAP, um/s): the average velocity of the sperm head along its average trajectory; the percentage of linearity (LIN, %): the ratio between VSL and VCL; mean amplitude of lateral head displacement (ALH, m): the average value of the extreme side-to-side movement of the sperm head in each beat cycle; total motility (TM, %); progressive motility (PR, %); rapid (R, %), and middle rapid motility (MR, %) of spermatozoa.

Statistical analysis

The results expressed as mean ± SEM were analyzed using Statview 4.02 software (Abacus Concepts Inc.,

Berkeley, CA, USA). The data were checked for normal distribution with Shapiro-Wilk test. Differences in motility parameters, between the control and different concentrations of IMD, were determined using a one-way ANOVA followed by Fisher's test. Values were considered significant when $P < 0.05$.

Results

Traditional analysis of sperm motility

Motility percentage and motility index

Under the current experimental conditions, exposure to different IMD concentrations reduced systematically sperm motility percentage (Figure 1) comparatively to the control group (78.5 ± 4.47%). This effect was statistically significant whatever the considered concentration: 1 μM (33.07 ± 7.37%), 10 μM (24 ± 6.67%), and 25 μM (8.46 ± 3.11%) ($P < 0.001$; $P < 0.05$, and $P < 0.001$, respectively). Motility index was significantly higher in the control group (4) with gametes exhibiting circular and progressive movement. Sperm treated with IMD at the concentration of 1 and 10 μM showed motility index equal to 2 and sperm treated with 25 μM an index of 1.

Sperm motility analyzed with CASA system

Percentages of total motility, progressive motility

TM, progressive motility (PR), rapid (R), and middle rapid spermatozoa (MR) are presented in Figure 2. No significant difference was observed between the control group and the tested treatments, with the exception of rapid spermatozoa (R) where the control group showed the highest percentage (3.8 ± 0.05%).

Kinematic parameters (VCL, VAP, VSL, LIN, ALH, BCF)

The impact of IMD treatments on VCL, VSL, VAP, BCF, ALH, and LIN are presented in Figure 3. All parameters are significantly higher in the control group, except for VCL and ALH, where the control group showed lower values than 1 μM IMD treatment. Compared to the control, the results indicated that IMD deteriorates all the other CASA motility variables.

Sperm velocities (VSL and VAP) have expressed the same tendency with a significant difference between all groups. The highest values were observed in the control group (21 ± 0.32 μm/s and 8.19 ± 0.16 μm/s), respectively.

Sperm of the control group showed a progressive movement materialized by high VSL (8.19 ± 0.16 μm/s). The impact of IMD on sperm motility was particularly expressed at the concentration of 25 μM with the lowest VSL (2.28 ± 0.23 μm/s). Among all the three concentrations of IMD, the lowest values of VCL were observed at the concentration of 10 μM (17.69 ± 0.33 μm/s) and 25 μM (17.66 ± 0.6 μm/s). The highest value was found at the concentration of 1 μM (21.98 ± 0.4 μm/s) followed by the control group (21 ± 0.32 μm/s). The average path velocity (VAP) is largely dependent on IMD concentration, the highest value was observed in the control group (13.98 ± 0.2 μm/s) and the lowest at the concentration of 25 μM of IMD (6.13 ± 0.2 μm/s). A significant difference was recorded ($P < 0.05$) between all tested concentrations.

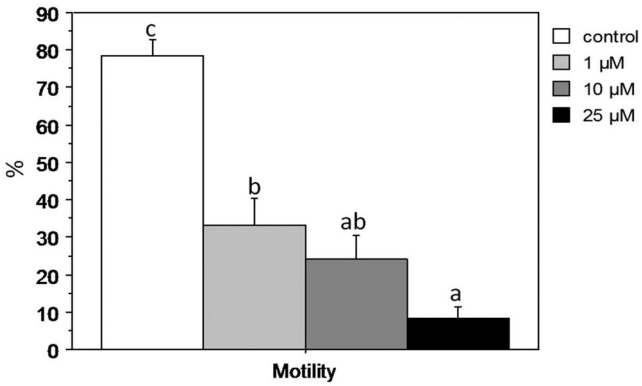

Figure 1. Effect of IMD on drones sperm motility (percentage ± SEM) after *in vitro* exposition to different concentration (1, 10, and 25 μM). Different letters indicate significant differences (ANOVA, $P < 0.05$).

Concerning BCF, the highest values were observed in the control group (2.46 ± 0.05 Hz) while the lowest ones were found at the concentration of 25 μM of IMD (0.73 ± 0.056 Hz). With regard to LIN, results demonstrated remarkable differences between the control and the different IMD treatments.

Discussion

Sperm motility is an important factor determining semen quality and nowadays computer-CASA allows objective evaluation by avoiding the subjectivity related to the operators. These automated systems offer the best gametes visualization in a shorter time with a large set of data detecting subtle variations in sperm quality (Klimowicz et al., 2008; Mortimer, 2000). In the current study, CASA was used to assess the direct effect of IMD on bee sperm, but also to report the normal gametes motility parameters measured in the control group.

In the treated sperm, the traditional microscopic analysis revealed a negative effect on motility percentage in a dose-dependent manner. Sperm from the control showed exclusively circular movements, when only immotile or straight-moving gametes were observed in IMD-treated sperm. When compared to the control, motility falling was 50% at the lowest dose (1 μM) but reached 70% at 25 μM. These results are in agreement with those of Ben Abdelkader & Barbouche, (2015) investigating the effects of four insecticides including IMD.

When using CASA, several quantitative parameters, including VCL, VAP, VSL, STR, LIN, ALH, and BCF, considered as potential indicators of sperm quality and vigor (Duty et al., 2004; Schettgen et al., 2002), expressed IMD damages.

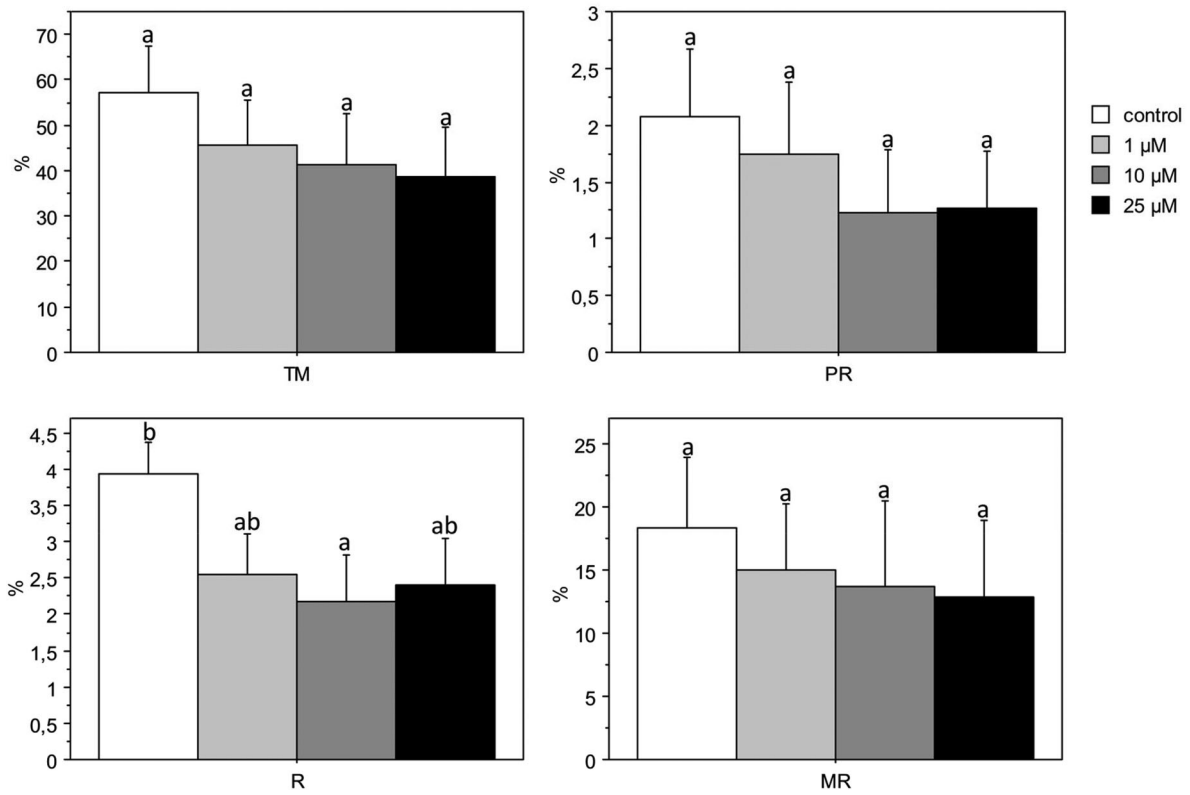

Figure 2. Percentages (Mean ± S.E.M.) of total motility (TM), progressive motility (PR), rapid (R) and mean rapid motility (MR) after *in vitro* exposition of drones spermatozoa to different concentrations of IMD (1, 10, 25 μM). Different letters indicate significant differences (ANOVA, $P < 0.05$).

The results showed that sperm motility was altered in the three tested doses and the values of VSL, VAP, LIN, BCF, STR, and ALH declined in a dose-dependent manner. Significant impairments were observed at the highest concentration (25 μM), where motility was practically inhibited. Ciereszko et al. (2017) reported *in vivo*, the same significant decline in drones exposed to a higher concentration of IMD (200 μM). This suggests strongly that sperm motility may be affected by neonicotinoids in a range of concentrations, and in turns impacts bee fertility.

CASA parameters measured in the control group could serve as reference values in the studied area, drones spermatozoa velocities obtained in the present study appear to be significantly lower than those reported in other animal species (Frazer et al., 1999; Iguer-ouada & Verstegen, 2001; İnanç et al., 2018). Such low activity could be, in part, involved in the long life span of bee spermatozoa, particularly when stored in the spermatheca.

We can assume that the alteration of sperm motility and viability is due to oxidative stress generated by IMD causing mitochondrial and enzymes activities damages. In fact, it had been shown that pesticides cause significant oxidative stress across a wide range of animal taxa including insects (Chakrabarti et al., 2015; Qiao et al., 2005). Especially, in honey bee, different studies have demonstrated that some neocotinoides (IMD and Adetametride) generate intensive oxidative stress (Balieira et al., 2018). Under excessive generation of reactive oxygen species (ROS), spermatozoa interact by an intrinsic apoptotic pathway involving caspase activation, oxidative DNA damage, nuclear fragmentation, mitochondrial ROS generation, and loss of mitochondrial membrane potential (MMP) (Aitken et al., 2016).

Moreover, spermatozoa are known to be well endowed with polyunsaturated fatty acids (PUFAs) (Jones et al., 1979) and are consequently more exposed to lipid peroxidation and motility alteration (Methorst & Huyghe, 2014). Also, limited defensive enzymes make spermatozoa more vulnerable to oxidative stress (Aitken et al., 2016).

In addition, xenobiotics such as pesticides are important factors involved in the alteration of MMP reducing consequently energy production. In this respect, Ruiz-Pesini et al. (1998) showed that mitochondrial damages induce a progressive loss of motility, and more recently Ciereszko et al. (2017) showed, in honey bee, significant interactions between sperm viability and MMP.

We can also hypothesize that when spermatozoa are exposed to pesticides, the production of ATP is altered by lowering enzymatic activities, including catalase (CAT), glutathione *S*-transferase (GST), and superoxide dismutase (SOD). These enzymes are involved in drone spermatozoa survival by holding the balance between reactive oxygen species (ROS) (Gavella et al., 2009;

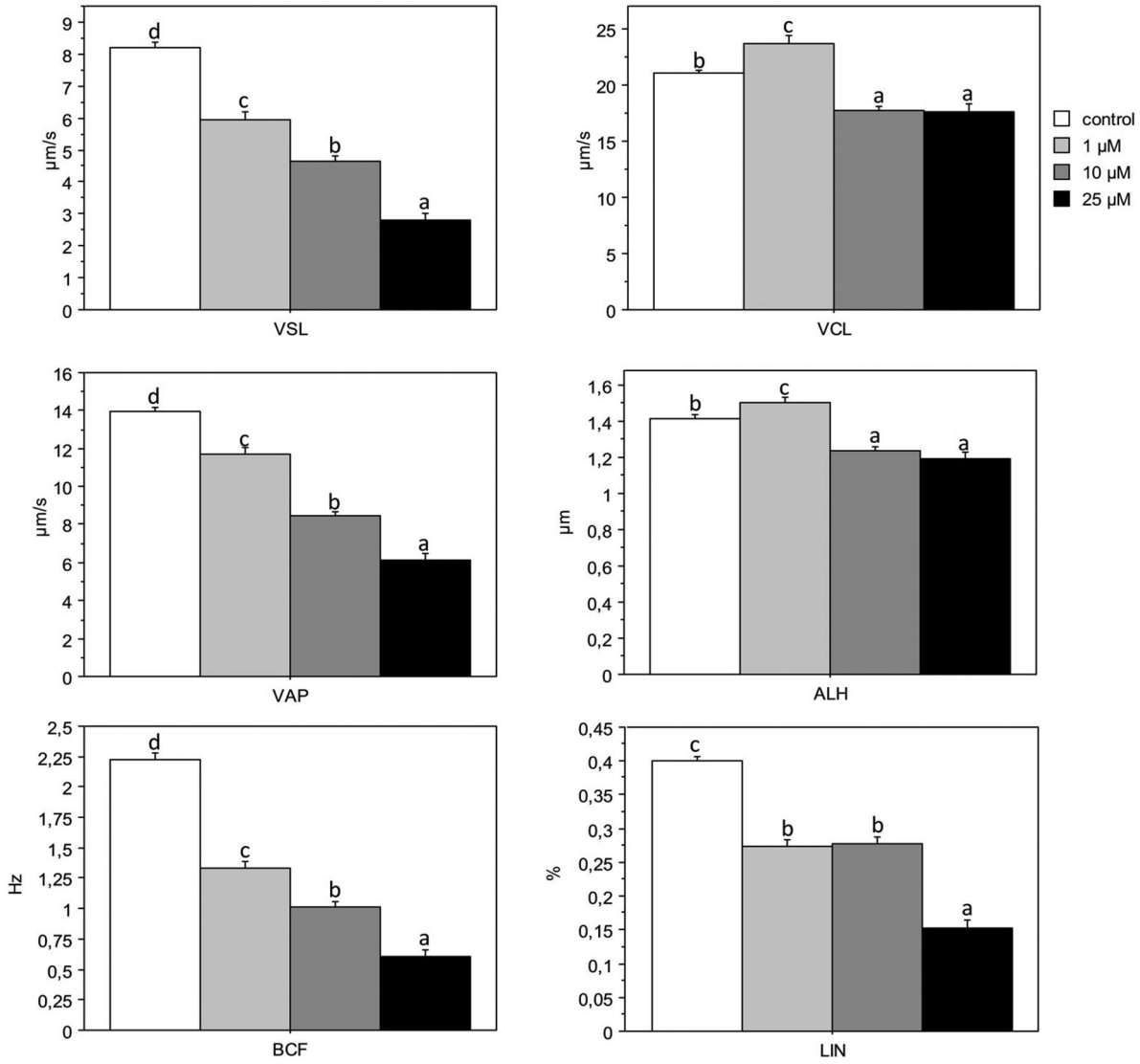

Figure 3. Curvilinear velocity (VCL), straight linear velocity (VSL), average path velocity (VAP), linearity percentage (LIN), amplitude of lateral movement of the head (ALH), and beat cross frequency (BCF), after *in vitro* exposition of drones spermatozoids to different concentrations of IMD (1, 10, 25 μM). Different letters indicate significant differences (ANOVA, $P < 0.05$).

Wegener et al., 2012). In this respect, Ben Abdelkader et al. (2013) have found a lower SOD level in drone semen exposed to pesticides.

The current *in vitro* results highlighted the direct impact of IMD on different sperm parameters, this could be the underlying mechanism involved in reproductive disorders both in drones (Ciereszko et al., 2017) and spermatheca stored gametes (Chaimanee et al., 2016). This hypothesis could be further investigated by inseminations of bee queens with IMD contaminated semen.

In conclusion, the current study showed that IMD impacts directly sperm cells at very low concentrations. The quantitative evaluation, using CASA systems, provided more accurate and objective information on sperm impairments. The normal bee CASA parameters are provided which could serve as standards in the studied area. Future investigations could be dedicated to the existing relationships between CASA parameters and fertility outputs.

Disclosure statement

No potential conflict of interest was reported by the authors.

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