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RESEARCH ARTICLE

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The effect of mixed honeybee drone semen on sperm quality characteristics: A preliminary study

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Following copulation, sperm of multiple honeybee (*Apis mellifera* (A.m.) Linnaeus (Apidae)) drones are mixed inside the spermatheca of a queen, and sperm behaviour in this environment is of importance for sperm survival and successful fertilization. The polyandrous mating system of honeybees further may allow for cryptic female choice and sperm competition to select for specific sperm traits to enable successful reproduction. This preliminary study therefore examined multiple sperm quality parameters in mixed drone semen. Thirty-one drone ejaculates were collected and split to be mixed with one and two other drones' semen. A computerised sperm analysis system was used for analysing sperm functionality parameters, at baseline and 60 minutes. Morphology measurements included head, tail, and total sperm length. Sperm concentration was significantly higher in three-drone samples compared to other groups ($p < 0.001$), while comparisons of sperm functional and morphometric parameters did not reveal differences. However, after 60 minutes, mixed semen samples displayed changes in terms of sperm functionality. The motility percentage significantly decreased in two-drone samples ($p = 0.004$). In contrast, three-drone samples had consistently lower motility and kinematic parameters at baseline, but following an hour it was the only group to show improvement in sperm progressive motility, swimming speed, and kinematics. Sperm concentration correlated with the majority of functionality parameters, while, morphological measurements did not. Sperm functionality changes observed in this study, can be attributed to a combination of factors, including, mixing of drone semen, sperm concentration, and time allowing for potential sperm interaction.

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INTRODUCTION

In polyandrous insect species, a female copulates with multiple males and thus receives sperm from several males. Particularly in the order Hymenoptera, polyandrous mating increases genetic diversity and subsequently colony health and fitness. The honey bee (*Apis mellifera (A.m.)* Linnaeus (Apidae)) is an example of hyperpolyandry, where in some subspecies a queen can mate with as many as 77 drones (Withrow and Tarpy 2018; Delaplane et al. 2021). The beneficial effect of honey bee polyandry on colony health and fitness have been shown in the improved productivity or task performance by workers (Delaplane et al. 2021), such as foraging, food collection, brood rearing efficiency, and increased brood survival (Mattila and Seeley, 2007; Delaplane et al. 2021). Polyandrous colonies further seem to be larger in size and have increased brood production (Mattila and Seeley 2007).

Another suggestion for the polyandrous mating system in the honeybee has been to prevent sperm depletion (Delaplane et al. 2021), thus, to avoid the queens' spermathecae to run out of sperm. Accordingly, copulation with multiple mates could ensure a sufficient supply of sperm possessing specific traits to enhance fertilisation success in the long-term. For example, the South African subspecies, *A.m. capensis* Esholtz, is a species with smaller drones, that produce lower sperm numbers, and has queens possessing a larger spermatheca, and therefore potentially requires higher mating frequencies compared to European subspecies, *A.m. carnica* Pollman, to provide a sufficient sperm supply for a lifetime (Kraus et al. 2004). It may well be explained that the large number of sperm received following multiple mating can serve as a buffering mechanism to further ensure that there is a sufficient number of sperm with optimal quality and potentially fertilising ability (sperm competition within the spermatheca).

Contrary to queens, honeybee drones only mate once and after copulation the fate and survival of sperm depends on the queen (Den Boer et al. 2010). It is well-known that 4 to 6 million sperm (3–5% of all drones' sperm and 5–10% per individual drone's sperm) reaches and is stored in the spermatheca, while the rest is expelled (Baer 2005; Gençer and Kahya 2020; Liu et al. 2020) through the bursa copulatrix (Gençer et al. 2014). Finally, only 1 to 1.6 million sperm are used to fertilise eggs in the years to come (Baer 2005). Considering the amount of sperm stored and used, and that sperm has to be viable for years, indicates that sperm quality also matters (Baer 2003; Stürup et al. 2013). Therefore, researchers have further suggested and investigated the presence of sperm competition (Moritz 1986; Woyciechowski and Król 1996; Shafir et al. 2009). Sperm competition *per se* may be driven by pre- and post-mating factors, including male-male competition and cryptic female choice (Fitzpatrick and Lüpold 2014).

In the case of post-mating, both intra-oviductal and intra-spermathecal sperm competition have been suggested in the honeybee (Gençer and Kahya 2020). Sperm competition may further occur when sperm leaves the spermatheca, entering and leaving the spermathecal duct to reach, penetrate, and finally fertilise the egg (Harbo 1990). Moreover, sperm competition in the case of polyandrous mating, often result in the improvement of sperm possessing specific traits advantageous for fertilisation (Pearcy et al. 2014). Sperm quality traits to consider in terms of fertilisation capability include progressive motility, swimming speed, viability, sperm morphology and/or specific sperm component dimensions (e.g. length of the sperm head and tail, and total sperm length) (Fitzpatric and Lüpold 2014; Pearcy et al. 2014). In-depth understanding of insect sperm kinematics is however limited. Sperm kinematic or motion parameters usually include sperm velocity parameters i.e. curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP); velocity ratio parameters i.e. linearity (LIN), straightness (STR) as well as wobble (WOB); sperm wobble characteristic parameters i.e. amplitude of lateral head displacement (ALH) and beat chain frequency (BCF) (Lu et al. 2014; WHO 2021). Sperm kinematics is widely used to assess mammalian sperm performance with the aid of advanced technology such as computer-aided sperm analyses (CASA) systems, which track the sperm head to determine its movement (Hook and Fisher 2020; WHO 2021). Applying such technologies to assess insect sperm, however, is rather difficult considering its much smaller head and long flagella; explaining the lack of such detailed analyses.

Following copulation, honeybee sperm are mixed (Borsuk et al. 2018) and form dense sperm bundles in the spermatheca (Wegener et al. 2014), and its behaviour changes after several hours, to form groups of circular swimming sperm (Tofilski et al. 2018). Such behavioural changes can be indicative of sperm-sperm interactions or sperm cooperation, factors that may also play a very important role in fertilisation success in insects (Lüpold et al. 2012; Pearcy et al. 2014). For example, sperm cooperation in some insects may result in changes in motility and swimming speed to enhance migration through the reproductive tract (Pizzari and Foster 2008; Lüpold et al. 2012; Pearcy et al. 2014; Liberti et al. 2018). However, increased motility and velocity might eventually impair viability, which is important for long-term sperm storage. Sperm movement is associated with increased metabolism and requires sufficient energy production. Mitochondria produce adenosine triphosphate (ATP), but also reactive oxygen species (ROS), that are important for regulation of sperm movement. However, overproduction of ROS, that may occur as a result of increased motility, can eventually lead to oxidative stress (Reinhardt 2007) if exceeding the cells antioxidant capacity (Abdelkader et al. 2018). Oxidative stress in turn may cause sperm cell damage, and ultimately lead to deterioration of sperm structure and function, and finally cell death (Reinhardt 2007; Abdelkader et al. 2018). For example, in two studies, *in vivo* exposure of adult honeybee drones (Abdelkader et al. 2018), as well as the *in vitro* exposure of honeybee sperm to insecticides (Abdelkader et al. 2015), resulted in sperm with increased metabolic activity as demonstrated by increased production of ATP. However, in both studies, the upregulated metabolic activity increased sperm mortality and thus decreased sperm viability (Abdelkader et al. 2015, 2018).

In the majority of studies, the *in vitro* mixing of honeybee drone semen seems to have no effect on sperm viability (Shafir et al. 2009; Tofilski et al. 2012; Gençer et al. 2014). Sperm viability also does not seem to differ among the lateral oviducts and spermatheca and within the spermatheca after four hours, the time period during which sperm migration from the oviducts to the spermatheca is at its highest in cases of artificially inseminated queens (Gençer and Kahya 2011). Knowledge about changes in sperm behaviour and or quality as a result of mixing with multiple drones' semen during and after copulation in honeybees remains limited to research investigating changes in swimming patterns and viability. Furthermore, it is also not clear which sperm traits (structural and functional) are most advantageous to participate in fertilisation of eggs.

In monandrous (i.e. the male is polyandrous and not the queen) Hymenopteran species such as the majority of bumble bee species, and solitary bees (Baer 2003), sperm plugs are formed by males after mating which limit chances of additional mating by females. Subsequently only one male's sperm will be reaching the spermatheca without exposure to other males' sperm. With this mating behaviour, sperm competition within the female reproductive tract is less likely to occur in these species (Baer et al. 2006; Strobl et al. 2019).

In this preliminary study, we aimed to provide more clarity on *A.m. capensis* drone sperm quality traits and how mixing of multiple drone's semen may influence overall sperm behaviour and or quality. We analysed a wide range of sperm quality parameters of individual drones and mixed semen *in vitro*. The first aim determined whether mixing of different drones' semen alter sperm functionality characteristics (motility parameters and kinematic parameters), and the second aim determined whether structural characteristics (sperm components) influence observed sperm functionality or behaviour.

MATERIALS AND METHODS

Husbandry and honeybee drone collection

Sexually mature *A.m. capensis* drones were randomly collected from three unrelated colonies in an apiary site located in the Stellenbosch area, Western Cape, South Africa during October 2022. Drones that were collected from the same colony on a given day would have been 50% related to each other because they have the same queen mother. The reason for collecting drones in this manner was to limit the contribution of genetic variation/diversity on results observed. Colonies were maintained according to standard apicultural practices (Swart 2001). Drones were collected during late morning hours before their daily flights, and defecating drones were excluded from the study to avoid contamination of semen samples. 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

Semen was immediately obtained from drones following manual ejaculation (Cobey et al. 2013) following the methods of Murray et al. (2022). Each semen sample was diluted in a microcentrifuge tube containing 12 µl Kiev buffer solution (Özkök and Selcuk 2020) (pH 8.3). Samples were kept at 37 °C using a dry bath (Bench mark Scientific, Whitehead Scientific (Pty) Ltd, South Africa) and analysed within one hour. Semen volume was determined from individual drones using a weighing method used to measure human semen volume (WHO 2021); weighing a microcentrifuge tube, containing 12 µl Kiev buffer solution, before and after adding a collected semen sample, using a micro-balance scale (Nimbus analytical balance, Adam Equipment South Africa (Pty) Ltd, Johannesburg).

Each diluted semen sample was split into three parts, of which one part was the control (individual drone semen), while the other two parts were mixed with one or two other related drones' semen to create groups consisting of two-drone and three-drone mixed samples, respectively. All three groups consisted of the same volume of semen, i.e. 6 µl each. Samples were thoroughly mixed by pipetting it up and down five times (Eckel et al. 2017). To exclude genetic influences as a confounder for sperm behaviour,

the individual drones from each colony were mixed together. Sperm concentration was determined for each group using results obtained from motility analyses (using samples stained with SYBR14) and will be discussed further in the following section.

Analysis of semen parameters

Motility and kinematics

A total of 31 individuals, 19 and 12, two- and three-drone semen samples respectively, were analysed for motility and kinematic parameters. In order to determine sperm motility manually, using a motility index scoring system (Murray et al. 2022), diluted semen samples of individual and mixed drones were analysed by loading 2 µl into a Leja slide (10 µm chamber depth) (Leja Products B.V., Nieuw-Vennep, The Netherlands) and viewed after two minutes (Inouri-iskounen et al. 2020) using a 20× objective and phase-contrast microscopy on a heated stage (37 °C) (Nikon Eclipse 50i IMP, South Africa). In brief, the manual motility index scoring, based on motility and sperm swimming patterns:1– no motility, 2– vibrating sperm but no progressive motility, 3– individual, circular and progressively forward-moving sperm, 4– < 7 groups of helical swimming sperm, and 5– > 7 groups of helical swimming sperm in the microscopic field of view (Murray et al. 2022). Based on previous work by Yaniz et al. (2019) showing the highest percentage of motile sperm and circular swimming sperm at 60 minutes after loading, we chose to assess samples at baseline (T0) and 60 minutes (T60).

Sperm motility was also assessed using a computer-aided sperm analysis (CASA), Sperm Class Analyzer (SCA)® (version 6.5.0.44) and fluorescence system according to the method of Murray et al. (2022). A 5 µl drop was placed onto a glass cover slide and the chamber depth equated to 10 µm as calculated by SCA®. The configuration settings in the SCA® Motility module was as follows for detecting different swimming speeds: Static (µm/s) < 10; Medium (µm/s) > 10 < 65; Rapid (µm/s) > 100. A minimum of 100 sperm was analysed per sample and motility parameters were assessed. The following parameters were determined: percentage of immotile sperm (IM) (no tail beating detected); percentage of motile sperm (total motility (TM)) (tail beating is detected) (Mortimer et al. 2015); percentage of sperm with progressive motility (PR) (STR > 75% (SCA® configuration)); percentage of sperm with non-progressive motility (sperm with a beating tail but STR < 75% (SCA® configuration)); classes of progressively swimming sperm (rapid-(RP), and medium progressive (MP) sperm); percentage of rapid, medium and slow swimming sperm (Mortimer et al. 2015)

Kinematic parameters assessed using CASA included: VCL (µm/s), VSL (µm/s), VAP (µm/s), LIN (%), STR (%), WOB (%), BCF (Hz) and ALH (µm). For ease of reference a short description will be given for the different kinematic parameters: VCL – time-averaged velocity of the sperm head along its curvilinear path; VSL – time-averaged velocity of a sperm head along the straight line between its first and last points in the path; VAP – time-averaged velocity of the sperm head along its smooth curved (average) path; LIN – linearity of the curvilinear path (VSL/VCL × 100); STR – linearity of the average path (VSL/VAP × 100); WOB – oscillation of the curvilinear (actual) path about the average path (VAP/VCL × 100); BCF – average rate at which the curvilinear path crosses the average path; ALH – magnitude of lateral displacement of the sperm head about its average path (Lu et al. 2014; WHO 2021).

Sperm concentration of SYBR14-stained samples, as previously mentioned, was analysed using a 10 µm depth glass cover slide and a digital Makler® chamber (available as a tool in CASA, SCA®) was superimposed on captured fields. Sperm concentration was calculated as shown in the formula below. Different dilution factors used as for the groups were as follows: 48-fold dilution for individual, 96-fold dilution for two- and 144-fold dilution for three-mixed samples:

Sperm concentration (million/µl) =
(Sperm counted in 5 chambers × 2) × (dilution factor)]/1000

Morphology

In order to determine sperm morphometric dimensions, samples of each group were further diluted, using a 10-fold dilution in Kiev solution and stained with BrightVit (a nigrosine eosin (NE)-based stain) (Delfran, Johannesburg, South Africa) in a 1:1 ratio (two-fold dilution) in an Eppendorf tube and incubated at 37 °C for 15 minutes. After incubation, 5 µl of the stained sample was used to make a duplicate smear on microscope slides and left to dry (Microptic, 2021). Sperm were viewed using bright field optics (Nikon Eclipse 50i microscope with a 20× objective) and captured with a Basler a CA 1300–200uc camera mounted on the microscope. Sperm morphometry was analysed in manual mode using the SCA® Morphology module. The sperm head and tail as well as total sperm length were measured. A minimum of 100 sperm was measured per group.

Statistical analysis

Statistical analyses were performed using MedCalc® Statistical Software version 20.111 (MedCalc Software Ltd, Ostend, Belgium). In order to compare sperm motility, kinematic and morphology parameters of individual drones with mixed drone samples, one-way analysis of variance (ANOVA) was performed for parametric data, while a Kruskal Wallis test was performed for non-parametric data. For ANOVAs the Levene's test for equality was considered, if this test was significant ($p < 0.05$) a non-parametric test was performed. To determine the difference in results over time for each group, the repeated measures ANOVA test was performed for parametric data, and the Friedman's test for non-parametric data. Parametric data was presented as mean and standard deviation (SD), and non-parametric data as median (25th and 75th percentiles). To test for correlations between motility and or morphology, as well as sperm concentration and motility and kinematic parameters, a Pearson's correlation test was performed for parametric data and a Spearman Rank correlation test for non-parametric data. Only samples with complete data for both time intervals, T0 and T60, were included in the statistical analyses. To determine variance in sperm component lengths among males in each group, the coefficient of variance (CV) was calculated:

CV = (SD/mean) × 100

Multivariate visualisation methods

For multivariate statistical visualization analyses (Andrews plots) Statgraphics 19® Centurion Version XIX (Statgraphics® 2022, Statgraphics Technologies, Inc., obtained from Dittrich and Partner, Solingen, Germany) was used. A multivariate visualisation technique, i.e. Andrews plots were applied to enable us to detect subtle differences in variables among the groups at T0 (baseline) and T60. To construct Andrew's plots variables as well as a grouping code to identify the samples, as in this case, must be selected. A further major advantage of Andrews plots is that it allows comparison of many variables in the same analysis. In this method, variables are combined for each group and Andrews plots then draw a line for each row with complete data. Results obtained from Andrews plots are further presented as peaks and valleys to show subtle differences among groups with regards to variables selected (Statgraphics® StatPoint, Statgraphics Technologies, Inc. 2013).

RESULTS

Baseline semen characteristics

The mean semen volume obtained from individual samples was 0.60 ± 0.30 µl per ejaculate and the mean sperm concentration was 3.63 ± 2.23 million/µl. The sperm concentrations obtained confirmed the dilution of the different groups' samples. Sperm concentration significantly differed among groups and was significantly higher in three- (8.81 ± 3.37 million/µl) compared to two-drone (5.26 ± 1.59) and individual samples (3.63 ± 2.23) ($p < 0.001$) (Figure 1).

Effect of mixing drone semen on sperm motility parameters

All groups had a motility index score of 5 at baseline (T0) and T60, which indicates that most samples had more than seven groups of helical (circular) swimming sperm per microscopic field of view as per manual motility classification used (Supplementary Table 1). For motility parameters, as determined using CASA and SCA®, also no significant differences were observed between individual and mixed drone samples (Supplementary Table 2). The relatively high total motility percentages of all groups agreed with high motility index scores at both time intervals. At baseline, although not significantly so, individual (88.1%) and two-drone samples (86.9%) displayed a higher total motility percentage compared to the three-drone samples (79.1%) ($p = 0.592$) (Figure 2). However, after 60 minutes, the percentage of total motile sperm in two-drone samples significantly decreased to 71.5% ($p = 0.004$). Furthermore, as a consequence, the total percentage of immotile sperm significantly increased in the two-drone samples after 60 minutes (T0 = 13.1%; T60 = 28.5%) ($p = 0.004$) and remained the same in three-drone samples over time (T0 = 20.9%; T60 = 21.2%).

At both time intervals, the majority of sperm in all groups were non-progressive (NP) and exhibited slow swimming sperm. The percentage of progressive swimming sperm, including rapid (RP) and medium (MP) progressive swimming sperm, as well as sperm swimming with rapid and medium speeds, made up only a very small percentage of each group's sperm (Supplementary Table 2).

In two- and three-drone samples, progressive swimming sperm increased alongside decreased NP sperm percentages over time, however, the two-drone samples resulted in a significantly decreased percentage of NP sperm (T0 = 81.9%; T60 = 66.4%) ($p < 0.001$) after 60 minutes. Slow speed swimming sperm also significantly decreased in the two-drone samples ($p < 0.001$) after 60 minutes (T0 = 78.7%; T60 = 64.7%). In three-drone samples the decrease in slow swimming sperm was accompanied by proportional increases in progressive motility, rapid or medium speed swimming sperm, but not in the case of two-drone samples (Supplementary Table 2). Compared to other groups, three-drone samples therefore appeared to improve after an hour, displaying enhanced progressive motility and swimming speeds.

Effect of mixing drone semen on sperm kinematic parameters

As for the motility parameters, no significant differences were found between individual and mixed drones' samples for any of the kinematic parameters at the time intervals, and neither between time intervals within groups (Supplementary Table 3). Despite no significant differences it is evident that three-drone samples' sperm adjust its swimming behaviour over time as seen in the increased velocity (VCL, VSL, VAP) and path of swimming (LIN, STR) compared to individual and two-drone samples.

Formal statistics did not show significant differences among groups for motility and kinematic parameters, hence Andrews plots were applied to detect subtle differences in variables among the groups at T0 and T60 (Figure 3). Variables included for this multivariate method were motility parameters: % PR, % MP, % Rapid and % Medium swimming sperm; velocity parameters: VCL and VSL. Peaks and valleys shown in the Andrews plots clearly show separation among the various mixed semen groups and these differences were emphasised when several sperm traits were combined.

In addition, significantly positive, but weak correlations were observed between sperm concentration and majority of the motility and kinematic parameters, except for percentage RP and slow swimming sperm (T0 and T60), and percentage rapid swimming sperm at T60 (Supplementary Table 4). We assumed that sperm concentration remained constant over time, and applied Spearman rank correlation tests for T60 data too, which provided similar correlation results as at T0.

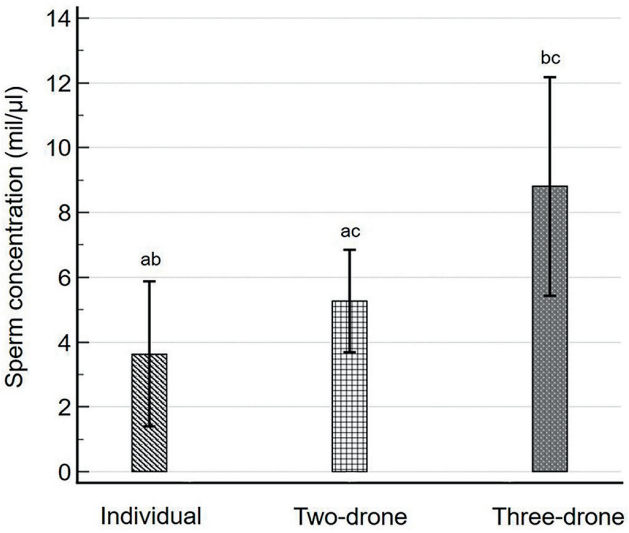

Figure 1. Comparison of sperm concentration in individual and mixed semen groups at baseline (mean ± SD). Individual samples (***n*** = 31), Two-drone samples (***n*** = 17) and Three-drone samples (***n*** = 12). Means with the same superscripts [abc] indicate significant differences between groups.

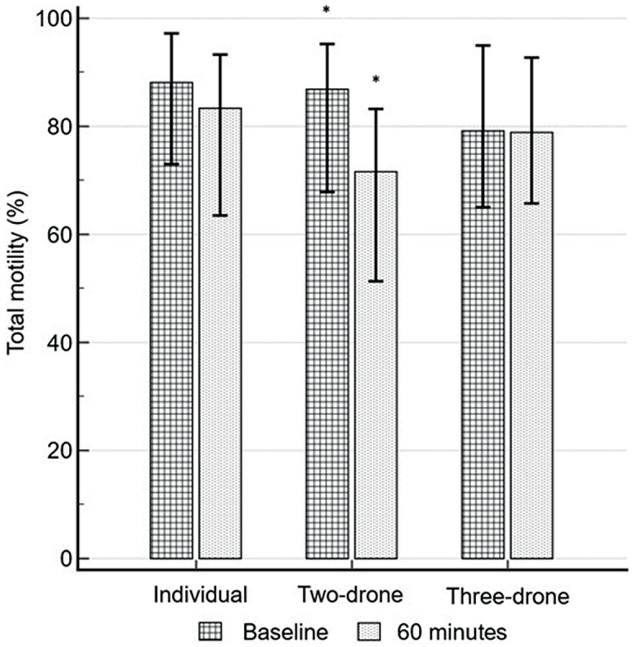

Figure 2. Changes in total motility percentage within mixed semen groups over time (baseline and 60 minutes) (median and 25,75% tiles). *Indicates significant differences within groups between baseline and 60 minutes.

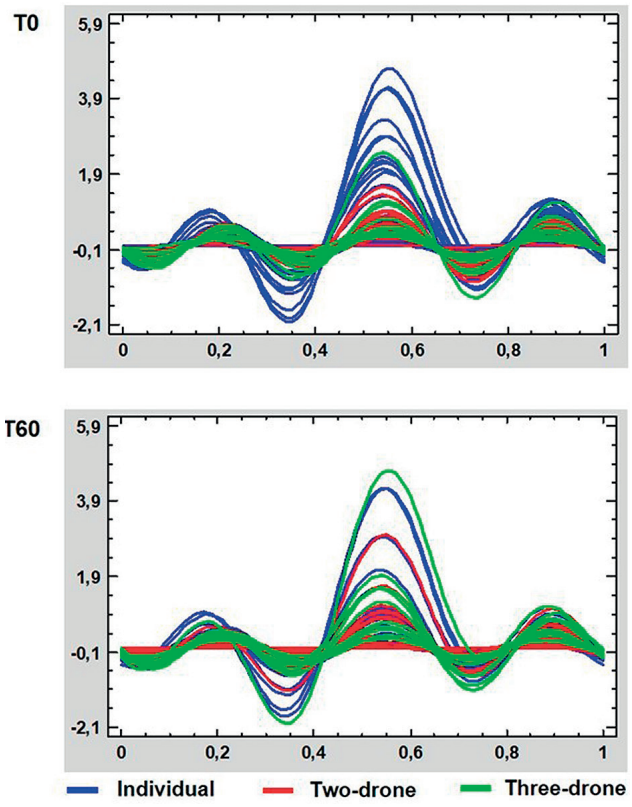

Figure 3. Andrews plots presenting the differences among individual and mixed drone semen at time intervals for combined motility and kinematic variables. The six variables included in the plots were total progressive motility (%), medium progressive motility (%), rapid and medium swimming speed (%), as well as kinematic velocity parameters VSL and VCL. VCL, curvilinear velocity; VSL, straight-line velocity; T0, baseline; T60, 60 minutes.

Effect of sperm morphology on sperm functionality

No significant correlations were found between sperm components, and motility or kinematic parameters. Regarding sperm component length variance among males in each group, the three-drone sample's CV for head length [7.13 (5.27– 8.06)%] was significantly lower compared to the individual [8.58 (6.80–11.2)%] and two-drone groups [9.10 (8.11–9.72) %] (p = 0.04). There were no significant differences in the CV's among the groups for the other sperm components. Furthermore, there were no differences in sperm component measurements among groups (Supplementary Table 5).

DISCUSSION

This study provides new information on changes in honeybee drone sperm functional and structural parameters following *in vitro* mixing. Tofilski et al. (2018) reported changes in sperm movement over time in the spermathecae of artificially and naturally inseminated queens; observations revealed no visible forward movement of sperm for the first 8 hours, with only a small fraction that showed fast forward movement. After 16 hours, however, coordinated circular movements were observed and very slow-moving groups of circular swimming sperm. Although our observations were terminated after 60 minutes following mixing of semen, our results are largely in agreement with the observations made by Tofilski et al. (2018) showing that in mixed drone semen groups the majority of sperm were NP and slow swimming, and displayed groups of circular swimming sperm.

Total motility was initially the highest in individual and two-drone samples, but markedly decreased in two-drone samples after 60 minutes, while minor decreases were noted in individual and three-drone samples over time. This finding supports observations made in *A.m. carnica* drones, where the mixing of two mature drones' semen resulted initially in very active sperm, but this activity later disappeared (Borsuk et al. 2011). Unfortunately, these authors did not provide a specific time frame of evaluation. Furthermore, in other insects such as the leaf-cutting ant, *Acromyrmex echinatior* Forel (Formicidae), a male's own seminal fluid has shown to increase sperm motility to a lesser extent, to preserve viability, while when mixed with a rival male's seminal fluid motility was greatly enhanced (Liberti et al. 2018).

The percentage total motility of a sperm population comprises the percentage PR and NP sperm. In the two-drone samples the percentage of NP and slow swimming sperm decreased over time without a proportional increase in progressive or faster swimming sperm, as would be expected. Instead, the percentage of immotile sperm increased (> 50% increase) alongside a decrease in percentage of motile sperm over time. It thus appears as if the mixing of two related drones' semen negatively affected sperm quality in this study. Sperm motility may be affected by the sperm cell's integrity, i.e. membrane integrity or permeability, that if compromised will result in leaking of important substances required for movement, such as ATP. Ultimately a reduction in ATP will result in immotile or less motile sperm. The presence of superoxide dismutase (SOD) in semen also has an important role in sperm survival and motility, functioning as an antioxidant. Therefore, if oxidative stress caused by the overproduction of ROS exceeds the antioxidant proteins in semen, sperm function can be compromised (Reinhardt 2007; Abdelkader et al. 2014, 2018). It is therefore possible that sperm in the two-drone samples were compromised due to oxidative stress.

As we did not investigate sperm viability over time, we cannot confirm that the increased percentage immotile sperm constituted an increased number of dead sperm. Den Boer et al. (2010), although obtaining sperm from seminal vesicles, have shown that sperm survival in six different insect species, including the honeybee and leaf cutter ants (*Atta colombica* Guerin-Meneville and *Acromyrmex echinatior*), increased if sperm were treated with its own seminal fluid, but decreased when an individual male's sperm is exposed to the seminal fluid of a related (brother) or unrelated male of the same species. The authors suggested that seminal fluid seems to contain substances that can induce incapacitation, thus reduce sperm viability, but also increase sperm survival. However, in the case of mixing two unrelated drones' semen, after storing samples for four days at 25 °C, Shafir et al. (2009) observed a non-significant higher viability percentage compared to individual drone samples, while Tofilski et al. (2012) did not find any difference in sperm viability once assessed after collection. In studies where more drones' semen was mixed, a much lower viability percentage was seen in four-drone semen samples (< 50%) compared to individual samples (Collins 2004), but in the case of mixing 5–8 (Collins 2003) or 8–10 (Gençer et al. 2014) semen samples, viability was not affected. In addition, Tofilski et al. (2012) have demonstrated increased particles in two-drone samples and suggested that it could be a result of sperm competition whereby different drones' seminal fluid can cause coagulation in order to immobilise the sperm of another. We did not observe coagulation of samples in this study.

Discrepancy in observations for sperm viability could possibly be resulting from studies using different methods for sperm collection, storage, and preparation, that may cause sperm damage (Collins 2004; Shafir et al. 2009; Gençer et al. 2014). It should be noted that sperm viability among individual drones can also vary (Collins 2004). Furthermore, several other factors have been associated with reduced sperm viability, for example, drone age. It has also been suggested that environmental factors such as seasonal differences, floral resources for nutrition, and particularly exposure to pests and pesticides, may even have a

greater effect on sperm viability (Rangel and Fisher 2019; Yaniz et al 2020; Morais et al. 2022). Especially the Varroa infestation rate of colonies as well as miticides used in colonies against Varroa should also be considered as possible factors that can affect drone sperm viability (Collins 2004; Rinderer et al. 1999). Varroa infestation rates of colonies in this study were not determined.

Sperm viability is higher in the spermatheca than in ejaculates which indicates that sperm viability is important for selection (Lodesani et al. 2004). Sperm viability is important in both the drone and the queen, but mechanisms to preserve sperm viability appears to differ. For example, the amount of proteins (enzymes) involved in metabolism differ considerably between spermathecal and seminal fluid, with the former containing far more substances to support sperm survival (Collins et al. 2006; Baer et al. 2009). Within the spermatheca sperm viability seems to be maintained by densely packed sperm that reduce the effect of dilution, and in turn decrease osmotic stress and endogenous respiration. As a result, metabolism of densely packed sperm is reduced (Reinhardt 2007). The relationship between sperm viability and motility inside the spermatheca is further of importance because sperm viability is also maintained by limiting motility. Compared to drone semen, the concentration of sodium and potassium inside the spermatheca is much higher, and is suggested to reduce sperm motility (Verma 1973). The presence of immotile sperm appears to be associated with older queens when sperm numbers and hypertonicity is reduced (Al-Lawati et al. 2009).

Nevertheless, reduced sperm viability of mixed drones' semen might have implications for particularly artificial insemination (AI) considering that, compared to naturally mated queens, sperm viability in AI queens reduces to a greater extent over time (Tarpy and Olivarez 2014; Lodesani et al. 2004). Reduced sperm viability can pose a threat to queen reproductivity as she may become a drone layer (unfertilised eggs). However, it has also been shown that in the case of AI, sperm with a viability of as low as 42.6% is still sufficient for queens to lay a sufficient amount of fertilised eggs (Collins 2000).

In contrast to the effect of two-drone samples on sperm movement, mixing of three drones' semen seem to improve sperm movement over time. For example, in this group, the decrease in percentage NP and slow swimming sperm over time was accompanied by increased percentages PR and faster swimming sperm, with majority of results being higher compared to individual and two-drone samples. Furthermore, this is the only group in which all kinematic parameters (although not significantly) increased over time, with increases particularly in VSL, LIN, and STR, with the latter two parameters dependent on VSL and VCL, VSL and VAP, respectively. In the honeybee, higher sperm motility percentages have been associated with increased motility parameters including progressive motility, and rapid motility as well as kinematic parameters VCL, VSL and VAP, and LIN, STR in individual drone's samples collected from seminal vesicles (Inouri-Iskounen et al. 2020) and ejaculates (Murray et al. 2022).

We suggest that the changes observed in sperm motility and kinematic parameters of three-drone samples in this study is likely to be related to a combination of factors, including sperm concentration, sperm cooperation, and time. This group had a much higher sperm concentration, which positively correlated with functional parameters. A higher sperm concentration could also have increased viscosity, and in turn increased sperm movement, as observed in the spermatheca (Tofilski et al. 2018). Al-Lawati et al. (2009) have further speculated that the hypertonicity inside the spermathecae can be responsible for the circular movement of sperm, and it is possible that with the higher sperm concentration in three-drone samples hypertonicity has increased. In other insects, for example, the humpback fly (Curtis and Benner 1991) and desert ant (Pearcy et al. 2014) increased viscosity of media used, resulted in increased sperm velocity. The higher sperm concentration could also have contributed to better sperm cooperation. Sperm cooperation has previously been observed in other insects of the Hymenopteran order, such as the eusocial desert ant (*Cataglyphis savignyi* Dufour) where dozens of sperm aggregate to form sperm bundles, and resulted in a 50% higher sperm velocity versus that of individual sperm. Such cooperative behaviour has further been suggested to increase the chances of an individual male to contribute to a larger number of sperm reaching and being stored in the spermatheca (Pearcy et al. 2014). Lastly, changes in honeybee drone sperm movement and motility have also been ascribed to time that elapsed between observations (Tofilski et al. 2018; Yaniz et al. 2019), as time may allow sperm to arrange itself (Tofilski et al. 2018).

It should however be considered, as previously mentioned, that the environment inside the queen's reproductive tract and spermatheca is different than in ejaculates. In case of successful mating or insemination, only a certain number of sperm is stored, and this sperm number further seems to be the same regardless of the number of drones' semen used for artificial insemination as shown by Woyciechowski and Król (1996). In *A.m. capensis* queens, for example, the sperm concentration in spermathecae seems to range between 3–11 million per spermathecal volume of about 1 µl (Buys 1990). In this study all groups' sperm concentration fell within this range, and it is not known how sperm movement will be affected inside the spermatheca with such varying sperm concentrations in this species. The large variation in sperm concentration observed in this study agrees with previous findings in the same species (Murray et al. 2022). Also, *A.m. capensis* drone sperm appears to be shorter than other subspecies such as Carniolan drones (Gontarz et al. 2016), which may further affect sperm movement in such an environment. Moreover, inside the spermatheca sperm is exposed to spermathecal fluid and anaerobic metabolism compared to aerobic metabolism in ejaculates (Liu et al. 2020).

Structure relates to function, and motility in insect sperm may therefore be affected by its length (Fitzpatric and Lüpold 2014). In *Drosophila melanogaster* Meigen (Drosophilidae), for example, sperm polymorphism is present, with the longer sperm swimming slower compared to short sperm and this provided the longer sperm with an advantage to resist sperm displacement by the female from her reproductive tract (Lüpold et al. 2012). However, in this study, sperm component dimensions did not differ among the three groups, and no correlations were found between motility parameters and sperm components. In other hymenopteran species, such as the wasp *Dahlbominus fuscipennis* Zetterstedt (Eulophidae), five different types of sperm are present in the seminal vesicles of males, but sperm in the spermathecae are uniform in size (Lee and Wilkes 1965). Although seasonal changes have been noticed in honeybee sperm dimensions (Gontarz et al. 2016), the presence of sperm polymorphism in sperm reaching and stored in the spermatheca of the honeybee following copulation is yet to be described. The shorter sperm of *A.m. capensis* drones may have different sperm movements compared to longer sperm of other subspecies.

A limitation of this study is that individual drone body size was not assessed, which has been previously shown to influence sperm concentration or sperm number per ejaculate in honeybee drones (Schlüns et al. 2003; Bratu et al. 2022), as well as the presence of abnormal sperm in ejaculates (Bratu et al. 2022). However, according to results obtained from previous experiments in our laboratory, body weight does not seem to have a negative effect on sperm concentration.

CONCLUSIONS

We conclude that findings obtained for motility and kinematic parameters in this study can in part be ascribed to the combined

effects of mixing drone semen, sperm concentration, and sperm arrangement over time, but not necessarily genetics. However, it is important to note that sperm mixed *in vitro* is not subjected to cryptic female choice, by which only a small percentage of sperm received from multiple drones after mating is selected to be stored in the spermatheca.

Although there are environmental factors that may affect queen quality, drone semen quality plays a very important role in queen health and her reproductivity because of inducing post-mating changes. These changes further affect the colonies' health and performance (Brutscher et al. 2019). Therefore, our findings may particularly be of relevance for AI programmes and beekeepers, by evaluating drone sperm quality (such as sperm concentration, motility and viability) as an indicator or biomarker for queen and subsequently colony health. In the case of AI, evaluating the pool of drone sperm collected prior to insemination can help to ensure a queen is inseminated with sperm of good quality.

We suggest that to better understand the mechanisms contributing to changes induced by mixing of drone semen, further studies investigating multiple sperm characteristics, including different sperm concentrations, the presence of ROS and mitochondrial function is required as well as a study of sperm traits in the spermatheca of the queen. Additionally, in order for the beekeeping industry to deploy the routine investigation of drone sperm, easy to use, and cost-effective methods to analyse drone sperm should be developed.

SUPPLEMENTARY MATERIAL

Supplementary tables are available alongside this article in a separate file.

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ETHICAL CLEARANCE

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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.

DISCLOSURE STATEMENT

The authors report there are no competing interests to declare.

AUTHOR CONTRIBUTIONS

Retha C.M. Kotzé was involved with the conceptualization and funding of the study, as well as supervising the research. She was also responsible for performing analyses and writing the original draft of the manuscript.

Gerhard van der Horst was involved with the conceptualisation and methodology of the study, as well as supervision of the research. He was also responsible for reviewing and editing of the manuscript.

Janice F. Murray conducted the investigation, and assisted with the analysis of data, and data curation. She was also responsible for reviewing and editing the manuscript.

Anja Duminy was involved with the conceptualisation and methodology of the study, and conducting the study. She also reviewed and edited the manuscript.

Mike Allsopp was involved with the conceptualisation and supervision of the field research and was also involved with the reviewing and editing of the manuscript.

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