In vitro maintenance of drones and development of a new software for sperm quality analysis facilitate the study of honey bee reproductive quality

Running title: New methods for the study of reproductive capacity in the honey bee drones

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This study aimed to develop a laboratory method that allows the *in vitro* maintenance of honey bee drones for several days while preserving their reproductive capacity and to create a new open-source software for the automatic analysis of their sperm quality. Three experiments were performed. The first experiment was designed to validate the new open-source software named CASABee for sperm quality assessment specifically designed for the honey bee. The software was able to identify motile and static spermatozoa with high precision. Results showed a high correlation between the results of sperm quality obtained both manually and by the CASABee system (0.95 and 0.96 for sperm motility and concentration, respectively, $p < 0.001$). In the second and third experiments, the effect of *in vitro* maintenance of drones without attendant workers for four days on their ejaculatory capacity and sperm quality, respectively, was evaluated. Survival rate was 98.68 %, 89.48 %, 75.93 %, and 60.97 % on average on days 1, 2, 3, and 4 after capturing, respectively. A high proportion of the drones (80.37 % on average) were able to ejaculate providing semen, and there were no significant differences in the ejaculatory capacity and sperm quality of drones on the different days of *in vitro* maintenance, except for sperm viability, which decreased slightly on day 4 (71.71% vs 82.8% on day 0, $p<0.05$). It was concluded that the new CASABee system and the method for laboratory maintenance of honey bee drones facilitate the study of reproduction in this species.

Keywords: *Apis mellifera*, drones, sperm quality, CASA system, survivability, ejaculation

introduction

Honey bee (*Apis* mellifera) drones are genetic reservoirs of the bee colony, which invests a considerable amount of its resources in their care and nurturing during the reproductive season. Despite the relevance of drones for reproduction and their high sensitivity to biotic and abiotic stressors (Boot et al. 1995; Tanner et al. 2012; Fisher and Rangel, 2018; Fisher et al., 2018; Metz et al., 2021; McAfee et al. 2022), there are relatively few studies focused on them, especially when compared to those carried out on workers. Two of the aspects that greatly limit the study of honey bee drones are the difficulties in maintaining them *in vitro* and the lack of specific methods for the automatic analysis of sperm quality in this species.

It is frequently considered that workers are required to provide food to the drones via trophallaxis (Williams et al. 2013), and very few studies have been conducted on the *in vitro* maintenance of drones without the presence of attendant workers (Jaycox 1961; Adam et al. 2010; Abou-Shaara and Elbanoby 2018) and the effect of this on their reproductive quality (Adam et al. 2010). The maintenance of drones without workers in the laboratory facilitates their management, avoiding the risks of stinging and horizontal transmission of pathogens from the workers. However, there is an urgent need to develop more appropriate methods for the *in vitro* maintenance of drones, which show greater sensitivity to laboratory conditions than that of workers (Williams et al. 2013). In order to evaluate the effects of *in vitro* maintenance of honey bee drones on their sperm production and quality, it is first necessary to develop more objective evaluation methods for sperm quality, like the computer-assisted sperm motility analysis (CASA-Mot) systems for mammals (Yaniz et al. 2018; 2020a).

The study of sperm quality in *Apis mellifera* is of great interest for both basic and applied studies, although considerably less research on this topic has been undertaken in this species when compared to other animals (Yaniz et al. 2020a). For example, sperm motility is one of the most widely used sperm quality parameters in mammals (Yaniz et al. 2018), while in the honey bee, it has only been assessed in a few studies (Yaniz et al. 2020a), probably because its determination in this species is still subjectively performed, typically using a 4-6 grade score, according to the percentage of motile cells estimated subjectively. The efficient computerized methods developed for the automatic analysis of sperm motility in mammals (Yaniz et al. 2018) are not useful in the case of honey bee drones, given their sperm morphology, with a sperm head hardly distinguishable from the tail (Al-Lawati et al., 2018; Yaniz et al. 2020a). In a recent study, however, the use of SYBR14 and a conventional CASA system has been proposed as an alternative for the assessment of sperm motility in this species (Murray et al. 2022). This method has the advantage of providing results of sperm kinematic parameters, but it also has several limitations, such as the use of high magnifications, which increases the difficulty of focusing all the cells at once and reduces the number of spermatozoa analyzed per field (Murray et al. 2022). Also, the need to stain the cells with fluorochromes and use expensive equipment, the possible fading of fluorescence while tracking the sperm motility over time, and the possible effect of fluorochromes on sperm motility may also limit the usefulness of this method. Consequently, the development of specific automated methods for computer assisted sperm motility analysis is of great interest to the study of the honey bee and other related insect species.

The aim of this study was to develop a laboratory method that allows the *in vitro* maintenance of honey bee drones for several days while preserving their reproductive

capacity and to create new specific software for the automatic analysis of the sperm quality in this species.

Materials and methods

Animals

The experiments were carried out during the beekeeping season (March-June 2021and 2022) and included drones reared in 30 honey bee (*Apis mellifera iberiensis*) colonies from three apiaries (8-12 colonies/apiary) in northeastern Spain. Colonies were housed in Langstroth (2 apiaries) and Jumbo (1 apiary) hives. In order to increase variability, an attempt was made to minimize genetic relationships between the colonies used in the study.

Mature flying drones were manually collected in the afternoon of days with good weather on their return to the hive after blocking the entrance with a queen excluder. Drones were transported to the laboratory in hoarding polymethyl methacrylate-cages (outside measurement: 15 × 16 × 25 cm) with an absorbent paper at the bottom to absorb faeces and a 96-well standard microplate (well diameter: 5mm; well depth: 11 mm) filled with a syringe with honey diluted to 70% with water (Figure S1).

Collection of semen

Ejaculation was induced using manual procedures (Cobey et al. 2013). For this purpose, the first phase of eversion of the endophallus was induced under chloroform vapors, while the full eversion was completed by manual pressure of the abdomen. An insemination syringe (Peter Schley, Lich, Germany) was used to collect semen in a capillary tube.

Experiment 1. Validation of the new CASABee software

The first trial was designed to validate the new open-source CASABee software of sperm quality assessment specifically designed for the honey bee. For validation of the CASABee, 115 video sequences from different semen samples that vary in motility were used. Motile, static and total spermatozoa in each video were counted both manually (visual estimation by the same observer with the help of the ImageJ open-source software, available at http://rsbweb.nih.gov/ij/download.html) and by the CASABee system. For the manual counting, each video was opened with the ImageJ software and, using the Multi-point Tool, motile spermatozoa were individually marked and counted first, followed by the immotile spermatozoa. For a further guarantee of the precision of these measurements, several videos were counted two times in a blind manner and the results were coincident. All videos were randomly coded and both the CASABee and the manual analysis were conducted in a blinding manner. A representative sample of the videos used is available online (see Sample Videos at https://github.com/jodivaso/CASABee). The design and implementation of the CASABee software is provided in Supplementary Material 1. The code is publicly available at https://github.com/jodivaso/CASABee. This platform will allow researchers not only to download the software but also to be involved in and contribute to further developments. Software instructions have been uploaded to the Github repository. Results of sperm concentration and motility provided by the manual and automatic methods were compared.

Experiment 2. Effect of laboratory maintenance on ejaculation success

The second trial was designed to test the ejaculatory capacity of drones maintained in the laboratory for four days. Drones were captured from each colony as explained above. The cages with drones captured in the apiaries on Monday were maintained in an incubator at 31 ºC in the dark until Friday. The feeders with diluted honey were replaced every day.

In order to determine if this method of *in vitro* maintenance would allow a sufficient number of drones to be available during the different days of the experiment (Monday to Friday), a preliminary assay was carried out to evaluate the effect of laboratory maintenance on drone survival. Fourteen replications (120 drones per replicate) were performed.

In another 12 replicates (150 drones per replicate), the effect of *in vitro* maintenance on the ejaculatory capacity of the drones was evaluated. Ejaculation success was recorded every day between days 0 and 4 after capturing from a sample of 20 drones. Two hundred and forty drones (20 drones x 12 replicates) were evaluated each day of *in vitro* maintenance (day 0 to day 4, Monday to Friday).

Experiment 3. Effect of laboratory maintenance on sperm quality

In the third trial, the sperm quality of drones maintained in the laboratory was evaluated. The cages with drones were maintained in the same conditions as in Experiment 2. Semen was collected individually from a sample of 8 drones every day between days 0 and 4 after capturing for sperm quality assessment. Four replications were performed and the experiment included 160 drones in total.

Sperm motility assessment

After collection, the ejaculates were diluted in Kiev-BSA (Yaniz et al. 2019) to a final concentration ranging between 1 and 15 x 10^6 cells/mL, packaged in 0.5 ml tubes, and stored at 20-22 ºC until sperm quality assessment, which was performed in the first 30 min after collection. Three microliters of diluted semen were placed in a prewarmed Makler® chamber (MK; 10 µm deep; Sefi-Medical Instruments Ltd., Haifa, Israel). The chamber was maintained for 5 min at 35 °C on a heated stage before the analysis. Live video pictures were recorded at 60 frames per second using a set-up comprising an

Olympus BX40 microscope (Olympus Optical Co., Tokyo, Japan) equipped with a heated stage (35 °C), a 10× negative phase objective and a Basler digital camera (model acA1920 -155um; Basler AG, Vision Technologies, Ahrensburg, Germany). All the videos for sperm motility assessment were randomly coded so that the analysis of sperm motility with the CASABee software was conducted in a blinding manner.

Sperm viability assessment

Semen was diluted in Kiev buffer before evaluation. Sperm viability (membrane integrity, SV) was determined using a SYBR14-propidium iodide combination (Yániz et al. 2013). Samples were incubated in the dark at 35 ºC for 20 min and were processed and photographed as detailed in Yániz et al. (2013). At least 200 cells were examined per sample using the OpenCASA v2 software (Yaniz et al. 2020b). All the images for sperm viability assessment were randomly coded so that sperm viability with the OpenCASA was conducted in a blinding manner.

Statistical analysis

Statistical analyses were performed using the SPSS package, version 23.0 (IBM SPSS Statistics, Chicago, IL, USA). In the first experiment, the results of sperm concentration and motility from the visual and automated methods were compared using the Spearman's correlation test. The Bland–Altman test was carried out to study the agreement between the two different measurements (Bland and Altman 1986). A bias lower than 10% in the Bland-Altmann test was considered acceptable. In experiment 2, the Chi-square test was used to compare the ejaculatory capacity of drones on the different days of *in vitro* maintenance. In experiment 3, prior to the statistical analyses, an arcsine of the square root transformation of the dependent variables (sperm motility and sperm viability) was performed, and the normality of the distribution was then verified with the Kolmogorov–

Smirnov tests. Generalised linear model analysis was used in the analysis of the effect of time of drone maintenance on the dependent variables. The results of the main effects are shown as mean ± standard deviation (SD). The statistical significance level (alpha) was set at 0.05.

Results

Experiment 1

A total of 115 videos containing about 4,934 spermatozoa were processed, of which most of the motile spermatozoa (98.8%) showed a circular shape while most static spermatozoa (99.4%) showed a linear shape. The CASABee software was able to identify motile and static spermatozoa (Figure 1). The default values of the parameters worked well in most cases (110 of the 115 videos analyzed). In this study, the images were optimized using negative phase contrast microscopy, in which sperm appear white against a black background (Figure 1).

Sperm motility and concentration values were obtained manually (visual estimation by an observer) and by CASABee. Results compared using Pearson's correlation test showed a high correlation (Table 1). A good agreement between both measurement systems was revealed on the basis of the Bland–Altman test for motility variables, and a less good but still acceptable agreement was achieved for sperm concentration (Table 1).

Experiment 2

Drone survival rate was 98.68 %, 89.48 %, 75.93 % and 60.97 % on average on day 1, 2, 3 and 4 after capturing, respectively, so that the number of captured drones necessary to evaluate the ejaculatory capacity was adjusted to 150 drones per replicate.

There were no significant differences in the ejaculatory capacity of drones between the different days of *in vitro* maintenance (Table 2), and a high proportion of the drones (80.37 % on average) were able to ejaculate by providing semen (Table 3). Figure S2 represents the ejaculation success rates obtained in the 12 replicates (colonies) during the different days of *in vitro* maintenance.

Experiment 3

There were no significant differences in sperm quality between the different days of *in vitro* maintenance, except for sperm viability (Table 2), which was lower on day 4 than on days 0 and 3 (Table 3). Figure S3 represents average sperm quality obtained in the four replicates (colonies) during the different days of *in vitro* maintenance.

Discussion

The quality of the semen produced by the drones determines the reproductive success of the queen, the level of productivity of the colony and even its survival (Pettis et al. 2016). It has been even described that the drone semen can even modulate several aspects of queen biology, such as ovary activation, pheromone production, and subsequent worker retinue behavior (Brutscher et al., 2019). Semen quality is also a key aspect that determines the success of instrumental insemination (Collins 2000; Collins 2004). Given its relevance in sperm transport and fertilization, sperm motility is one of the most widely used sperm quality parameters in mammals (Yaniz et al. 2018). In the honey bee, sperm motility allows migration to the queen's spermatheca and subsequent egg fertilization, and its study has shown a better prediction ability of *in vivo* performance after artificial insemination of queens than that of other parameters of semen quality (Wegener et al. 2012). Despite this, sperm motility in the honey bee has only been assessed in a few

studies (Yaniz et al. 2020a), probably because its determination in this species is still subjectively performed.

In a previous study (Yaniz et al. 2019), we made a great effort to standardize the conditions for the analysis of sperm motility in honey bee drones. The viewing chamber where the semen is placed, the diluent and the time of the analysis had a great impact on the results obtained. We observed that the addition of bovine serum albumin (BSA) to the semen using a Makler chamber reduced sperm adherence to the glass surface, allowing a better estimation of sperm motility. Under these conditions, most motile spermatozoa acquired a circular shape after 5 min of incubation at 35ºC, while the static spermatozoa retained a linear shape. Based on these findings, we have developed the new open-source CASABee software program, specifically designed for the automatic analysis of sperm motility and concentration in honey bee drones.

CASABee was able to automatically measure sperm motility and concentration of a semen sample with high precision. The optimal sperm concentration for sperm motility assessment using CASABee ranged between 5 and 15 x 10^6 sperm/ml. At higher concentrations, there may be problems in the detection of static sperm, which may hide within the circles of the motile sperm, and in the detection of motile sperm, which might merge forming circles containing various cells difficult to differentiate. In contrast, at lower concentrations, CASABee usually performs well, but the low number of spermatozoa analyzed per video reduces the interest of automatic analysis. It is also important for the analysis to have quality images, with sufficient contrast between the cells and the background and avoiding artifacts. To the best of our knowledge, this is the first software able to analyze sperm motility and concentration in the honey bee using phase-contrast images. There was an attempt to use a commercial CASA system to

evaluate sperm motility in the honey bee (Inouri-Iskounen et al. 2020), but the authors did not provide convincing evidence or explanations to be able to conclude that this CASA system, based on the detection of sperm heads, works properly with this species. As explained above, sperm heads are indistinguishable from their tails in honey bee drones (Yaniz et al. 2020a).

The evaluation of sperm motility and concentration in honey bee drones may be of interest in both routine sperm analyses and experimental studies. The CASABee has the following advantages when compared to the manual assessment of sperm concentration and motility. First, it is fast and accurate, allowing analysis in a shorter time. The time required for the analysis of a video sequence of 60-frames is about 10-20 s (range 8-40 s), but several videos may be processed in a single step, after which the operator can check, process and save the results of each processed video immediately. Second, the software is compatible with different cameras and video formats, so that usually no additional equipment is required. Third, the same software may be used by different labs, allowing the standardization of the technique. Finally, CASABee is flexible, because it allows access to algorithms, so that adaptations to specific necessities may be undertaken by different research groups. The results were strongly correlated with visual counting of motile and total spermatozoa when using a Makler chamber. Nevertheless, this software could also be suitable for other different counting chambers, since it allows users to set the depth of the chamber and the resolution of the image. Thus, the module automatically calculates, from the number of counted sperm, the sperm motility percentage and the concentration in millions of cells per milliliter. If the initial concentration of the sperm sample is high and requires dilution to avoid overlapping, the dilution factor can be included in the text box for the sperm concentration of the undiluted sample.

In the first versions of the software, the detection of motile spermatozoa was more robust than that of static ones, since when the latter overlapped, the software considered the group as a single event. To avoid this problem, CASABee automatically divides the total length of each detected static sperm by the mean sperm length adjusted in the settings. More sophisticated algorithms may be designed to separate and count individual static sperm, but the time required for the analysis would be increased and this simplified approach provides satisfactory results.

In the second part of this study, a method for laboratory maintenance of honey bee drones preserving their reproductive function was described. It has been described that drones are more sensitive to abiotic stressors than workers (McAfee et al. 2022), so that *in vitro* conditions may have a great effect on them. Despite its relevance, only a few studies have evaluated the possibilities of laboratory maintenance of honey bee drones. It is generally assumed that drones should be maintained *in vitro* accompanied with nurse workers collected off brood frames (Williams et al. 2013). The presence of attendant workers can prolong the survival of drones in laboratory cages (Abou-Shaara and Elbanoby 2018), but increases the risk of stings and of horizontal disease transmission (Williams et al. 2013). Our goal was not to maximize drone survival but to develop a method to ensure the availability of reproductively active drones in the laboratory for several days avoiding the use of worker bees. This was considered important because the management of live bees in the laboratory is complicated in some instances, particularly when dealing with bees with marked defensive behavior, like the *Apis mellifera iberiensis* used in this study.

Initial works reported low drone longevity, averaging about 3 to 5 days, in cages without worker bees when fed with sucrose syrup or sugar candy (McIndoo 1914; Phillips

1922; Oertel et al. 1953). The latter suggested that drones may not be able to invert sucrose as do worker bees, and this could explain, in part at least, the short survival obtained. In fact, Jaycox (1961) prolonged *in vitro* survival of immature drones using specific feeding devices with honey and kept them between 31 and 34°C, but few data on drone survival were provided. In agreement with this, Abou-Shaara and Elbanoby (2018) observed that mature drones fed with honey candy survived longer than those fed with sugar candy. However, drone survival without attendant workers was relatively low using honey candy (Abou-Shaara and Elbanoby 2018) or diluted honey (Adam et al. 2010) as food supplies: mature drones only survived up to 4 days, with high mortalities on day 2 and successive days. Clearly improved results were obtained in the present study, with high drone survival on day 4 using diluted honey. The design and management of the feeder is very important, as drones are unable to groom their bodies and, if they become sticky, they will be immobilized and quickly die (Jaycox 1961). In the present study, 96-well standard microplates placed at the bottom of the cage were used as feeders. Special care was taken to avoid overfilling the wells with diluted honey, and the presence of drones caked with food was not observed. This was not the case in the study of Adam et al. (2010), where the presence of drones caked with food and moisture was described, and this may explain the lower survival observed in this study which also used diluted honey. Drone survival could probably be improved using other food supplies, and more research is needed on this subject. For example, Adam et al. (2010) demonstrated that the addition of 1.25 % lyophilized royal jelly to the diluted honey increased drone survival, but further increases of this additive were contraindicated. It seems difficult to compare *in vitro* and *in vivo* longevity of drones, since drone lifespans in the colony seem to be highly variable, with means between 12 and 54 days (Currie 1987).

To the best of the authors' knowledge, there is only one published paper evaluating the effect of drone laboratory maintenance on their reproductive function (Adam et al. 2010). The authors explained that ejaculation success was clearly reduced during subsequent days of drone *in vitro* maintenance. In the present study, however, no clear reduction in the ejaculatory capacity was observed during the four days of drone laboratory maintenance. Discrepancies may be associated to the different protocols used for *in vitro* maintenance and/or ejaculation. Adam et al (2010) described a decrease in the drone vigour during the successive days of maintenance in the laboratory, possibly explaining the reduction in ejaculatory success. In the present study, however, this reduction in drone vigour was not observed during the experiment.

In addition to maintaining the ejaculatory capacity and high survival rates, no differences were observed in the sperm quality of the drones during the four days in the laboratory, except for sperm viability, which slightly decreased on day 4. All these results greatly facilitate the study of reproduction in this species and open up the possibility of collaboration with other laboratories that do not have easy access to apiaries to work with fresh semen. To the best of the authors' knowledge, this is the first work in which the effect of maintaining drones *in vitro* on sperm quality has been studied.

In conclusion, the new CASABee system and the laboratory method for *in vitro* maintenance of honey bee drones without workers facilitates the study of reproduction in this and closely related species.

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Disclosure statement

The authors declare no conflict of interest.

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Data availability statement

The authors confirm that the data supporting the findings of this study are available upon reasonable request from the corresponding author.

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Figure caption

Figure 1. Examples of CASABee analysis. Phase contrast images from two video sequences of different sperm motility (a, c), and the resulting CASABee output (b, d), showing the classification of spermatozoa in motile (circles) and static (red lines).

Figure S1. Polymethyl methacrylate-cage used for *in vitro* maintenance of drones (a), cage with the device used to capture mature drones in the apiary (b), and cage containing drones (c).

Figure S2. Ejaculation success rates of mature drones maintained *in vitro* up to four days. Each color represents a replicate (colony).

Figure S3. Sperm quality of drones during laboratory maintenance showing the results of sperm motility (a) and sperm membrane integrity (b). Each color represents the average of a replicate (colony).