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ORIGINAL RESEARCH ARTICLE

A new fluorescent method to determine honey bee sperm motility parameters with computer-aided sperm analysis

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ABSTRACT

Honey bees are a keystone species, playing an important role in the food cycle. Improving honey bee reproduction will aid in the replacement of lost colonies. Fertility potential and reproductive health are dependent on semen and sperm quality. Current data on drone semen parameters are limited to semen volume, sperm concentration, sperm viability, and the assignment of sperm motility grade scores. The assessment of drone sperm motility is of importance to determine fertility potential and colony health. This study aimed to establish a quantitative and a qualitative method to measure drone sperm quality, and the fertility potential of *Apis mellifera capensis* subspecies of South Africa. Firstly, an improved five-point semi-quantitative manual motility index score was used to classify drone sperm motility. Secondly, it was possible to accurately analyse drone sperm motility qualitatively using a fluorescent technique in conjunction with a computer-aided sperm analysis (CASA) system. Manual motility index scores corresponded with total motility percentages as determined by using a CASA system. Furthermore, low values for motility kinematic parameters, particularly velocity parameters, were obtained in samples with both low motility index scores and low total motility percentages. Additionally, total sperm motility percentage and velocity parameters positively correlated with sperm total progressivity. This study provides in-depth data on honey bee drone sperm motility and motility kinematic parameters, which can serve as a reference for future studies on honey bee sperm and possibly related species.

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Honey bee drone; reproduction; sperm quality; sperm motility; motility kinematics; fluorescence; CASA

Introduction

Honey bees are the most consistent cross-pollinators of crops, hence their importance for both pollination and production of commercial products (Potts et al., 2010). Declining honey bee populations may have adverse ecological effects, threatening the entire agricultural field, including beekeepers (Biesmeijer et al., 2006). Colony health, survival, reproductive quality of the queen, and successful breeding, are dependent on drone semen quality (Ciereszko et al., 2017; Yániz et al., 2020). Particularly sperm quality plays a vital role in colony reproductive health, given that poor sperm quality impairs both queen and drone reproduction (Brutscher et al., 2019; Yániz et al., 2020). Therefore, establishing a method to routinely determine sperm quality will assist to improve reproduction.

Following mating, sperm is temporarily stored in the queens' lateral oviducts where only a fraction (about 2.5%) of sperm received during mating is transferred into the spermatheca for long-term storage (Baer, 2005) and thus requires sperm of optimal quality to survive and participate in fertilization. Drone semen quality assessment mainly includes semen volume, sperm concentration, and viability (Yániz et al., 2020). Comprehensive sperm quality analysis, however, requires the assessment of multiple sperm characteristics, relating to potential fertilization success (Ciereszko et al., 2017; Yániz et al., 2020). For example, sperm motility and morphology, are important indicators of sperm quality and fertilization success (Abu et al., 2012; Quartuccio et al., 2020; Yániz et al., 2020), and poor sperm motility, low sperm concentration, and abnormal sperm morphology may contribute to low fertilization rates (Larson-Cook et al., 2003). Sperm motility is required for migration to the spermatheca and for fertilization success (Yániz et al., 2020), and the predominantly helical (circular) swimming pattern of honey bee drone sperm, as demonstrated by Tofilski et al. (2018) inside the spermatheca of the queen, is considered an indicator of good sperm quality (Yániz et al., 2020). However, in commercial settings, honey bee sperm motility assessment is not the norm.

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Sperm motility, generally assessed by phase-contrast microscopy, typically includes the assignment of a grade score based on the percentage of motile sperm in a semen sample, and sperm motility patterns (Inouri-Iskounen et al., 2020; Locke & Peng, 1993; Yániz et al., 2020). In addition to the total sperm motility percentage, sperm motility kinematic parameters are equally important, as it also relates to sperm migration through the female genital tract (Robayo et al., 2008). Recently, Inouri-Iskounen et al. (2020) used conventional phase-contrast microscopy and a CASA system to perform honey bee drone sperm motility analysis and reported very low values for velocity parameters [straight line velocity (VSL) and curvilinear velocity (VCL)]. However, CASA systems determine accurate X and Y sperm coordinates using the sperm head as a reference point (Lu et al., 2014), and as drone sperm heads are indistinguishable from their tails, sperm motility analysis with CASA is not feasible using phase-contrast microscopy, unless performed in combination with fluorescent staining and microscopy (Yániz et al., 2020). CASA analyses, routinely used in mammals, are yet to be developed in honey bees, and data regarding the quantification of sperm motility and kinematic parameters in honey bee drones, is very limited (Ciereszko et al., 2017; Yániz et al., 2020). Kinematic parameters commonly used for analysing sperm motility include VCL, VSL, average path velocity (VAP), the amplitude of lateral head displacement (ALH), linearity (LIN), straightness (STR), wobble (WOB), and dance (DNC) (Supplementary Table (ST) 1) (Dunson et al., 1999). These parameters measure specific quantitative aspects of sperm motility and can be used to identify sperm motility classes (i.e. progressive, rapid, medium, and slow swimming sperm). An individual sperm track is shown in Supplementary Figure (SF) 1, and further illustrates how the respective kinematic parameters are calculated.

The entire sperm population can further be divided into subpopulations, namely rapid-, medium- and non-progressive, determined by the sperm swimming speed (VCL) and progressivity (STR) of individual sperm (Maree & Van der Horst, 2013).

Understanding honey bee drone reproduction, sperm biology, and specifically sperm functionality, is important owing to its strong relationship with fertilization success. Accordingly, developing a technique to routinely provide objective and quantitative information on honey bee sperm functionality, specifically motility, will enhance honey bee breeding programs and artificial insemination (AI) techniques applied to propagate honey bee populations (Cobey et al., 2013). The study, therefore, aimed to determine drone sperm quality, focusing on evaluating sperm motility and kinematics using a fluorescent method and CASA, and will contribute to the quantitative and qualitative establishment of honey bee drone sperm quality assessment and honey bee reproduction in general.

Materials and methods

Husbandry and honey bee drone collection

Honey bee colonies were maintained according to standard apicultural practices. Free-roaming, sexually mature drones (*A. mellifera capensis*) (Couvillon et al., 2010), were randomly collected from 10 colonies, at the Agricultural Research Council Plant Protection Institute (ARC-PPI) (Stellenbosch, South Africa). The colonies were located close to each other and as they were not marked, some level of drifting was possible, however, previous research conducted on *A. mellifera capensis* drones, has indicated that surprisingly low levels of drone drift took place for this species (unpublished). The colonies were genetically unrelated and were all trapped swarms from the wild population found in and around Stellenbosch, Western Cape, South Africa. During drone harvesting, we mostly collected drones from a single colony on any given day, but from different colonies over time. Thus, for the drones we collected from the same colony, the drones will be 50% related to each other (each having half of their mothers' genes), while drones collected from different colonies are unrelated to each other. While we did not specifically analyse differences between colonies we believe that we measured greater potential variability. However, superficially there were no specific differences noted between colonies.

Drone collection occurred during the morning of warm spring days, before their daily flights. Defecating drones were minimal and excluded from laboratory analysis. Ethical approval was obtained from the Animal Research Ethics Committee of the University of the Western Cape, South Africa (Ethics Reference Number: AR 17/5/3).

Drone semen collection and preparation

Manual ejaculation (Collins, 2005) of 200 drones was performed on-site immediately after drones were collected from colonies. Semen was collected using a positive displacement pipette (1 µl) with sterile tips, followed by immediate dilution in 6 µl Kiev buffer solution (0.3 g glucose, 0.41 g potassium chloride, 0.21 g sodium bicarbonate, and 2.43 g sodium citrate-2 hydrate, in 100 ml of distilled water, final pH = 8.3) (Collins, 2005). Samples were incubated at 37 °C (based on the typical body temperature of the honey bee) (Esch, 1976) until

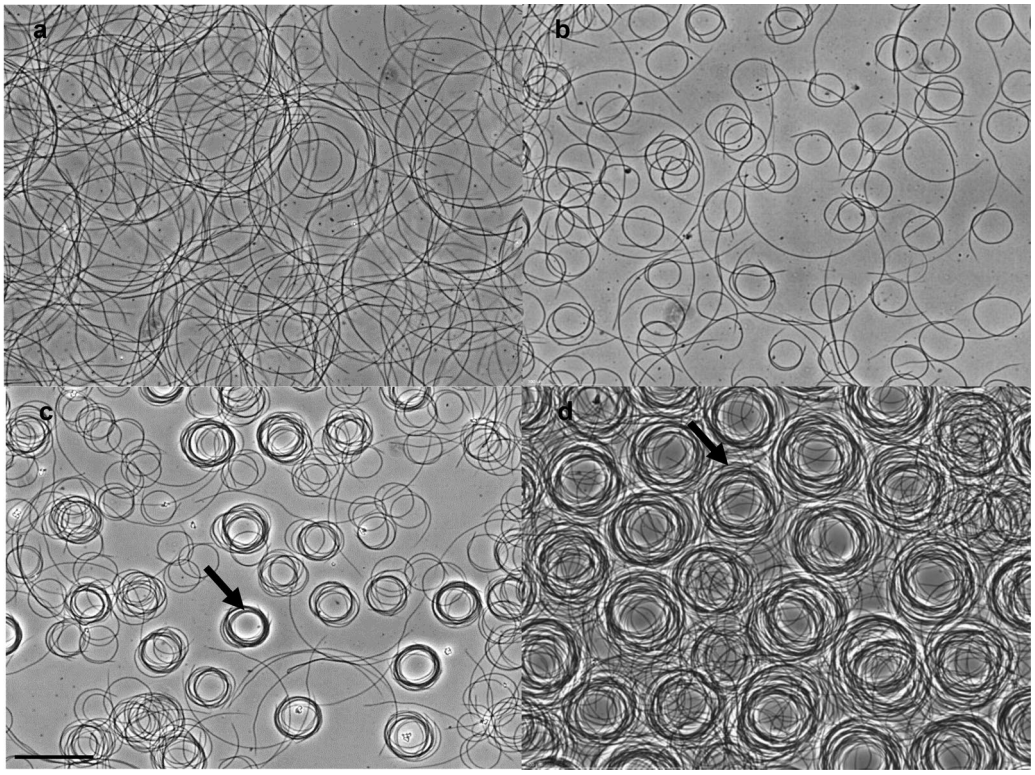

Figure 1. Semi-quantitative manual motility index classifications using phase-contrast microscopy. a = motility index 2, b = motility index 3, c = motility index 4, d = motility index 5. Arrows in c and d indicate relay swimming sperm, a collective term used to refer to many sperm swimming together in a helix (SF 2). Scale a-d = 100 μm.

analysed. To simulate an *in vivo* spermathecal environment of densely packed sperm, the initial dilution of samples was kept to a minimum, taking into account further two-fold dilutions during the staining procedures that follow (12X dilution in total) (Tofilski et al., 2018). Analyses were performed within one hour of semen collection (Yániz et al., 2019). Semen volumes collected from drones were relatively small, approximately 0.5 μl. Semen volume was determined by means of weighing an Eppendorf tube, before and after a semen sample was added, using a Nimbus® Analytical balance scale (Adam Equipment S.A. (Pty) Ltd, Kempton Park, Johannesburg, Republic of South Africa), a method routinely used to measure human semen volume (World Health Organization, 2010). Following semen volume assessment, samples were evaluated microscopically and all mucus-contaminated samples were excluded from any assessments, thus volume measurements relate only to samples not containing mucus. Sperm concentration was assessed using a manual method in combination with a CASA system as explained in section 2.3.

Analysis of sperm parameters

Sperm functionality (motility) was firstly determined semi-quantitatively using motility grade scores and secondly, qualitatively, by using a CASA system.

Manual assessment (semi-quantitative)

Extracted semen samples were assessed by loading 2 μl of diluted semen into a 4-chamber, 10 μm deep Leja slide (Leja Products B.V., Nieuw-Vennep, The Netherlands); allowing one to two minutes for sperm adjustment (Inouri-Iskounen et al., 2020). Sperm motility was then observed using phase-contrast microscopy with a 20X objective. All motile sperm were categorized manually using a motility index score, illustrated in Figure 1.

While there is the well-established motility classification system of Locke and Peng (1993), there was a need for modification to take into consideration helical swimming groups of sperm, as described in previous studies (Borsuk et al., 2011; Tofilski et al., 2018). The adjusted classification system provides a more quantitative assessment of sperm motility that is taking sperm movement or swimming patterns into account. Motility index scores were assigned as follows: 1- no motility, 2 - vibrating sperm but no progressive motility, 3 – individual circular- and progressively forward-moving sperm, 4 - less than seven groups of helical swimming sperm (relay swimming sperm) (SF 2), and 5 - more than seven relay swimming sperm groups in the microscopic field of view. Fifty-one samples were used for sperm functional analyses. Videos demonstrating the motility indices are presented in Supplementary Videos (SV) 1-4.

Computer-aided sperm analysis (automated and qualitative) assessment

Automated sperm motility analysis is based on sperm head centroid recognition (Lu et al., 2014). In most insects, and accordingly, honey bee sperm, the head (0.4 – 0.5 μm) has approximately the same width as the rest of the sperm (0.7 μm) (Peng et al., 1993) and with bright field/phase contrast microscopy the head cannot be clearly defined or recognized for CASA tracking (Yániz et al., 2020). In this study fluorescence microscopy, has therefore been employed and a fluorochrome (SYBR14) for nuclear material has been used. The possible impact of the SYBR14 fluorochrome on sperm functionality was validated with a preliminary, unpublished, study (Murray, 2019, unpublished Honours thesis), which entailed the manual motility assessment of unstained sperm samples at 20X objective in a 10 μm deep Leja chamber, using image analysis and the hand-tracking of sperm heads for every frame (50 frames) per second. In the case of both methods (SYBR14-stained and unstained sperm), sperm swimming patterns remained the same over the entire analysis period of at least half an hour (See the comparison in SF 2).

A SYBR14 (L-7011, LTC Tech, Fairland, South Africa) stock solution was prepared using a 50-fold dilution in DMSO (Sigma-Aldrich, Johannesburg, South Africa) from which a fresh working solution was prepared before sperm analysis (5-fold dilution in Kiev buffer solution). A 1:1 ratio of diluted semen and SYBR14 working solution were used for staining and incubated for 10 minutes at 37 °C. A 4 μl suspension drop (translating to a chamber depth of 8.3 μm, as determined by the CASA, Sperm Class Analyser (SCA®) system) was placed on the glass cover slide and left undisturbed for one to two minutes at 37 °C until analysis, to allow sperm cells to adjust. Chamber depth of the glass cover slide was determined by selecting the coverslip size and exact drop volume in the SCA® Motility module in order for the system to automatically equate a specific chamber depth. The chamber depth of the glass cover slide is in close proximity to the 10 μm deep Leja slide.

Sperm concentration and motility were assessed using the Motility module of SCA® (version 6.5.0.44, Microptic S.L., Barcelona, Spain). The CASA SCA® system was equipped with a Nikon Eclipse 50i Fluorescence Microscope (IMP, Cape Town, South Africa) and a Basler aCA 1200-1300uc USB 3.0 digital camera (Basler AG, Ahrensburg, Germany), specially designed by the manufacturers for fluorescence microscopy (SV 5 and 6). Owing to the complexity of honey bee swimming patterns, CASA captured most (90 to 98%), but not all sperm, and for accurate determination of sperm concentration, we used a semi-quantitative procedure forming part of the SCA® system. Sperm concentration was determined semi-automatically by superimposing a digital Makler chamber on the SCA® analysis field of view, and subsequently counting the number of sperm in ten of these Makler chamber fields. The sperm count was then multiplied by the dilution factor and expressed as million sperm/ml, according to Makler chamber instructions but also in sperm/μl.

The configuration settings of the SCA® system for sperm motility were as follows: Frame rate = 50 images/sec; Optics = Fluorescence microscopy; Chamber = Glass cover slide; Species = Invertebrate. A minimum of 100 sperm per sample was analysed using a FITC filter and 40X fluorescence objective with a 0.7 intermediate lens (B-2A Nikon: ex450-490; DM 505; BA 520) translating to a 28X objective magnification. Based on the total percentage motility obtained from CASA, SCA® three motility categories were established for analysed samples: > 79% motility, > 60-79% motility, and > 20-59% motility. The analysis included sperm motility and kinematic parameters (SV 6 illustrates analysed sperm tracks using CASA).

Statistical analysis

Statistical analysis was performed using MedCalc software (version 19.5.3, Mariakerke, Belgium). For normally distributed motility and kinematic parameters, ANOVA tests were performed (Levene's test, $p > 0.05$). Results that encompass an F-ratio signify all normally distributed data. Significant differences ($p < 0.05$) in the ANOVA table were further analysed using Scheffé's post-hoc test for pairwise comparisons. A Kruskal-Wallis test was performed for data that were not normally distributed (Levene's test, $p < 0.05$). Correlation tests performed included a Pearson correlation test (normally distributed data) and Spearman Rank correlation test (not normally distributed data).

Kinematic parameter cut-off values for VCL were determined using receiver operating characteristic (ROC) curve graphs, to distinguish between different subpopulations. Parameters that displayed both high sensitivity and specificity were used to determine cut-offs for the various subpopulations. The identification of sperm subpopulations using ROC curves, aided in the identification of good quality sperm, based on motility.

Results

Baseline sperm parameters

A. mellifera capensis drone sperm concentrations ranged between 1.10×10^6 – 16.5×10^6 million per μl. Baseline sperm motility results (Table 1), indicated that both the manual motility index scores, total

Table 1. Baseline drone sperm motility index (manual motility quantification) and the total sperm population motility percentage (determined by CASA), (mean ± SD).

	Mean n = 51	> 79 % Motility n = 38	> 60-79% Motility n = 7	> 20-59% Motility n = 6	P-value	F-ratio
MI	4.11 ± 1.11	4.32 ± 1.04^{a}	4.14 ± 1.22^{b}	2.83 ± 0.41^{ab}	0.01	–
TM (%)	85.5 ± 17.2	91.9 ± 5.34^{ab}	72.9 ± 6.71^{ac}	43.0 ± 6.33^{bc}	<0.001	2.09
TP (%)	27.5 ± 15.5	32.6 ± 13.9^{ab}	17.5 ± 8.44^{a}	6.37 ± 2.75^{b}	<0.001	–
RP (%)	5.18 ± 5.02	6.23 ± 5.31^{a}	3.05 ± 2.40	0.98 ± 0.97^{a}	0.003	–
MP (%)	22.3 ± 11.6	26.4 ± 9.80^{ab}	14.4 ± 7.02^{a}	5.39 ± 2.37^{b}	<0.001	–
NP (%)	56.1 ± 13.9	59.2 ± 13.3^{a}	55.4 ± 6.99^{b}	36.7 ± 6.70^{ab}	<0.001	8.97
Rapid (%)	10.3 ± 8.66	12.4 ± 8.92^{a}	6.14 ± 3.94	2.03 ± 1.88^{a}	0.001	–
Medium (%)	24.6 ± 11.7	29.2 ± 8.91^{ab}	16.2 ± 7.27^{a}	5.50 ± 3.14^{b}	<0.001	25.3
Slow (%)	48.6 ± 13.9	50.3 ± 14.6^{a}	50.5 ± 7.62^{b}	35.5 ± 6.27^{ab}	0.04	–

Parametric data are presented as mean and standard deviation (SD). ^{a,b,c} Means with the same alphabetical letters in the same row differed significantly. The dash symbol (-) indicates no data obtained from non-parametric Kruskal Wallis tests where data were not normally distributed (Levene's test $P < 0.05$).MI, Motility index; TM, Total motility; TP, Total progressivity; RP, Rapid progressive; MP, Medium progressive; NP, Non-progressive.

Table 2. Baseline drone sperm kinematic parameter measurements (mean ± SD) across three different motility percentage categories determined by CASA.

Parameters	Mean n = 51	> 79 % Motility n = 38	> 60-79% Motility n = 7	> 20-59% Motility n = 6	P-value	F-ratio
VCL (μm/s)	50.3 ± 11.4	52.9 ± 11.5^{ab}	43.1 ± 6.78^{a}	42.3 ± 8.60^{b}	0.02	4.4
VSL (μm/s)	30.4 ± 7.2	32.6 ± 6.84^{ab}	24.5 ± 3.57^{a}	23.4 ± 3.24^{b}	<0.001	9.21
VAP (μm/s)	39.5 ± 9.07	42.3 ± 8.48^{ab}	31.9 ± 4.49^{a}	30.1 ± 4.38^{b}	<0.001	10.4
LIN (%)	59.2 ± 6.73	61.2 ± 5.66^{ab}	52.7 ± 7.44^{a}	54.2 ± 5.63^{b}	0.001	8.49
STR (%)	72.7 ± 4.56	73.8 ± 4.39^{a}	68.6 ± 4.19^{a}	71.1 ± 2.99	0.01	4.94
WOB (%)	77.3 ± 6.82	79.7 ± 4.62^{ab}	70.1 ± 8.58^{a}	70.5 ± 6.89^{b}	<0.001	14
ALH (μm/s)	1.78 ± 0.40	1.82 ± 0.42	1.71 ± 0.36	1.63 ± 0.28	0.51	0.69
BCF (Hz)	15.8 ± 1.71	16.3 ± 1.39^{a}	13.7 ± 1.69^{a}	14.8 ± 1.43	<0.001	11.6
DNC (μm^2/s)	185 ± 79.7	198 ± 84.0	140 ± 52.8	141 ± 50.7	0.12	2.22

Parametric data are reported as mean and standard deviation (SD). ^{a,b} Means with the same letters in the same row differed significantly as obtained from ANOVA. VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; DNC, dance.

motility percentages (CASA), and sperm progressivity significantly decreased alongside decreasing motility categories.

Motility index scores correlated with total motility percentages ($r = 0.51$) and with sperm progressiveness ($r = 0.47$). Furthermore, a positive correlation was also observed between total motility percentages and sperm total progressiveness ($r = 0.62$) (ST 2); both these parameters decreased simultaneously from the highest to the lowest motility percentage categories as shown in Table 1. Results obtained for the majority of kinematic parameters were significantly higher in the > 79% than > 20-59% motility category, excluding ALH ($p = 0.51$) and DNC ($p = 0.12$). No significant differences were observed between the > 60-79% and > 20-59% motility categories. Majority of the kinematic parameters also decreased as the motility percentage decreased, including velocity parameters (VCL, VSL and VAP) (Table 2). Our findings furthermore highlighted a statistically significant positive correlation between velocity parameters and sperm total progressiveness (VCL, $r = 0.86$; VSL, $r = 0.91$; VAP, $r = 0.92$) (ST 2).

Cut-off values for drone sperm kinematic parameters for different motility categories

ROC curves, based on kinematic parameters, VCL, VSL, VAP, ALH, resulting in sensitivity and specificity values of less than 60%, were excluded (SF 3). For this study, VCL as a cut-off value was sufficient to determine motility quality. Cut-off values obtained from ROC curves were applied, and the settings for VCL in the Motility module of SCA® were adjusted as follows: slow < 50 > medium < 70 > rapid. For all kinematic parameters, significant differences were observed between subpopulations (ST 3). Velocity parameters (VCL, VSL and VAP), were significantly higher in rapid progressive subpopulations (VCL = 99.1 μm/s (± 23.0), VSL = 69.8 μm/s (± 13.7) and VAP = 78.9 μm/s (± 15.0)) ($p < 0.001$) compared with other subpopulations. The same tendency was observed for the majority of other parameters. For BCF, the medium progressive subpopulation had a significantly higher value of 17.1 ± 2.07 Hz compared to rapid (15.7 Hz ± 3.74) and non-progressive subpopulations (15.3 Hz ± 2.01).

A visual representation of honey bee drone sperm tracks obtained from the Motility module of CASA, SCA®, is illustrated in Figure 2.

Sperm tracks constructed by CASA and SCA® for randomly selected rapid, medium, and non-progressive swimming sperm were reconstructed into 3 D tracks and presented in SF 5 (Van der Horst & Sanchez, 2016). Each sperm track can be verified as playback one frame at a time by utilizing the software of the SCA® and CASA system and accordingly confirming that the constructed path represents the actual sperm swimming path.

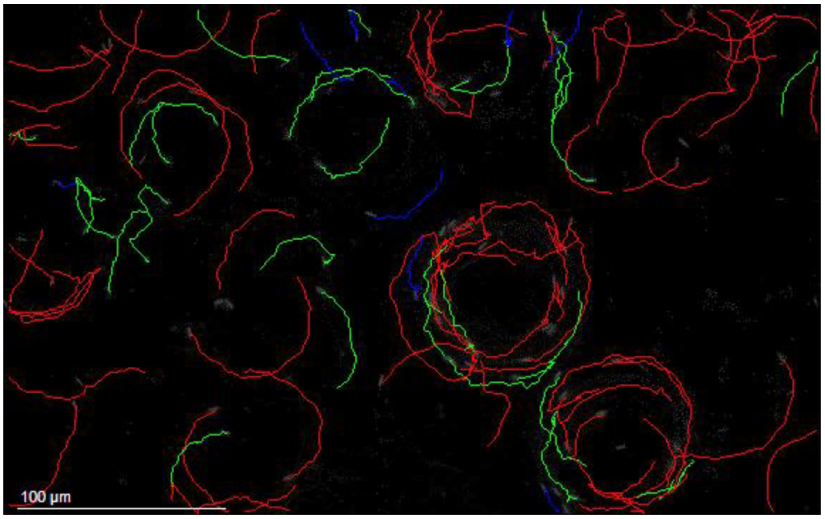

Figure 2. Honey bee drone sperm motility tracks of all subpopulations detected using CASA, SCA® in fully automated mode. Red tracks - rapid swimming sperm; Green tracks – medium swimming sperm; Blue tracks – slow swimming sperm (In SF 4, different colours for swimming tracks have been chosen to provide better contrast between tracks for those readers who cannot distinguish between the red and green tracks).

Kinematic parameter values for the entire sperm population were grouped into rapid, medium, and slow swimming sperm. These categories refer to sperm swimming speed and are based on VCL (µm/s). Results across the three swimming speeds differed significantly (ST 4).

Velocity measurements for medium swimming sperm were more than 50% lower compared to the rapid swimming sperm, VCL= 185 µm/s (± 34.5), VSL= 118 µm/s (± 21.4), and VAP = 139 µm/s (± 21.9) versus VCL= 62.5 µm/s (± 5.52), VSL = 44.6 µm/s (± 7.05), VAP = 53.6 µm/s (± 5.76) ($p < 0.001$). The same pattern was seen for slow swimming sperm compared to medium swimming sperm. Medium swimming sperm showed significantly higher values for LIN, STR, WOB, and BCF compared to rapid and slow swimming sperm. Furthermore, ALH and DNC values were significantly higher for the rapid swimming sperm compared to the medium and slow swimming sperm.

Discussion

This study established a quantitative and qualitative method to determine honey bee drone sperm quality, mainly focusing on sperm motility and the data obtained is important as a baseline for future research on honey bee sperm motility and sperm quality assessment.

The sperm concentration of *A. mellifera capensis* drones was similar (1.10×10^6 – 16.5×10^6 million per µl) to previously reported results in this subspecies (7.9 million per sample that averaged about 1.1 µl) (Buys, 1990) and European subspecies (6-12 million sperm per ejaculate) (Duay et al., 2002; Rhodes et al., 2011) despite a smaller semen volume (0.5-0.7 µl) produced by *A. mellifera capensis* drones than what is reported for European subspecies (0.4 – 2.4 µl) (Rousseau et al., 2015). Furthermore, it has been shown that sperm concentration may vary greatly among drones, even within the same breeder line and colony (Koeniger et al., 2005). We also suggest that the large variation in sperm concentration among drones within a colony is rather a function of differential sperm qualities.

In agreement with previous studies, three different sperm swimming patterns were observed namely, single helices, progressively forward snake-like swimmers, and groups of helical swimming sperm, also described as circular moving whirls of sperm (relay swimmers) (Borsuk et al., 2011; Tofilski et al., 2018). Findings from our manual tracking in a preliminary study of drone sperm motility, have shown that groups of relay swimming sperm demonstrate significantly higher swimming speed values than single sperm swimming progressively forward or in a single helix (Murray, 2019, unpublished Honours thesis). These findings were confirmed with fluorescence and CASA in this study.

Manually determined motility index scores decreased significantly with decreasing motility percentages. The mean motility index of 4.11 (± 1.11), i.e. less than seven groups of relay swimming sperm in a sample, indicated that the majority of sperm were highly motile (85.5% motility), which is in agreement with total sperm motility (± 80%) reported for African honey bee drone subspecies, *A. mellifera intermissa*, assessed using phase-contrast microscopy and CASA (Inouri-Iskounen et al., 2020). Furthermore, semen samples with a high motility index (4.32 ± 1.04) corresponded with a high percentage motility (91.9% ± 5.34) (CASA); therefore, the

presence of relay swimming sperm is often associated, but not necessarily, with a high percentage total motility.

Previous attempts to determine honey bee drone sperm motility and kinematics using manual or CASA techniques obtained sperm motility parameter results that appear to be considerably different from our findings. For example, velocity kinematic parameters determined manually (VCL range = 19 − 32 μm/s) (Al-Lawati et al., 2009) and using CASA (VCL = 21 μm/s, VSL = 8.19 μm/s, VAP = 13.98 μm/s) (Inouri-Iskounen et al., 2020), were very low compared to results obtained in this study (VCL= 50.3 μm/s, VSL = 30.4 μm/s, VAP = 39.5 μm/s). The latter values however corresponded with results from our previous experiments, using a manual method (VCL = 56.81 μm/s and VSL = 42.73 μm/s) (Murray, 2019, unpublished Honours thesis). It should be noted that accurate tracking of sperm when using CASA requires a fluorescence method, as demonstrated here. Furthermore, SYBR14, used for staining sperm heads for CASA motility analysis in this study, has been previously used on insect sperm such as *Drosophila melanogaster* (Radhakrishnan & Fedorka, 2011), and honey bees (Yániz et al., 2020) but for the purpose to determine sperm vitality (distinguish between live and dead sperm).

The CASA analysis of honey bee sperm motility parameters reported in the literature to date is not a reflection of using a consistent point of reference such as the sperm head, and also accordingly not bringing it in line with the well-tested centroid head-tracking approach used for 99% of CASA studies. The only other real valid approach is semi-automatic "hand tracking", as we have done in an earlier study (Murray, 2019, unpublished Honours thesis) or by flagellar analysis, which is currently not possible for insect sperm (Van der Horst, 2020).

Additionally, our results have shown a positive correlation between total motility percentage and total progressivity, indicating that honey bee sperm groups, swimming in a helical fashion, also swims progressively forward. The percentage of progressive forward movement of sperm is an essential indicator of sperm quality and fertility since successful insemination and reproduction depend on sperm movement (Tofilski, 2014). Therefore, to determine fertility potential for routine sperm analysis as well as AI success, it is important to consider sperm motility parameters such as progressivity (by determining subpopulations) and swimming speed when analysing honey bee semen quality.

Furthermore, assessing honey bee sperm motility in a sample with minimum dilution is challenging, yet it is necessary to replicate *in vivo* conditions, especially in the case of helical swimming groups of sperm with very long flagellae. The dilution used in this study was similar to what was used by Tofilski et al. (2018) for AI and presents the same circular swimming patterns as demonstrated by them. It is well-known that in other animals, great care must be taken to prevent over dilution (Wilcox & Clark, 1962), in agreement with the findings of Halak et al. (2020) who illustrated that over dilution changes the swimming pattern of honey bee sperm.

Honey bee drone sperm motility and movement remain sensitive to sample dilution, diluent composition, deposition time, chamber type and depth, and these factors should be considered carefully when determining honey bee sperm motility (Taylor et al., 2009; Yániz et al., 2019, 2020). Yániz et al. (2019) raised a valid point relating to chamber depth, however, it has been shown extensively that sperm typically swim 1 μm above their surrounding surface (Nosrati et al., 2015), and prefer smaller areas given the hydrodynamic interaction between migrating sperm and its surrounding surfaces (Suarez & Wu, 2017). In addition, it is of benefit to allow for sperm to swim in a chamber depth that equates to or is similar to the diameter, for example, of the spermathecal duct lumen (approximately 8-12 μm), in the female reproductive tract (de Camargo & Mello, 1970).

Given the structural nature of honey bee sperm, important considerations related to the above-mentioned aspects are the limitations in terms of using sufficiently high magnification for adequate sperm head fluorescent detection and using a specific chamber type and depth, particularly when assessing motility using a CASA system. The narrow honey bee sperm head posed a limit to the amount of fluorescence that could be detected, and the only way to produce a strong enough fluorescence signal for CASA detection in this study was to use a 40X objective in conjunction with an intermediate tube lens of 0.7X, translating to an objective magnification of 28X. Unfortunately, sufficient detection of sperm heads using CASA was not always possible when using a 10 μm Leja slide in conjunction with these magnifications; hence a glass slide and a 0.17 mm thick coverslip were used, which allowed for successful head detection by CASA.

Finally, we realize that some studies make use of sperm stored in the spermatheca as opposed to sperm ejaculated by drones, and that the question may arise whether the presence of oxygen in ejaculated sperm or the absence thereof in the spermatheca may influence sperm motility. However, we believe that sperm motility will be retained under both conditions, becausethe low-oxygen environment in the spermatheca supports glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity without

increased production of reactive-oxygen species (ROS) (Al-Lawati et al., 2009; Paynter et al., 2017), that has been associated with reduced motility in mammals (Elkina et al., 2011). Furthermore, there is also an abundance of glycolytic enzymes present in the spermathecal fluid after mating to the benefit of sperm adenosine triphosphate (ATP) production (Paynter et al., 2017).

Conclusions

It is not possible to accurately perform normal head centroid CASA analysis on insect sperm unless it is in fluorescent mode. This study provides for the first time a method to accurately quantify the percentage of sperm motility and sperm kinematics such as velocity, of honey bee sperm, using a CASA system in combination with fluorescence microscopy.

The technology used in this study provides a fast and effective method for beekeepers to determine sperm quality and colony health to ultimately improve honey bee reproduction. The ability to determine drone sperm quality qualitatively will further help to improve AI success by selecting sperm samples of optimal quality and also holds the potential to select optimal genetic lines to improve colony performance.

Disclosure statement

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Data availability statement

All data generated or analysed during this study are included in this manuscript and its supplementary information files.

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