Evaluating Cape honey bee sperm quality and the *in vitro* effect of miticides on sperm functionality and structure

JANICE FAITH MURRAY

Thesis submitted in partial fulfilment of the requirements for the degree *Master of Science* in Medical Bioscience at the University of the Western Cape

Supervisor: Dr C Kotze

Co-supervisor(s): Prof G van der Horst

Mr M Allsopp

30 September 2021

DECLARATION

I, the undersigned, declare that the work contained in this thesis, *"Evaluating Cape honey bee sperm quality and the in vitro effect of miticides on sperm functionality and structure"* is my own work and has not been previously submitted for any degree or examination at any other university. All the sources I have used or quoted have been indicated and acknowledged as complete references.

Full Name: Janice Faith Murray

Date: 30 September, 2021

DEDICATION

This thesis I dedicate to my parents, Japie and Rosalynd Murray. I am grateful for your prayers, support and sacrifices. Thank you for the good examples set and for always believing in me.

I thank my God every time I remember you.

ACKNOWLEDGEMENTS

"Entrust your efforts to the Lord, and your plans will succeed." - Proverbs 16:3

Thank you Lord, for Your blessings on me.

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My family, for their continuous support.

ABSTRACT

INTRODUCTION AND AIM

Apis mellifera (honey bees) are essential pollinators and thus considered a keystone species. Reproductive success is therefore important to the survival of the species, with drone sperm quality playing a vital role in fertilization success and colony health. Drone sperm quality is determined by multiple aspects including both structural and functional parameters. Particularly, motility, a functional parameter of sperm, plays an important role in reproductive success by determining the ability of sperm to move in the reproductive tract of the queen and to participate in fertilization. However, for the South African honey bee subspecies, the Cape honey bee (*Apis mellifera capensis Escholtz*), data on sperm quality parameters, are lacking.

Drone health and sperm quality are affected by several environmental factors, including pests such as the *Varroa destructor*, regarded as one of the main contributors to collapsing colonies particularly in Western honey bees (*Apis mellifera*). Additionally, despite limited data, the use of miticides to combat mites may also affect drone sperm quality. Furthermore, assessment of drone sperm quality in terms of functionality is limited to a few parameters only, and the application of modern techniques such as computer aided semen analysis (CASA) to accurately analyse motility parameters, as in human and invertebrate sperm, are yet to be established for honey bee drone sperm. Therefore, the aim of this study was to establish both a qualitative and quantitative method to determine sperm quality, particularly sperm functionality, using a CASA system and to determine the effect of miticide exposure on drone sperm quality.

MATERIALS AND METHODS

The study included data from 153 semen samples of sexually mature drones collected from 10 different colonies. A total of 51 samples were assessed to determine baseline sperm motility and kinematic parameters using CASA and a further 102 drones, to investigate the impact of *in vitro* amitraz (an active ingredient in commonly used miticides) exposure on sperm quality parameters including sperm functionality (motility and kinematics) and structural characteristics (vitality and morphology).

Honey bee drone samples were firstly assessed to determine gross sperm motility semi-quantitatively by using conventional phase-contrast microscopy and classifying samples by assigning a motility index

score, based on motility and swimming patterns (e.g. from immotile to many groups of circular swimming sperm), to each sample. Secondly sperm motility and kinematics were assessed qualitatively by using a fluorescent method and CASA. Sperm motility and kinematic parameters were obtained using a fluorescence assay (SYBR14) and analysed using fluorescence microscopy with CASA and Sperm Class Analyser (SCA) ® Motility module. Motility characteristics obtained from CASA, were compared with gross sperm motility results. To determine the *in vitro* effect of amitraz on drone sperm quality, samples were exposed to three different concentrations of amitraz (125 µM, 250 µM and 500 µM). Results obtained for motility and kinematic parameters were compared among different concentrations and with control samples (unexposed). Viability of amitraz-exposed and control samples were determined using a dead/live staining kit (SYBR14 and propidium iodide); analyses were obtained using fluorescence microscopy with CASA and SCA ® Vitality module. Morphological differences of sperm treated with amitraz concentrations were investigated using BrightVit stain and analysed using CASA and SCA ® Morphology module.

RESULTS

Cape honey bee drone sperm concentrations ranged between 1.10 x 10^6 - 16.5 x 10^6 million per µl and a significantly high average sperm total motility percentage of 85.5% (± 17.2) (n = 51) was obtained at baseline. Motility index scores corresponded with percentage motility categories as determined by CASA. For example, the mean motility index score of 4.11 (± 1.11) (n = 51) corresponded with a high motility percentage (85.5 % motility) category (> 80% motility). Furthermore, although not significantly so, kinematic parameters such as curvilinear velocity (VCL), appeared to decrease as the percentage total motility of a sperm sample decreased. In addition, low values for velocity kinematic parameters were also seen in samples with low motility index scores (3 and less). The initial kinematic parameters obtained, were then used to determine cut off values for slow (VCL = 50 µm/s) and medium-rapid (VCL > 75 µm/s) swimming sperm; finally resulting in the establishment of sperm subpopulations (slow-, medium- and rapid progressive).

Regarding the effect of different amitraz concentrations (125 µM (n = 13), 250 µM (n = 18) and 500 µM (n = 20)) on sperm quality parameters, no significant effect on motility and kinematic parameters was observed. However, for velocity parameters (VCL, straight-line velocity (VSL) and average path velocity (VAP), values seemed to decrease in a dose-response manner. Viability was significantly lower when exposed to 250 and 500 µM amitraz compared to the control and those exposed to 125 µM amitraz.

Furthermore, amitraz concentrations did not significantly affect sperm morphology (head, and tail lengths). In addition to the usual sperm components observed with BrightVit staining, a structure, which resembles mitochondria, stained purple in dead sperm. The length of this mitochondrial structure was significantly shorter for sperm exposed to 250 and 500 μM amitraz, compared to the control.

CONCLUSION

This study reported for the first time, on accurately measured motility and kinematic parameters using a fluorescence stain and CASA, and the findings in this study can therefore be used as reference for future honey bee sperm quality studies. Furthermore, it is evident that very high amitraz concentrations reduce sperm viability and may have adverse effects on sperm structure.

KEYWORDS: Honey bee, Drone, Reproduction, Cape honey bee, Sperm quality, Sperm structural characteristics, Sperm motility, Sperm kinematic parameters, Sperm viability, CASA

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percentage
<	Less than
>	Greater than
±	Standard deviation
≥	Greater than or equal to
°C	Degree celsius
µl	Microlitre
µm	Micrometre
µm/s	Micrometre per second
µM	Micromolar
10^6 / ml	Million per millilitre
AI	Artificial insemination
A.m	*Apis mellifera*
ANOVA	Analysis of variance
AUC	Area under curve
CASA	Computer-aided sperm analysis
DCA	Drone Congregation Area
DNC	Dance
FITC	Flourescenin isothiocyanate
g	Grams
i.e.	In other words
IVF	In-vitro fertilization
LIN	Linearity
M	Mole
mg/L	Milligram per litre
mg/ml	Milligram per millilitre
Mil/ml	Million per millilitre
Min	Minute
ml	Millilitre
mM	Millimole

mmol/L	Millimoles per litre
mOsm/L	Osmolarity
n	Population size
NRF	National research foundation
pH	Expression of acidity/alkalinity
pH+	Positive phase contrast
SCA ®	Sperm class analyser
SD	Standard deviation
STR	Straightness
TM	Total motility
VAP	Average path velocity
VCL	Curvilinear velocity
vs.	Versus
VSL	Straight-line velocity
WOB	Wobble

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CHAPTER 1: INTRODUCTION

1.1 Background

Current literature reporting on the global rise in honey bee colony losses has raised public concern regarding the species' propagation and sustainability (Neumann & Carreck, 2010; Clermont, et al., 2014). Honey bees are considered to be the most important cross-pollinators of an estimated 75% crop species (Rhodes, et al., 2011), and are therefore considered an important keystone species (Breeze, et al., 2011). As a result, deteriorating honey bee populations may pose a future threat to the entire agricultural discipline; especially honey bee breeders (Potts, et al., 2010). Successful reproduction of honey bees relies on a healthy queen and drones (Delaney, et al., 2011; Tarpy, et al., 2012; Yániz, et al., 2020). However, literature available on honey bee drone reproductive biology, specifically comprehensive and routine analysis of drone semen quality, is restricted. Reports are commonly limited to physical drone health, superficial sperm parameter assessments, and factors compromising honey bee reproduction rates both naturally and artificially (Gençer, et al., 2014).

Drone semen quality is an important parameter as it relates to the longevity of the queen, given its vital role in queen reproductive health (Ciereszko, et al., 2017). Poor sperm quality with fertilizing implications may either result in the superseding of the queen or be the underlying cause for colony collapse (Cobey, 2007). Sperm quality assessment, therefore, plays an important role when determining colony health and performance (Johnson, et al., 2015; Ciereszko, et al., 2017; Yániz, et al., 2020). Until recently, research on drone semen quality only involved the assessment of parameters such as semen volume, sperm concentration, sperm viability, and an estimated percentage of total motility based purely on manual observational methods (Yániz, et al., 2020). However, thorough semen quality analysis requires a wide spectrum of sperm characteristics to be taken into consideration, discriminating between many aspects concerning cell integrity and functionality (Ciereszko, et al., 2017). Furthermore, reports on the functional aspects of honey bee drone sperm are restricted to the assignment of manual motility grade scores, total motility percentages, and sperm swimming patterns, mainly observational of nature (Tofilski, et al., 2018; Inouri-Iskounen, et al., 2020). Although recent literature proclaimed success in using computer-aided sperm analysis (CASA) to assess drone sperm motility, the technique used appears to be inaccurate (Inouri-Iskounen, et al., 2020). The accurate measurement of honey bee sperm motility and movement characteristics (kinematic parameters), using only negative phase-contrast microscopy, is not

possible given that CASA requires a reference point for tracking, such as the sperm head (Lu, et al., 2014). CASA, routinely used for sperm analyses in humans and domesticated animals, is yet to be developed in the honey bee (Yániz, et al., 2020). With the assistance of CASA and the sperm class analyser (SCA ®), routine sperm quality assessments of honey bee drone sperm can be performed objectively, with special emphasis on the accurate quantification of sperm functionality. Owing to the morphology of honey bee drone sperm, staining the sperm head cells with a fluorophore has been recommended for CASA systems to detect sperm movement and motility (Wegener et al., 2012; Yániz, et al., 2020).

Drone fitness and reproductive health, specifically its sperm quality, can be affected by several factors, such as age, genetics, seasonal changes, but more importantly, by the use of pesticides, especially in-hive miticide exposure, which is known to be a limiting factor of honey bee drone productivity and survival (Brittain, et al., 2010; Rhodes, et al., 2011; Czekońska, et al., 2013; Rousseau, et al., 2015). For example, amitraz, {N, N-[(methylimino) dimethylidyne] di-2, 4-xylidine}, a well-known acaricide and active ingredient of widely-used pesticides, is often used for tick and mite treatment in veterinary medicine (Avsarogullari, et al., 2006; Lee, et al., 2013). This liposoluble substance is quickly absorbed through the skin, which makes exposure to both human and animal species hazardous. Amitraz has furthermore shown the ability to induce DNA damage and cell apoptosis (Padula, et al., 2012). Studies investigating the effect of amitraz concentrations on honey bee sperm thus far, only determined its effect on sperm viability in queens (Chaimanee & Pettis, 2019) and drone sperm (Fisher & Rangel, 2018), omitting to report on the possible functional implications on drone sperm functionality, especially motility, and sperm structural changes, that in turn relates to sperm functionality. Honey bee drone sperm quality and the possible adverse effects of miticides thereon is thus a significant field of research interest especially for colony health, production, and ecological preservation (Yániz, et al., 2020).

Reduced colony performance, as a result of environmental factors, such as pesticide exposure, may lead to future colony collapses, and increased numbers of collapsing colonies, can further have a negative effect on pollination services (Biesmeijer, et al., 2006; Magal, et al., 2019). For example, irregular sperm parameters, as a consequence of chronic exposure to miticides, may result in infertility leading to inadequate reproduction (Ciereszko, et al., 2017; Yániz, et al., 2020). Therefore, understanding honey bee drone reproduction and its sperm biology are important to limit, but also to prevent losses. Particularly the relationship between drone sperm structural characteristics, sperm functionality, and its ultimate effect on colony performance warrants further research Although, the importance of drone sperm quality on colony

health and reproductive success in honey bees has gained more attention lately, research particularly focussing on sperm functionality and techniques to accurately and routinely measure motility parameters, is still, very limited (Yániz, et al., 2020).

Honey bee subspecies in Africa, including South African subspecies, where a large wild population is still present, seem to be more resistant to environmental factors (e.g. pests) (Strauss, et al., 2015), that have resulted in large colony losses in other countries where bee populations are mostly managed. For example *Varroa destructor*, for which South African honeybees are not treated for due to possible resistance (Strauss, et al., 2014). It can thus be postulated that the reproductive biology of African honey bee subspecies is not affected in such a negative manner. However, the growing southern African population resulting in increased losses of habitat, monoculture crops, and pollination demand, are just some of the environmental factors that may impact the reproductive biology of honey bee drones in the near future (Archer, et al., 2014; Pirk, et al., 2016).

Particularly data on South African honey bee drone sperm quality is currently lacking. This study, therefore, determined honey bee drone sperm quality of Cape honey bee (*Apis mellifera capensis Escholtz*) drones, by focussing on sperm motility and kinematics using fluorescence microscopy and a CASA system. In addition, the effect of three different concentrations of amitraz on sperm functionality and structure was also investigated to determine whether it has the potential to affect the sperm quality of Cape honey bee drones. The outcomes of this study provide data on the automated, fast and accurate assessment of insect sperm quality and the possible adverse effects of miticide exposure on colony health, contributing to the understanding of honey bee reproduction (both natural and artificially), colony treatment, and maintenance in general.

1.2 Aim and objectives

This main aim of this study is to establish a qualitative and quantitative method to determine Cape honey bee drone sperm quality, using a CASA system and to investigate the *in vitro* effect of amitraz on functional and structural drone sperm characteristics.

Specific objectives of this study include the following:

- To establish a technique to assess honey bee drone sperm motility by determining sperm motility and kinematics using a fluorescence method and a CASA system and compare with a manual motility index score
- To determine and compare the *in vitro* effect of three amitraz concentrations on sperm functional characteristics, i.e., motility parameters and motility kinematic parameters using a CASA system.
- To determine and compare the *in vitro* effect of three amitraz concentrations on sperm structural characteristics including dimensions of sperm components using the BrightVit staining method
- To determine and compare the *in vitro* effect of three amitraz concentrations on sperm viability using dead/live staining methods, SYBR14 and propidium iodide, and BrightVit.

1.3 Research team and contributions

All role players and their respective contributions to this study are listed in table 1.1.

Table 1.1 Research team members

Team member	Qualification	Role in study
Ms. J Murray	B.Sc. (Hons) Medical Bioscience	M.Sc. student: Responsible for protocol, literature review writing, collection and preparation of samples, analysis of objectives, implementation of methods, data capturing, statistical analysis, interpretation of results, writing up of dissertation
Dr. R Kotze	Ph.D. Nutrition	Supervisor of M.Sc. student: Provided guidance regarding writing of protocol, literature

		review and thesis overall, training student in methodology; guidance in data capturing, statistical analysis and interpretation of results
Prof. G van der Horst	Ph.D., Ph.D.	Co-supervisor of M.Sc. student: Provided guidance regarding writing of protocol, literature review and thesis overall, training student in methodology; guidance in interpretation of results
Mr. M Allsopp	M.Sc. Entomology	Co-supervisor of M.Sc. student: guidance regarding planning of study, writing of protocol and dissertation, training student in methodology and collection of samples, guidance in interpretation of results

1.4 Structure of the thesis

This thesis is presented in chapter format, and consists of six chapters. This thesis was prepared using the Harvard referencing format. Chapter 1 provides background information of the study and the motivation for the study. It further provides the aim and objectives, and the research team and their respective roles in the study. Chapter 2 presents an overview of the literature on honey bee reproductive biology, and the analysis of sperm quality. Chapter 3 explains the materials and methods as well as the experimental design of the study. In chapter 4 the results of this study are provided, starting with the baseline study, followed by the amitraz-dose response study results. In chapter 5, a detailed discussion and interpretation

of the results are provided as well as its relevance to currently available literature. This chapter finishes with a conclusion including main findings and new insights as obtained from this study. In the final chapter, chapter 6, limitations of the study are highlighted, and it also provides recommendations for future studies. The thesis ends with a reference list providing all references used in this thesis.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Long-term honey bee, (*Apis mellifera* (*A.m.*)) colony losses is a major cause for concern since it may pose a threat to the future availability of pollination services and ultimately food security (Breeze, et al., 2011). Continuous reports on colony infestations and losses from all parts of the world highlighted several shortcomings relating to knowledge on honey bee reproduction, more specifically drone reproductive health and semen quality assessment (Koeniger, 2005; Yániz, et al., 2020), and additionally how these factors impact queen reproductive health and fitness (Collins, et al., 2004; Delaney, et al., 2011; Tarpy, et al., 2012). It is however known that colony losses can be prevented by ensuring optimal drone, queen, and colony productivity (Amiri, et al., 2017).

Poor sperm quality and sexually incompetent queens are amongst the factors contributing to poor queen quality and colony losses (Spleen, et al., 2013; Amiri, et al., 2017). Sustaining optimal drone and queen reproduction is therefore important. Increasing reproductive quality will ensure sperm- and colony longevity. (Collins, et al., 2004; Delaney, et al., 2011; Tarpy, et al., 2012). The expiration of a queen's reproductive lifespan is furthermore determined by her ability to fertilize eggs; to do so adequate sperm stores are needed. Once sperm stores are depleted, it cannot be replenished since a queen mate only once, with multiple drones, therefore in most cases when sperm stores are depleted and a queen becomes reproductively unfit, supersedure (queen replacement) occurs (Cobey, 2007; Delaney, et al., 2011). This process involves the rearing of a new queen, in the presence of the unfit or incompetent queen (Butler, 1957). Therefore, sufficient sperm reservoirs are required, to prevent the stimulation of queen supersedure and colony collapse. Improving sperm quality will thus be beneficial to queen fertility, longevity, and ultimately colony productivity and performance (Tarpy & Olivarez, 2014). Drone sperm is stored in the spermatheca throughout the queen's entire reproductive lifespan, necessitating high concentrations of viable sperm and the ability to retain live sperm within the spermathecal environment (Collins, et al., 2004). Sperm quality and quantity are known limiting factors for successful reproduction (Schlüns, et al., 2004; Yániz, et al., 2020). Successful honey bee reproduction thus has an important role to ensure survival of the species and to ensure continuous, reliable pollination services. Therefore, research to improve the understanding of honey bee reproduction is important (Tarpy & Olivarez, 2014; Koeniger, et al., 2005).

2.2 Honey bee caste division

Honey bee species are well-known social insects. A renowned characteristic of such species is the division of labour amongst colony members, called caste division (Schwander, et al., 2010; Gordon, 2016; Yadav, et al., 2017). Insect castes, including honey bee species, are classified based on anatomical and physiological features. Honey bee colonies consist of a colony head, known as the queen, a sterile, female worker bee, and lastly the male bee, also known as the drone (Johannsmeier, 2001; Yadav, et al., 2017). Honey bee castes specifically, consist of the queen and worker bees, which differ in function, behaviour, and morphology. Distinguishing characteristics, including body size and features, between the queen, worker and drone, are illustrated in Figure 2.1.

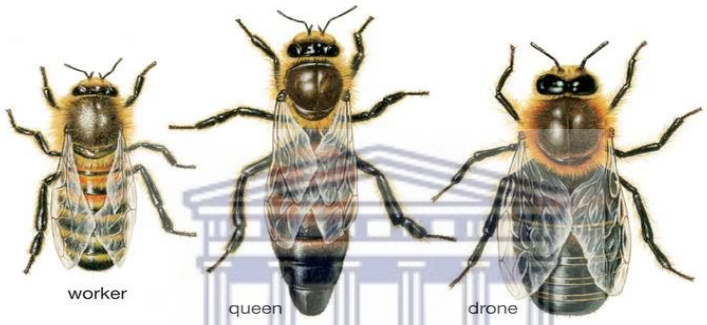


Figure 2.1 Characteristics of the honey bee (*Apis mellifera*) queen, worker and drone[Adopted from Rogers (2013)]

The queens and workers, result from diploid, fertilized eggs (Figure 2.2). The fate of a fertilized egg depends entirely on rearing conditions, specifically the nutrition provided during development. The development of a queen is owed to the amount of food provided and also the administering of royal jelly by worker bees during the larval developmental stage (Buttstedt, et al., 2016). This is a type of brood nutrient that contains large quantities of mandibular gland secretions (Winston, 1991; Yadav, et al., 2017).

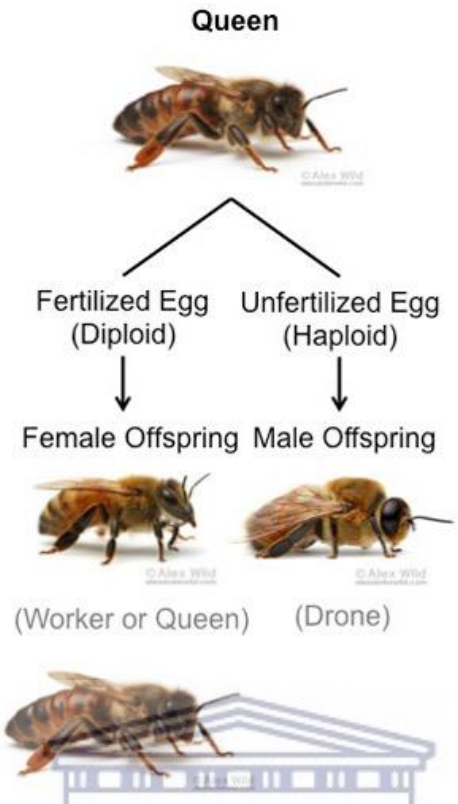


Figure 2.2 Illustration of sex determination in the honey bee [Adopted from Mortensen, et al. (2013)]

Queen development is noticeably shorter than those of workers (about 21 days) and drones (about 24 days), generally lasting up to 16 days (Johannsmeier, 2001). However, in African honey bees the queen and workers develop faster, the queen 14 days, and worker, 19-20 days (Ellis & Ellis, 2008). Upon emergence, its first instinct would then be to take control over the colony, this entails killing or destroying existing adult queens as well as prospective queen brood cells. Their mandibular pheromones furthermore inhibit and inactivate the female worker reproductive system, to prevent additional egg-laying of members other than the queen itself. Honey bee queens are therefore known as the egg-layer of the colony and are one of the key contributors to colony reproduction (Brutscher, et al., 2019).

Honey bees colony size range between 10 000 – 60 000 between winter and warmer, honey seasons. Other than the availability of food, efficient reproduction, including queen egg-laying and drone sperm quality, is important to ensure that these numbers are maintained (Johannsmeier, 2001; Tarpy, et al., 2014; Yániz, et al., 2020). The greater percentage of colony members consist of the female worker bees, responsible for nectar and pollen collection, hive maintenance, drone nursing, and colony, especially queen, protection (Johannsmeier, 2001).

Unlike the female castes, drones, develop from haploid, non-fertilized eggs (Figure 2.2), and compared with worker bees (diploid), it only has a genetic contribution from the queen (Johannsmeier, 2001; Page & Peng, 2001). On average, the honey bee drone larval development stage lasts up to 24 days until emergence (Page & Peng, 2001; Rangel & Fisher, 2019). Drones are reared solely during reproductive periods, which is typical during the warmer seasons of the year. Drone rearing is exorbitant given that they do not contribute to hive maintenance and are thus only produced when needed for reproduction (Johannsmeier, 2001; Rangel, et al., 2013).

A drones' main purpose is to participate in mating flights where they copulate with virgin queens. A drone mates only once in its lifetime, and is therefore known to be semelparous, while the polyandrous queen mates with multiple drones per mating flight (Fuchs & Moritz, 1999; Withrow & Tarpy, 2018). Honey bee queens and drones are both responsible for colony growth; hence it is important to preserve their reproductive health (Brutscher, et al., 2019).

2.3 South African honey bee subspecies

South Africa is home to two western honey bee (*Apis mellifera*) subspecies, i.e., *A.m. capensis* ("Cape honey bee"), and *A.m. scutellata Lepeltier* ("African or Savannah honey bee") (Hepburn, et al., 1998). The Cape honey bee originates in the western and southern Cape regions of South Africa where they feed on the fynbos biome (green area in Figure 2.3), meaning small or fined leaves, a form of vegetation, which is present only in the southern tip of Africa (Du Preez, 2014). The rest of South Africa, especially the northern regions, is home to the African bee, *A.m. scutellata* (Hepburn & Crewe, 1991; Johannsmeier, 2001).

Figure 2.3 Geographic distribution of the Fynbos biome in South Africa where the Cape honey bee is typically found [Adopted from Mortensen, et al. (2013)]

The latter subspecies present with undesirable, aggressive characteristics, which involve unwarranted swarming and hive absconding, hence they are commonly nicknamed the "killer bee". Despite their aggressiveness and often unwanted traits, they remain important pollinators of widely ranged crops and for honey production (Johannsmeier, 2001). The Cape honey bee is less aggressive and has the distinctive reproductive capability to produce diploid eggs, meaning they can produce either male or female offspring without mating, a unique method of reproduction called thelytokous parthenogenesis (Figure 2.4) (Neumann & Moritz, 2002). This type of behaviour enables them to completely take control over a colony often so targeting other honey bee sub-species (Johannsmeier, 2001; Hepburn & Crewe, 1991). This unique reproductive feature sets the Cape honey bee apart from all other subspecies (Johannsmeier, 2001). Given the above-mentioned traits, such as thelytoky by the Cape honey bee and the pronounced defensive behaviour of the Savannah honey bee, both subspecies portray parasitic behaviour when found in non-endemic regions (Calis, et al., 2003).

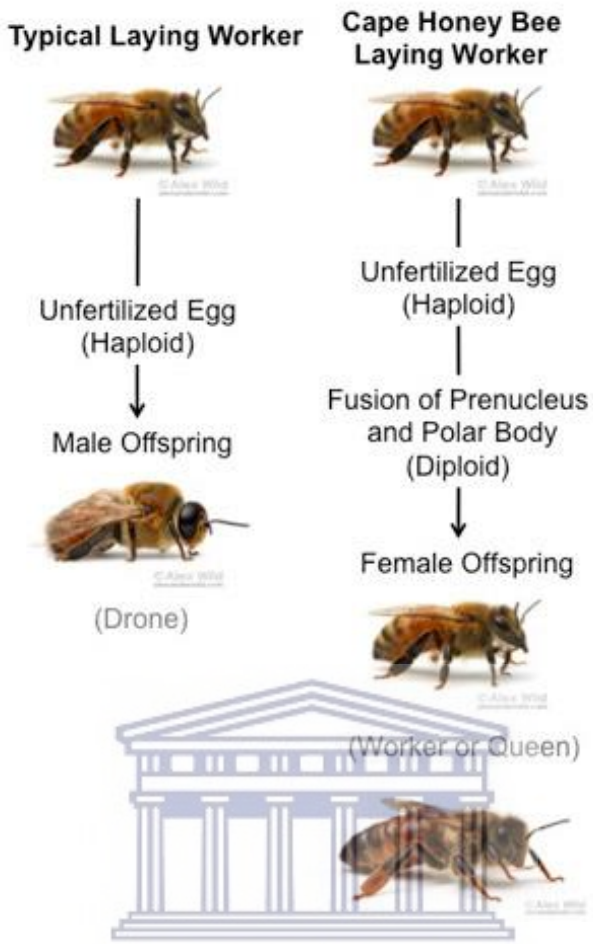


Figure 2.4 Diagram demonstrating thelytokous parthenogenesis, a reproductive trait of the Cape honey bee laying worker [Adopted from Mortensen, et al. (2013)]

Although the two honey bee subspecies in South Africa each have their distinctive habitats, their natural distributions do overlap in a region referred to as the hybrid zone (Hepburn, et al., 1998). In regions where they co-occur it is often difficult to qualitatively distinguish between the two, hence a set of standard morphometric features were established by Ruttner (1988), to distinguish between *A.m.* subspecies. Hepburn, et al. (1998) further quantified these features and established that it was only necessary to look at the following morphometric features: the length of cover hair, transverse wax plate length, pigmentation of the scutellum and scutellar plate, tergite colour, and wing angle.

In agreement with the findings of Hepburn, et al. (1998), Bustamante, et al. (2020) determined that tergite colour was a more accurate (97 % accurate) measure to discriminate between *A.m. capensis* and *A.m. scutellata*. Secondly, ovariole numbers are just as accurate since it also identifies the unique reproductive trait of *A.m capensis*, thelytoky; however, this method is less practical (Hepburn & Crewe, 1991; Bustamante, et al., 2020).

South African beekeepers can also easily identify established Cape honey bee colonies based on the following behavioural characteristics: worker bees producing female offspring, well-developed ovaries in egg laying-workers, and lastly queen-less swarms (Johannsmeier, 2001).

2.4 Drone reproductive development

The initiation of drone production typically occurs 3 - 4 weeks before queen rearing, as an endeavour to ensure the presence of adequate sexually mature drones during the mating season (Page, 1981). Newly emerged drones are nurtured by their sister-worker bees until they have reached sexual maturity, a common phenomenon amongst honey bee species (Stűrup, et al., 2013).

Drone reproductive tract

Honey bee drone reproductive organs entail the testes, vas deferens, seminal vesicles, mucus glands, and an ejaculatory duct. It furthermore consists of a copulatory organ, called the endophallus, used to deposit sperm into the queen bee's spermatheca during mating (Johannsmeier, 2001) (Figure 2.5).

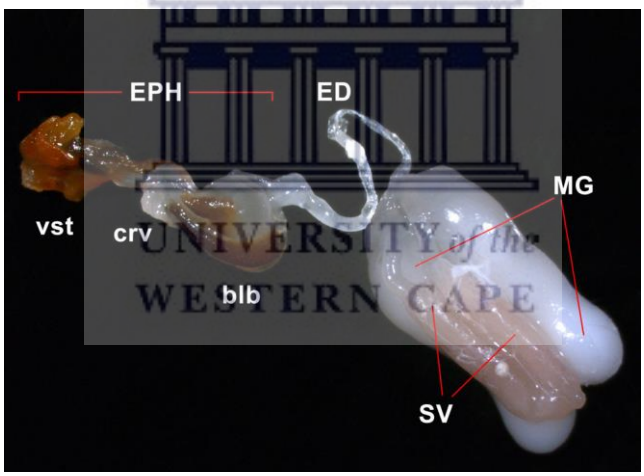


Figure 2.5 The honey bee drone reproductive system (testes were removed) [Adopted from Gençer, et al. (2014)] EPH, endophallus; blb, bulb; crv, cervix; vst, vestibulum; ED, ejaculatory duct; MG, mucus gland; SV, seminal vesicles

Spermatogenesis and spermiogenesis

The process of sperm production, spermatogenesis, occurs during the larval and pupal stages of the drone life cycle and is completed before adult drone emergence (Lago, et al., 2020). Spermatogenesis in honey bee drones occurs only once in its lifetime, therefore the adult drone emerges with a lifetime supply of sperm (Baer, 2005; Lago, et al., 2020). In newly emerged drones, sperm are located in the

testes and migrates to the seminal vesicles between days 3 - 8 post-emergence. Sperm migration marks the start of the sperm maturation process (spermiogenesis), which is completed on the eighth day post-emergence. Mature sperm are now retained in the seminal vesicles until the sexually mature drone (8 days old and above) participates in a mating flight with a virgin queen bee (Woyke & Jasinski, 1978; Locke & Peng, 1993; Mazeed & Mohanny, 2010). Honey bee males are considered a neglected gender (Koeniger, 2005); this is evident in the limited available research on drone testis development and spermatogenesis, and a lack of in-depth results on these processes (Bishop, 1920; Baer, 2005; Lago, et al., 2020). It is only recently that Lago, et al. (2020) provides detailed insights on the mitotic and meiotic phases of drone spermatogenesis and the role of insect reproductive hormones thereon.

During early larval development male and female honey bees share many architectural characteristics. The distinction between the two sexes is only visible upon the entry of metamorphosis, during which spermatogonia enter the first phase of meiosis. This meiotic phase starts once the drone brood cell is capped (day 11), and is completed once the larvae develop into a red-eyed pupa (day 18). The appearance of the pupal molt marks the beginning of the second meiotic phase during which the spermatids undergo spermiogenesis, this sperm maturation phase is completed in the pharate-adult drone (Lago, et al., 2020). Insect reproduction in general is regulated by two important hormones namely the juvenile hormone, mainly responsible for caste division, and ecdysteroids (Santos, et al., 2019; Swevers, 2019). Juvenile hormone levels gradually increase during the first phase of meiosis, occurring simultaneously during larval to pupal transition (Holometabola), and levels peak during Holometabola (Belles, 2020). Contrary, ecdysteroid levels rise after the larval to pupal transition, therefore this hormone is associated with sperm maturation, which occurs in the second phase of meiosis when spermatocytes become spermatids. Thereafter, ecdysteroid levels decrease. Soon after the peak in ecdysteroids, spermatids ultimately differentiate into mature sperm cells (Lago, et al., 2020).

The second phase of meiosis in drone spermatogenesis is concluded in Figure 2.6, while Figure 2.7 illustrates the process of spermiogenesis.

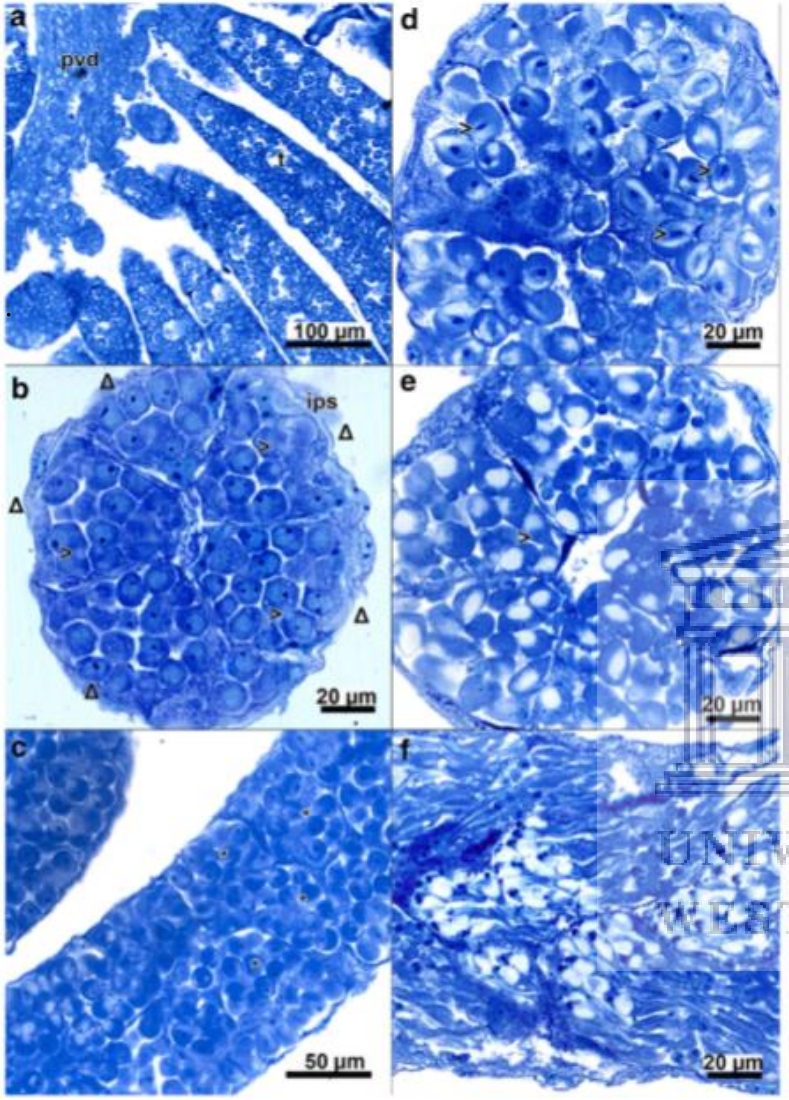


Figure 2.6 Meiotic division during spermatogenesis in the honey bee

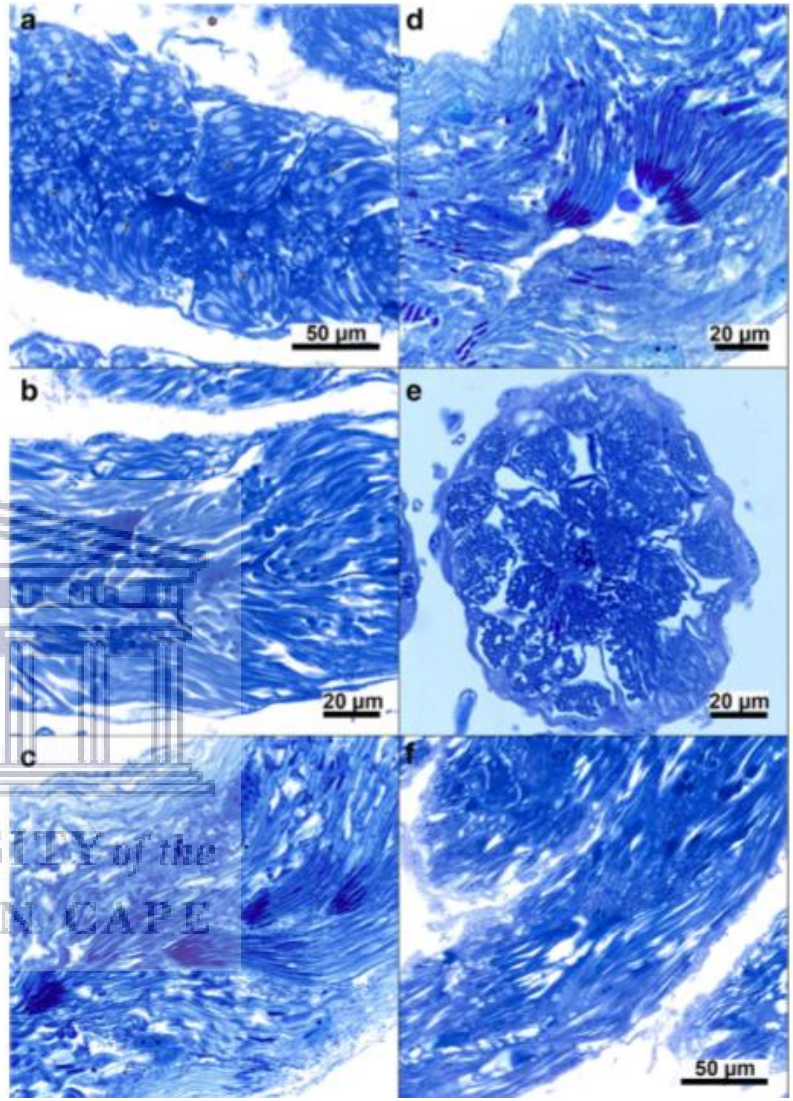


Figure 2.7 Spermiogenesis in the pharate-adult honey bee

[Adopted from Lago, et al. (2020)]

Honey bee mating

On a typical mating flight, the young, polyandrous, virgin queen mates with approximately 37–77 drones at drone congregation areas, which die post-copulation (Withrow, et al., 2018; Lago, et al., 2020). Conversely, in the Cape honey bee, the number of drones a queen mate with may range between 25 - 30 drones (Johannsmeier, 2001). Drones emit a gland-produced scent, initiating the formation of a drone congregation area (DCA), which is typically 30 – 200 meters in diameter and 5 – 40 meters, above ground (Johannsmeier, 2001; Yániz, et al., 2020). An average of 11 000 drones, from an unknown number of colonies, meet at mating sites where they interact with a virgin queen (Brandstaetter, et al., 2014; Villar, et al., 2018). Since congregation areas are mid-air, mating generally involves relatively lengthy flights. The duration of these flights range between 15 – 30 minutes, thus adult drones require good flying power and ultimate flight fitness (Duay, et al., 2002; Hrassnigg & Crailsheim, 2005). However, to date, the important role of the drone in the mating flight, and honey bee reproductive biology, in general, has not received adequate attention (Woyke, 2016; Lago, et al., 2020).

Mating is initiated when the drone approaches the queen by directing its abdomen towards the queen's open sting chamber, during which the drone's abdominal muscles begin to contract. The natural mating process involves seven stages as illustrated in Figure 2.8, while Figure 2.9 provides an overview of the process of a drone mating with a queen. During the first phase early abdominal contractions take place, and the copulatory organ of the drone, called the endophallus, partially evert into the open sting chamber of the queen. At this stage, the drone becomes completely paralyzed in the head and thorax, while abdominal contractions continue to initiate the second phase of eversion (Koeniger, et al., 2014; Woyke, 2016).

The second phase entails the contraction of the mucus glands, pushing mucus into the ejaculatory duct which in turn forces semen forward in the bulb. The continuation of muscle gland contractions further pushes glandular epithelium into the now mucus-filled ejaculatory duct, filling up the posterior part of the bulb. At this stage, higher pressure is required for full eversion and successful insemination to occur (Koeniger, et al., 2014; Woyke, 2016). Once fully everted, the mucus hardens when in contact with air (Bishop, 1920), and together with the exposed male genitalia, both aid in holding the drone in place during sperm transfer into the vaginal orifice (Koeniger, 1986).

Lastly, after successful copulation, remnants of the drone's copulatory organ are left behind in the open sting chamber of the queen; this is referred to as the mating sign, which is soon removed by the next copulating drone (Baer, 2005; Koeniger, et al., 2015). Mating activities are repeated until the mating flight is terminated. Figure 2.8 summarizes the stages of a honey bee mating flight.

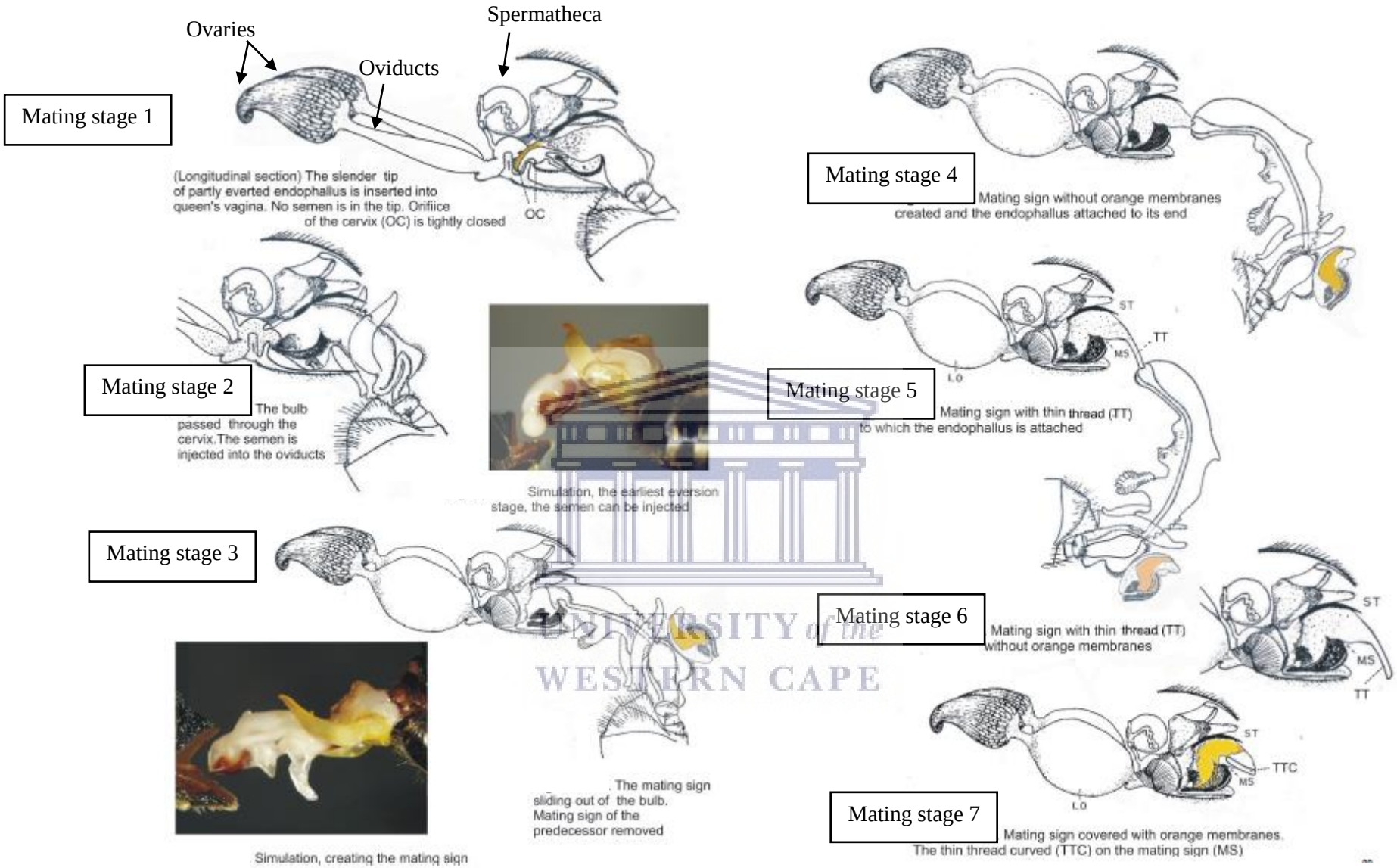


Figure 2.8 Seven stages of mating in the honey bee [Adapted from Woyke (2016)]

Figure 2.9 Overview of the drone and queen mating in flight [Adopted from Koeniger, et al. (2014)]

The drone determines the end of the mating flight, when failing to remove the mating sign left in the queen, by the previous drone, and the queen then will return to her colony for the onset of oviposition (egg-laying) (Woyke, 2016).

At the end of such a mating flight, the queen temporarily stores approximately 200 million sperm in her lateral oviducts, where sperm are pooled and mixed with the aid of abdominal contractions (Brutscher et al., 2019). Only a small fraction, 3-5 % of pooled sperm (approximately 4-6 million) is then transferred to the spermatheca for long-term storage, until egg-laying occurs (Ruttner, 1956; Baer, 2005). The number of sperm deposited into the spermatheca is suggestive of whether or not the queen should partake in another mating flight or is ready to initiate egg lying (Ruttner, 1956).

Drone reproductive success mainly depends on sperm quality, and furthermore on the number of eggs that they can fertilize (Ciereszko, et al., 2017). Their ability to do so is often restricted by the availability of a female mating partner as well as their reproductive health and development; this includes sperm production and quality, which is often adversely affected by environmental factors, impacting the transmission of sperm to the reproductive organs of the queen (Hayashi & Satoh, 2019). Compromised sperm quality in drones therefore often results in a low fertilization success rate. Poor

sperm quality and the ill reproductive performance of honey bee drones can further compromise queen reproductive health and colony fitness, and growth, in general (Rangel & Fisher, 2019).

2.5 Hone bee drone sperm structure

General and ultrastructure

Honey bee sperm are filamentous cells with an indistinctive head and a gradually thinning tail end (Phillips, 1970; Peng, et al., 1993; Line-Neto, et al., 2000). The structural components, in general, are comparable to those of other insect species. A typical insect germ cell consists of the acrosome, nucleus, transitional centriole adjunct, and a flagellum (Lino-Neto, et al., 2000).

Figure 2.10 illustrates the general structural components of a typical insect sperm. Like insect sperm structures in general, the sperm of honey bee drones are extremely elongated with a total sperm length of 250 - 270 µm (Phillips, 1970).

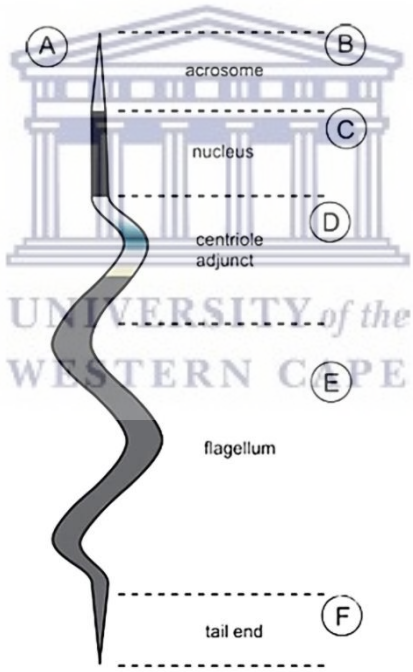


Figure 2.10 Schematic illustration of the structural organization of pterygote insect sperm and its ultrastructure. (A) A filiform insect spermatozoon (B) Acrosome (C) Nucleus (D) Centriole adjunct (E) Flagellum (F) Tail [Adapted from Werner, et al. (2008)]

Sperm head region

The honey bee drone sperm head region is relatively small, with a length of 10-12 µm, and are indistinguishable from the tail given their narrow width of 0.5 µm (Peng et al., 1993). The

asymmetrical head region contains firstly, the acrosomal complex followed by an electron-dense nucleus, which flattens anteriorly (Lensky, et al., 1979). The linear nucleus (5 µm in length) houses the densely-packed chromatin, lying posterior to the acrosomal complex (Peng, et al., 1993; Lino-Neto, et al., 2000).

The acrosomal complex is a tri-layered structure (Peng et al., 1993), which is formed by an outer plasma membrane and double-layered acrosomal vesicle underneath, which covers the perforatorium up until where it inserts into a deep fossa at the anterior nuclear end (Figure 2.11). At its posterior end, the acrosomal complex enlarges to form an insertion peg that fits into the nuclear fossa and here the acrosomal complex joins the nucleus (Peng, et al., 1993; Line-Neto, et al., 2000).

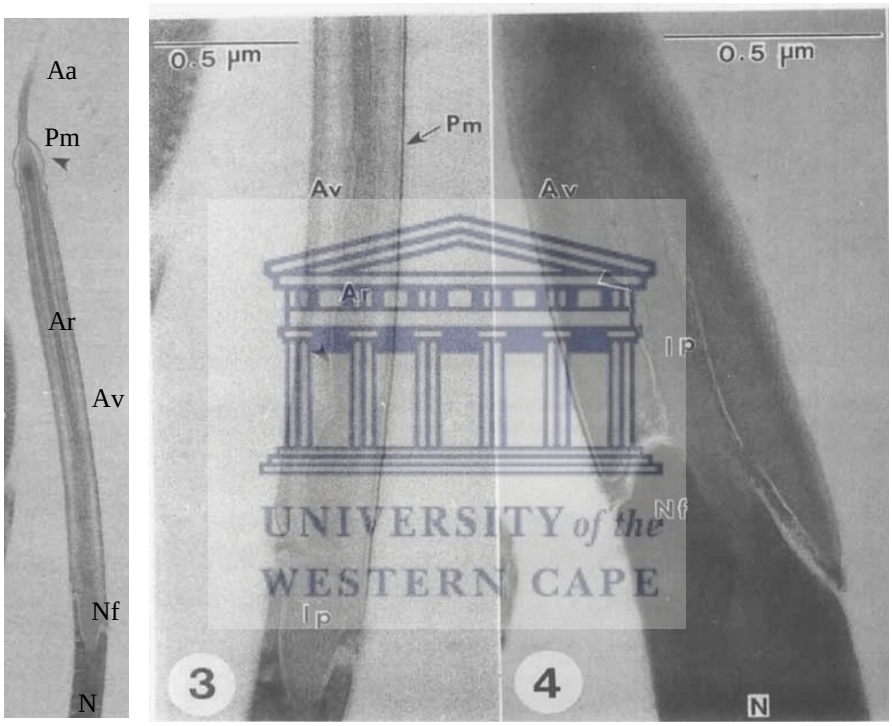


Figure 2.11 Longitudinal sections of the honey bee sperm, demonstrating the anterior head region and how the acrosomal complex insert into the nuclear fossa [Adapted from Peng, et al. (1993) & Lino-Netto, et al. (2000)] Aa, acicular apex; Pm, plasma membrane; Ar, acrosomal rod; Av, acrosomal vesicle; Nf, nuclear fossa; N,nucleus

Zone of overlap (neck region)

Contrary to mammalian sperm, insect sperm lacks a midpiece and is instead equipped with a zone of overlap (neck region), where the transition between the nuclear and tail region takes place (Peng, et al., 1993). The nucleus-flagellum transitional region is a complex area, with many uncertainties of its structural organization (Peng, et al., 1993).

In this region, the cone-like elongation of the nuclear pole (found in the head) is seen lying posteriorly next to the axoneme and mitochondrial derivatives of the tail ((Lensky, et al., 1979). At this intersection, the nucleus is enclosed by a funnel-shaped, membranous structure, which further contributes to the development of the axoneme (Peng, et al., 1993). Lensky, et al. (1979) further illustrated that the axoneme contains 9 outer fibers, 9 double tubules, and 9 accessory tubules, arranged according to the 9+9+2 pattern, with an electron-dense rod in each of the central and outer fibers. The axoneme additionally contains two triangular rods, positioned towards each of the mitochondrial structures, which both lie parallel to the axoneme. However, in the honey bee, it should be noted that in this transitional region there is firstly only one mitochondrial derivative that appears in the transitional region, partially attached to the anterior portion of the nuclear cone (Lensky, et al., 1979). This belief could be due to the one mitochondrial derivative originating more anteriorly than the second derivative (Peng, et al., 1993).

Tail region (Flagellum)

The flagellum, attached to the nuclear pole, consists of an axoneme arranged in a 9 + 9 + 2 microtubular pattern and a large pair of asymmetrical mitochondrial derivatives which lies parallel to the axoneme along with two accessory bodies. When observed in a cross-section, a large, oval, and smaller, round mitochondrial derivative can be identified respectively, with paracrystalline material found only in the large derivative. These structures differ in length, hence the tapered tail end (Lensky, et al., 1979; Peng, et al., 1993; Lino-Neto, et al., 2000). Additionally, Lensky, et al. (1979), illustrated that the two mitochondrial derivatives separate from the axoneme anteriorly, the reason remains unknown.

2.6 Honey bee semen quality: an overview

Honey bee drone semen consists of seminal fluid and sperm, of which the former has been associated with post-copulation changes in the queen and subsequent effects on the colony (Brutscher, et al., 2019). Seminal fluid is derived from the drones' accessory glands and secreted during ejaculation (King, et al., 2011). It is generally made up of a complex mixture of proteases, peptides, lipids, sugars, antioxidants, and antimicrobials, which all induce biological changes after copulation, and plays a crucial role in male fertility and the successful fertilization of a female egg (Brutscher, et al., 2019).

The post-copulation physiological and behavioural changes in the queen, owed to the seminal fluid contents, involve the onset of ovary development and ovulation (Brutscher, et al., 2019). Seminal fluid also has an impact on the queen's transcriptional regulation as well as pheromone modulation,

which is vital for in-hive communication between colony members, worker bee functionality, as well as egg-laying. The influence of seminal fluid contents on reproduction has been widely discovered in other species such as *Drosophila*; however, the impact thereof on the honey bee queen remains vague. Additionally, the high protein content in seminal fluid maintains sperm longevity and quality in the spermatheca, especially during long-term storage, by contributing to drone sperm structural integrity (King et al., 2011).

Semen also contains sperm and honey bee drones produce sperm only once in their lifetime, which is then stored until successful mating with a queen (Withrow, et al., 2018; Lago, et al., 2020). Sperm are then further expected to last for several years in the spermatheca of the queen following mating (Collins, et al., 2006). The successful insemination of the honey bee queen partially relies on the quality of sperm cells (Locke & Peng, 1993; Brutscher, et al., 2019). Yet, successful queen insemination does not guarantee productivity; in fact, several additional elements, such as sperm motility and viability, contribute to sperm quality (Yániz, et al., 2020). Retaining semen quality, particularly sperm vitality and motility, is important to ensure reproductive success, especially in artificial insemination programs (Collins, 2000, 2004; Cobey et al., 2013).

2.6.1 Indicators of honey bee semen quality

To date, the main indicators or predictors of semen quality in honey bee sperm reported on, involved mainly semen volume, sperm concentration, and sperm viability (Yániz, et al., 2020). Sperm motility of honey bee sperm, although to a lesser extent, has also been acknowledged as an indicator of semen quality more recently (Wegener, et al., 2012; Yániz, et al., 2019, 2020; Inouri-Iskounen, et al., 2020).

Semen volume and sperm concentration

Semen volume and sperm concentration, relate to queen fertility and semen quality as these are contributing factors of sperm reservoirs in the queen's spermatheca, where sperm are stored prior to fertilization (Schlüns, et al., 2004; Yániz, et al., 2020). Sperm concentration produced per drone is especially important in artificial reproduction as it relates to the amount of sperm that ultimately reaches the spermatheca of the queen (Yániz, et al., 2020). Furthermore, the depletion of sperm stores may result in the premature loss of a queen due to her inability to lay fertilized eggs (Delaney, et al., 2011; Tarpy, et al., 2012; Tarpy, et al., 2014).

The semen volume and sperm concentration in honey bee drones may vary according to body size, age, genetics, and seasonal changes (Rhodes, et al., 2011; Rousseau, et al., 2015). Variations may furthermore be owed to the type of subspecies. For instance, the Cape honey bee produces a smaller

semen volume than those honey bees of European decent, while the sperm concentration between these subspecies remains similar (Buys, 1990; Yániz, et al., 2020). However, the contribution of sperm and seminal fluid to ejaculate quality remains largely unknown and therefore requires further investigation.

Sperm motility and kinematics

Sperm functional characteristics such as motility, sperm swimming patterns, and movement characteristics, play an important role as it indicates the sperm's ability to migrate in the female reproductive tract, a process which requires long-distance traveling through the female reproductive tract, (Holt and van Look, 2004; Yániz, et al., 2020). Sperm should be able to firstly migrate from the drone endophallus into the spermatheca, and subsequently to the site of fertilization (Yániz, et al., 2020).

Honey bee sperm migration in the queen reproductive tract is an intricate practice, which involves a vacuum pump effect through queen abdominal muscular contractions and sperm movement (Rinderer, 1986; Meixner, et al., 1989). During copulation, sperm are placed in the lateral oviducts of the queen where it remains for 24 hours until sperm migrates to the place of storage, the spermatheca, where it is stored for up to 5 years, during which migration intermittently occurs to fertilize an egg (Rinderer, 1986). The preservation of sperm motility is therefore of utmost importance, especially in honey bees, since a low population of motile sperm can prevent migration in the female genital tract, an underlying cause of male infertility (Mortimer, 1994; Yaniz, et al., 2020).

Drone sperm motility is generally associated with different swimming patterns, presenting with more than one type of motility pattern (Hopkins & Herr, 2010; Ciereszko, et al., 2017; Yániz, et al., 2020). Commonly described swimming patterns include amoeboid, circular, synchronous, and progressively forward snake-like swimming sperm (Bishop, 1920; Rothschild, 1955; Borsuk, et al., 2011; Wegener, et al., 2012; Tofilski, et al., 2014; Tofilski, et al., 2018; Yániz, et al., 2019) (Figure 2.12).

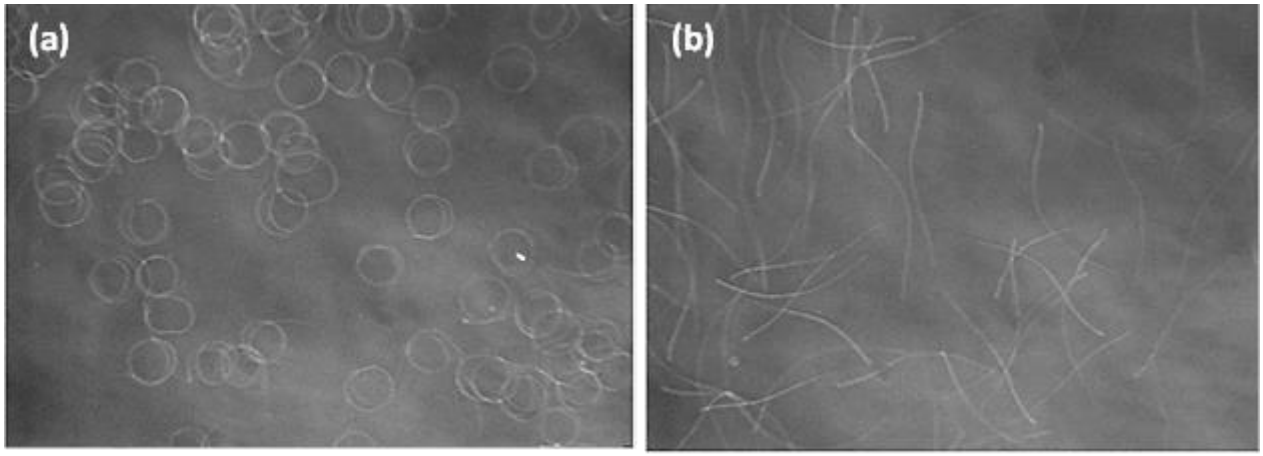


Figure 2.12 Micrographs of honey bee drone sperm presenting with (a) circular swimming sperm and (b) snake-like progressively forward swimming sperm [Adapted from Al-Lawati, et al. (2009)]

It has previously been believed that stored honey bee sperm are static in the spermatheca (Tofilski, et al., 2018). However, Tofilski et al., (2018) have demonstrated that there are groups of circular moving sperm inside the spermatheca of a freshly inseminated and naturally mated queen (Figure 2.13). Such circular groups of sperm in the spermatheca were located close to the spermathecal wall and thus closer to the spermathecal duct for easy release and ultimately fertilization. It is suggested that these circular whirls of sperm appear to be the stronger sperm, of better quality, compared to the suspected weaker sperm in the centre of the spermatheca (Tofilski et al., 2018).

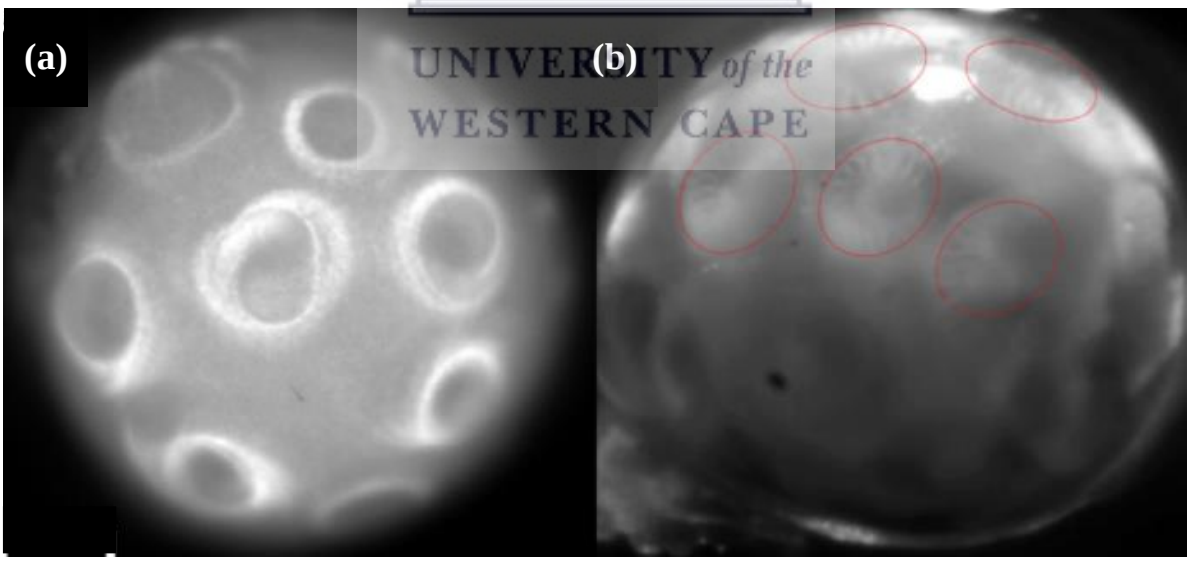


Figure 2.13 Groups of circular swimming sperm inside the spermatheca of (a) an instrumentally and (b) naturally inseminated honey bee queen [Adapted from Tofilski, et al. (2018)]

The above phenomenon, of sperm swimming together in synchronized groups, appears to be a sperm swimming trait for insects (Pearcy, et al., 2014; Tofilski, et al., 2018). Pearcy, et al. (2014) reported on a similar trait in ant sperm, referring to this type of behaviour as team swimming. In addition, it is suspected that these sperm-sperm interactions are instigated as a result of peculiar female mating behaviour, such as polyandry, which initiates sperm competition (Pearcy, et al., 2014). It was further established that team swimming in ant sperm increased sperm velocity and further allowed for increased sperm storage in the queen's spermatheca (Pearcy, et al., 2014).

Swimming patterns are suspected to interchange between a double-helical swimming pattern and a linear, snake-like trajectory once they encounter the narrowing ducts of the female genital tract (Werner, et al., 2008). Furthermore, during storage in the spermatheca, sperm also have shown to interchange between swimming patterns. As a result of sperm storage time, Al-Lawati, et al. (2009) determined that drone sperm stored in the spermatheca changes from a circular to straightened swimming pattern as a result of long-term storage periods, of approximately 2 years. The change of swimming patterns in the spermatheca can also be ascribed to the ratio of spermathecal fluid vs. sperm concentration that increases as a result of sperm used for fertilization, and thus can result in more diluted sperm, which also affects its swimming patterns (Halak, et al., 2020)

Several reasons have been suggested for the circular sperm movement in insect species, for example, that it could be due to the sperm structure, specifically sperm with abnormally long tails, and a behavioural characteristic to prevent entanglement during storage. It is furthermore believed to be a mechanism used to release individual sperm from its place of storage when ready for fertilization (Al-Lawati, et al., 2009),

Honey bee sperm also seems to uncoil itself from a coiled or circular swimming pattern (Figure 2.14), hence the different or interchangeable swimming patterns observed (Carreck et al., 2013; Tofilski, et al., 2018; Yániz, et al., 2020).

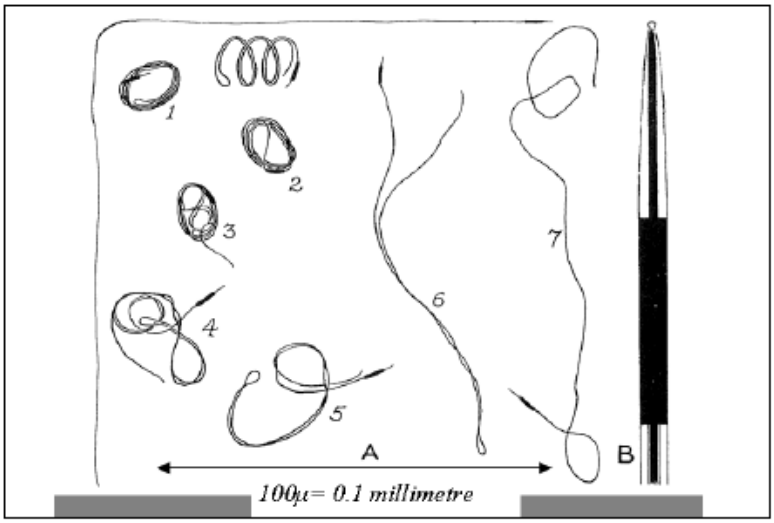


Figure 2.14 Depiction of the interchangeable honey bee swimming patterns [Adapted from Carreck, et al. (2015)]

Sperm swimming patterns and movement behaviour are often used as indicators of drone sperm motility quality. For example, the presence of synchronous, circular groups of drone sperm is associated with sperm of good quality which is most likely the ones to successfully fertilize an egg (Tofilski, et al., 2018; Yániz, et al., 2020). Circular swimming sperm is also believed to be associated with increased motility (Al-Lawati, et al., 2009; Yániz, et al., 2020).

There is furthermore a positive relationship between the quality of drone sperm motility and the reproductive performance of honey bee queens, including both total percentage motility and movement characteristics (kinematic parameters) such as sperm velocity (Wegener, et al., 2012).

When determining sperm motility quality, it is important to take kinematic parameters into account, as it indicates the quality of sperm functionality. Kinematic parameters are movement characteristics that provide a detailed description of the motility potential of the sperm cell; this includes information on sperm swimming speed, progressivity, head displacement, and tail vigor (David & Serres, 1981). In mammals, it is widely used to describe the sperm's migration potential through the female reproductive tract, given that parameters such as those indicative of velocity, are indicative of sperm movement and speed (Collins, et al., 2004; Robayo, et al., 2008). In humans, this kinematics furthermore describes the ability of sperm to penetrate through the cervical mucus, and since the velocity parameters are indicative of sperm movement it can be associated with sperm migration in the female genital tract (Collins, 2004). As in most species, honey bee sperm also require motility to

migrate through the queen's reproductive tract after copulation to first reach the lateral oviducts, and finally to reach the spermatheca after a period of time. During storage sperm also need to reach the eggs for fertilization via the spermathecal duct. The kinematics of sperm motility can therefore provide useful information regarding the ability of sperm to for example migrate in time to reach the spermathecal duct, before being expelled, and the ability to reach and penetrate the egg for fertilization. Determining the kinematic parameters when assessing honey bee semen quality for artificial insemination, is therefore important, as it provides a detailed report on the motility quality and fertilization potential of individual sperm cells and furthermore provides a good indication of reproductive success.

Sperm kinematic parameters, as determined by CASA, reported thus far, includes mainly velocity parameters, curvilinear velocity (VCL), which describes sperm speed by measuring the distance travelled from one point to another over time; average path velocity (VAP) which measures average velocity by tracking the roaming sperm head; and straight-line velocity (VSL), this parameter measures velocity using the starting point on the average path and the final destination reached; other kinematic parameters are percentage linearity (LIN), describing the curvature of the roaming sperm swimming pattern, calculated using a VSL/VAP ratio, amplitude of lateral movement of the head (ALH) which accurately describes the sperm head movement and behaviour; and beat cross frequency (BCF) which provides information on the sperm tail vigour (Liberti, et al., 2018; Inouri-Iskounen, et al., 2020).

Inouri-Iskounen, et al. (2020) recently assessed sperm motility and kinematic parameters in honey bee sperm using CASA, to measure the impact of *in vitro* imidacloprid exposure, a neonicotinoid insecticide used for agricultural purposes. The assessment of sperm kinematics has also been performed on ants that belong to the same family (Hymenoptera: Apidae) as honey bees, to measure the effect of seminal- queen reproductive tract fluid on sperm motility (Liberti, et al., 2018).

Although sperm motility and kinematics are widely-used parameters to determine the quality of sperm in mammals (Yániz, et al., 2018), it has only been assessed sporadically in honey bees (Yániz, et al., 2019). It is also important to note that sperm function, such as motility, is affected by sperm structure, and subsequently sperm quality (Maree, 2011), relationships which are still poorly understood in honey bee sperm. Motility, in general, is associated with other sperm parameters, including morphology and vitality (Abu, et al., 2012).

Sperm vitality

In honey bees, queen reproductive health and fertilization success mainly depend on viable sperm (the number of live cells) (Johnson, et al., 2015), since the premature death of sperm cells stored in the queen's spermatheca, will directly affect queen fertility and survival (Collins, et al., 2004). Drone sperm vitality further indicates colony performance, given that poor queen quality relates to low sperm vitality (Pettis, et al., 2016). In addition, the queen uses the same sperm reservoir for a prolonged period of approximately five years (Rinderer, 1986), hence the importance of honey bee sperm vitality (den Boer, et al., 2009). Honey bee semen samples generally present with an extremely high percentage of viable sperm cells, often exceeding 80% vitality with a range of 55 % to as high as 99 %, reported by various authors over the years (Collins, 2004; Yániz, et al., 2020).

2.7 Honey bee semen quality analysis

Comprehensive semen quality analysis requires the assessment of a wide spectrum of sperm characteristics as both structural and functional factors form part of sperm quality. For example, poor sperm motility, along with low sperm concentration and abnormal morphological characteristics may hamper sperm survival and functionality in general, finally resulting in low fertilization rates (Larson-Cook et al. 2003; Ciereszko et al. 2017).

2.7.1 Standard semen characteristic analysis

Semen volume and sperm concentration

Semen quality analysis of honey bee drones generally entails the measurement of semen volume and sperm concentration (Collins & Pettis 2001; Lodesani et al. 2004; Czekońska et al. 2013; Rousseau et al. 2015; Ciereszko et al. 2017; Yániz, et al., 2019, 2020). Honey bee semen volume is extremely small, and therefore difficult to measure. To accurately determine drone semen volume a precision syringe, (Gilmont micrometer syringe (Rhodes, et al., 2011) and Harbo Large Capacity Syringe (Rousseau, et al., 2015) or a metered capillary tube is required (Yániz, et al., 2020). The majority of honey bee drone species, on average, yield an ejaculate volume of 1 µL. However, between different species, these volumes range from 0.1 - 2.4 µL per ejaculate (Yániz, et al., 2020). *Apis mellifera capensis* drones produce smaller semen volumes compared to European species, with semen volumes varying between 1.01 - 1.29 µL per ejaculate (Buys, 1990).

Semen concentration is commonly determined manually, using a hemocytometer and a counting chamber (Collins & Pettis, 2001; Rousseau, et al., 2015). Ciereszko, et al. (2020) for the first time,

applied spectrophotometry to establish sperm concentration, using a 600nm wavelength, determining the sperm number in a sample through the amount of light absorbance.

Modern techniques such as automated image analysis have not yet been used for standard semen analysis in honey bees as the development of such a technique can be very taxing in terms of assessment time and accuracy (Delaney, et al., 2011).

Honey bee sperm concentrations reported are inconsistent amongst subspecies ranging between 2 to 9 million sperm per µL (Yániz, et al., 2020). Highly variant sperm concentrations can be a result of the techniques applied and the source of sperm used to determine sperm concentration. For example, some studies determine sperm count per ejaculate (Woyke, 2008; Rhodes, et al., 2011; Nur, et al., 2012; Rousseau, et al., 2015; Kairo, et al., 2017), while others determine sperm count from reproductive organs such as the seminal vesicles (Duay, et al., 2002; Schlüns, et al., 2003) and spermatheca of the queen (Collins, 2005). In addition, factors such as body weight, age, season, and genetics may also impact sperm concentrations, resulting in major discrepancies (Schlüns, et al., 2003; Rousseau, et al. 2015).

2.7.2 Sperm structural characteristics analysis

Morphology: General and ultrastructural characteristics

Given the uniform structural nature of honey bee drone sperm, staining is required to distinguish between the sperm components and to measure the length and width of each. Stains typically used to identify nuclear regions include a fluorescence-based assay, such as Hoechst or SYBR14 (Yániz, et al., 2020), a silver nitrate ($AgNo_3$) stain, or an eosin and gentian violet stain (Gontarz, et al., 2016) (Figure 2.15).

Both the nigrosin/eosin and $AgNo_3$ stains can be used to distinguish between the acrosomal and nuclear regions, as well as the flagellum. $AgNo_3$ is an alkaline-based stain that has an affinity for acidic chromatic proteins, and are thus able to further differentiate between sperm head components, by darkening the acrosomal complex from the lighter stained distal part of the sperm head, providing a more detailed morphological structure than the acid-based nigrosin/eosin stain (Gontarz, et al., 2016).

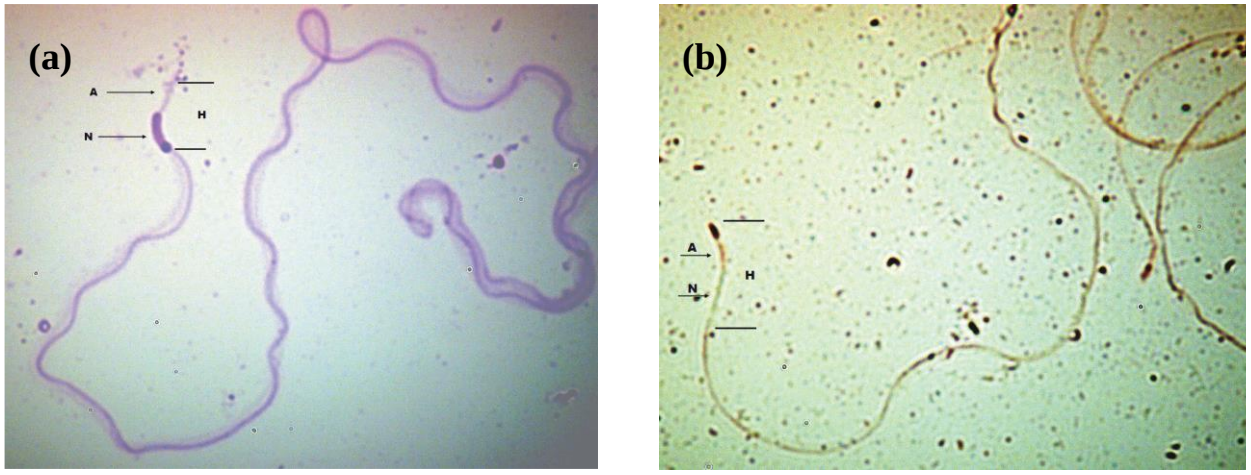


Figure 2.15 Honey bee drone sperm cells stained with (a) a nigrosin/eosin stain and (b) a silver nitrate ($AgNo_3$) [Adapted from Gontarz, et al. (2016)] A, acrosomal region; N, nucleus; H, head

In addition, BrightVit, an eosin-nigrosin stain developed initially for vitality analysis, has also been used to assess sperm structure in many species (Björndahl, et al., 2003). This staining technique is also recommended by Gontarz, et al. (2016) for honey bee sperm, however, it only differentiates between the sperm head and tail, and occasionally the mitochondrial structure in dead sperm (Figure 2.16) (Murray, 2019 unpublished), while silver nitrate provides further detail by distinguishing between the nucleus and acrosomal complex (Figure 2.16 (b)).

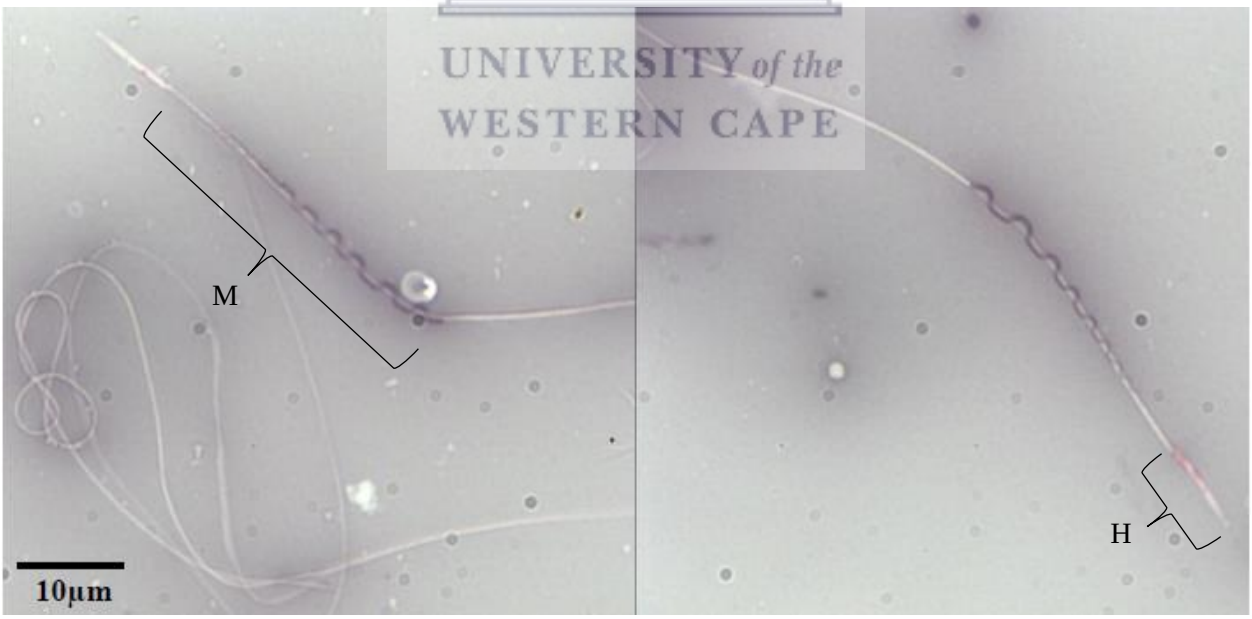


Figure 2.16 Non-viable honey bee sperm stained with BrightVit illustrating the sperm head and mitochondria [Adapted from Murray (2019)] H, sperm head; M, mitochondria

In addition to the above staining techniques used, transmission electron microscopy has also been widely used in the honey bee to assess the sperm ultrastructure (Peng, et al., 1993; Locke & Peng, 1993; Lino-Neto, et al, 2002). This allowed for the detailed identification of sperm structural components such as the mitochondrial derivates, acrosomal complex, and the nuclear region, and provides clarity on the transitional region located between the sperm head and tail (Lino-Neto, et al., 2000).

Despite the discoveries made regarding sperm components, structural significance remains vague as morphological abnormalities in honey bee drone sperm are seldom assessed (Yániz, et al., 2020). Evaluating sperm morphology is a vital test for sperm quality since it correlates with functional parameters, especially motility (Maree, 2011), and although honey bee sperm structure has been previously described, a relationship between sperm structure and functionality was not yet described (Yániz, et al., 2020).

Vitality

Sperm cell viability or vitality is based on the sperm cell's plasma membrane intactness (Yániz, et al., 2013). Vitality refers to the percentage of live sperm in a sample, including those that are immotile. It is therefore important to differentiate between cells that are non-viable and immotile, as sperm can be static yet viable (Prag, 2017). Furthermore, the percentage of viable sperm should always exceed the percentage of motile sperm in a sample.

In honey bee sperm, vitality analysis generally involves the use of fluorescence microscopy and flourochromes to distinguish between live and dead sperm (Yániz, et al., 2020). Common flourochrome stains used to analyse honey bee sperm vitality is SYBR14, Hoechst 33342, and acridine orange, which are often used in combination with the counterstain, propidium iodide (PI) (Yániz, et al., 2020). These stains target possess the ability to penetrate intact cell membranes, and particularly target the sperm head region. Live cells with intact membranes (viable sperm), stain as follows with differential stains used: green with DNA-targeted stains such as SYBR14 (Nur, et al., 2012) and acridine orange and blue with Hoechst 33342 (Yániz, et al., 2020) (Figure 2.17). The counterstain, Propidium iodide, easily penetrates the compromised membranes of non-viable cells, staining them red.

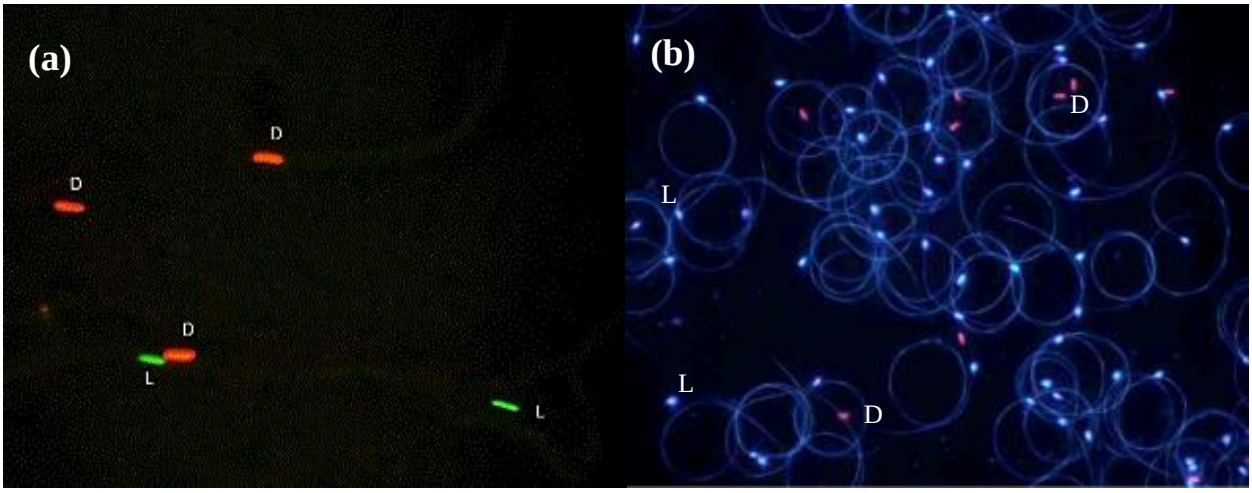


Figure 2.17 Honey bee sperm nuclei stained with flourochromes used for live/dead analysis. (a) SYBR14 (green = live) and PI (red = dead), (b) Hoescht (blue) and PI (red = dead) [Adapted from Nur, et al. (2012) and Straub, et al. (2016)] D, dead; L, live

As mentioned earlier, BrightVit has mostly been applied for vitality assessment in other animal species given that the acidic stain penetrates the compromised membranes of dead sperm, staining it purple while live sperm remain white. This stain can also be used to distinguish between live and dead honey bee sperm (Murray, 2019 unpublished); however, fluorescence microscopy in combination with manual sperm counting is the most common method, routinely used to determine honey bee sperm vitality (Yániz, et al., 2020).

Alternatively, a less popular technique used on honey bee drone sperm includes a hypo-osmotic swelling test using fructose and Sodium-citrate hypo-osmotic solutions with different osmolarities (50, 100, and 150 mOsm/kg) to determine plasma membrane integrity by looking at whether the sperm tail is coiled or not (Nur, et al., 2012) (Figure 2.18). Image analysis and flow cytometry have also been implemented recently, however, due to the discrepancies associated with these techniques, it is yet to be applied for routine quality assessment (Yániz, et al., 2019).

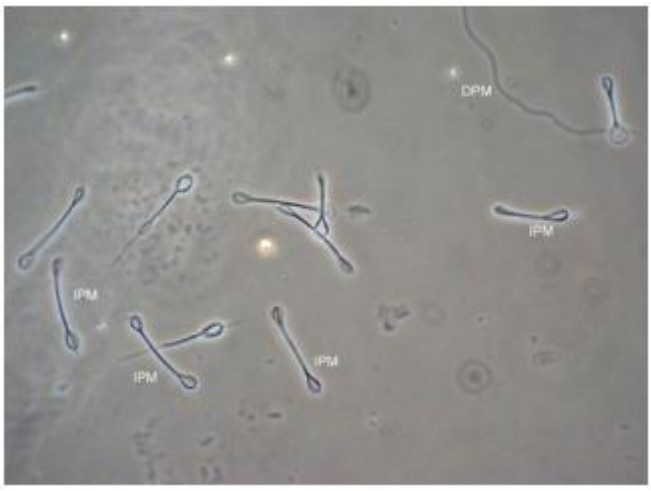


Figure 2.18 Hypo-osmotic testing used to determine honey bee sperm vitality [Adopted from Gontarz, et al. (2016)] IMP, Intact plasma membrane; DMP, damaged plasma membrane

Given the generally high vitality percentage (as high as 99%) of honey bee drone sperm, vitality assessment is rarely necessary. However, it remains an important indicator of sperm quality, especially when evaluating the impact of environmental factors such as seasonal changes, age, storage, disease, and pesticide exposure (Rangel & Fisher, 2019). In addition, vitality is often also measured during semen processing for artificial insemination purposes, especially when peculiar techniques such as centrifugation or the manual mixing of semen is applied in such assistive breeding programs (Shafir, et al., 2009; Wegener, et al., 2014).

2.7.3 Sperm functional characteristics analysis

Motility and kinematics

Sperm motility is the most commonly used parameter when establishing sperm quality, in mammals (Yániz, et al., 2018). The assessment of sperm motility quality in the honey bee widely involves an observational analysis, such as a motility grade score (Locke & Peng, 1993).

In most studies where honey bee sperm motility is determined the most common method used is phase-contrast microscopy (Inouri-Iskounen, et al., 2020) or manual methods to determine motility (Al-Lawati, et al., 2009), and in some cases, motility and swimming patterns are mainly observational of nature (Halak, et al., 2020; Yániz, et al., 2020) by using swimming patterns to determine motility (Locke & Peng, 1993). Motility grade scores, based on swimming patterns, are often used to classify motility (Verma 1978, Locke & Peng, 1993, Yániz, et al., 2020, Inouri-Iskounen, et al., 2020).

Locke & Peng (1993) established a motility grade score classification system, according to which they determined the motility quality of a drone sperm sample. Motility is classified as follows: a sample presenting with more than 50% progressive and circular moving sperm were assigned the highest-grade score, which is a 4; samples with less than 50% circular and progressively forward-moving sperm were classified as a 3; a 2 was assigned when samples were non-progressive, yet vibrating (>50%); a 1, when less than 50% vibrating and lastly a 0 were assigned to entirely static samples. Recently, the assessment of drone sperm motility quality slightly improved, involving the subjective estimation of percentage motile sperm and the expression of total percentage motility using image analysis (Wegener, et al., 2012, 2014; Ciereszko, et al., 2017; Alçay, et al., 2019; Yániz, et al., 2019,

The use of modern or advanced techniques, such as CASA, to assess motility and kinematic parameters, is widely used in other animal species, because (Maree & Van der Horst, 2013; Yániz, et al., 2018). Motility and kinematic parameters collectively provide a detailed description of sperm swimming behaviour, (Yániz, et al., 2018).

Unfortunately, the use of this technique has not been widely applied in the honey bee given the complex structural nature of its sperm and the sperm circular swimming pattern (Yániz, et al., 2020). Although attempts have been made to use CASA systems to determine sperm motility and kinematics in honey bees (Inouri-Iskounen, et al., 2020), results seem inaccurate as it is very difficult, and nearly impossible, to distinguish between the head and tail of honey bee sperm if only using phase-contrast microscopy. Yániz, et al. (2020) suggested that honey bee sperm should rather be stained using a Fluorescence-based assay to distinguish between sperm components, i.e. the sperm head and tail. This is indeed important as CASA determines a steady reference point for tracking, such as the sperm head (Mortimer, 2000; Lu, et al., 2014), and with fluorescence staining it will allow CASA to detect and track the head (Delaney, et al., 2011; Yániz, et al., 2020).

A detailed sperm functionality analysis using CASA includes the assessment of motility characteristics, providing motility mean values of the entire sperm population (rapid, medium, and slow swimming sperm) as well as sperm subpopulations (rapid-, medium- and non-progressive sperm) (Figure 2.19). Mean motility values provide an oversimplified report on the sperm motility of heterogeneous semen samples, overlooking the true impact of external factors such as drug treatments, therefore, looking at sperm subpopulations is crucial, since it optimizes the sensitivity of semen analysis (Abaigar, et al., 1999; Martinez-Pastor, et al., 2005; Maree & van der Horst, 2013). However, reports on honey bee drone sperm are often very basic, excluding a detailed evaluation of motility and kinematic parameters which accurately describe sperm swimming behaviour and associated velocities (Locke & Peng, 1993; Inouri-Iskounen, et al., 2020; Yániz, et al., 2020).

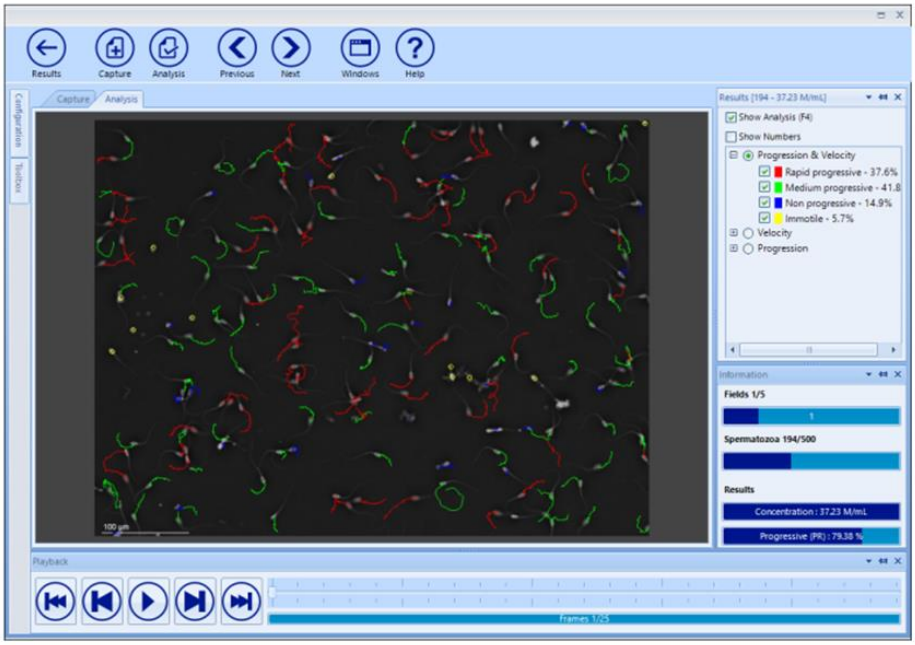


Figure 2.19 Human sperm motility assessment using CASA, SCA®, presenting CASA-generated sperm tracks and the classification of sperm motility parameters (green = medium progressive sperm; blue = non-progressive sperm; red = rapid progressive sperm) [Adopted from Microptic (2021)]

Furthermore, Table 2.1. provides the definitions or descriptions of sperm motility and kinematic parameters used in many animal species to determine sperm quality.

Table 2.1 Sperm motility and kinematic parameters defined

Parameters	Definition
Progressive motility (%)	Space gained of ≥5µm/s-1
Immotile (%)	No tail beating observed
VCL (µm/s)	Time-averaged velocity of a sperm head along its curvilinear path
VSL (µm/s)	Time-averaged velocity of a sperm head along the straight line between the first head/detection position and its last
VAP (µm/s)	Time-averaged velocity of a sperm head along its average path
STR (%)	Linearity of the average path (VSL/VAP)
LIN (%)	Linearity of the curvilinear path (VSL/VCL)
WOB (%)	A measure of fluctuation amid the actual path and the average path
BCF (Hz)	The average rate at which the curvilinear path crosses the average path
ALH (µm)	The magnitude of lateral displacement of a sperm head about its average path
DNC (µm^2/s)	Dance represents the two-dimensional surface area that a sperm traverses over in one second (VCL x ALH) (In SCA® 2 x ALH)

VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; STR, straightness; LIN, linearity; WOB, wobble; BCF, beat cross frequency; ALH, the amplitude of lateral head displacement; DNC, dance

Motion velocity values include VCL, VSL, and VAP, which are directly linked to sperm movement (Collins, et al., 2004). VCL entails the measurement of the average speed of a sperm head along its curvilinear path, the parameter with the highest value compared to others. VSL measures the distance from the initial sperm head point and its endpoint along a straight line. This parameter reflects the net space of progressive motility and will always have the lowest value amid the three velocity parameters. The third and last velocity parameter, VAP, refers to the average time of the sperm head along its average path. These three velocity ratios entail LIN expressed as VSL/VCL, STR expressed as VSL/VAP and WOB expressed as VAP/ VCL. Figure 2.20 gives a visual representation of these descriptions, also illustrating how it is calculated from an individual sperm track constructed by CASA.

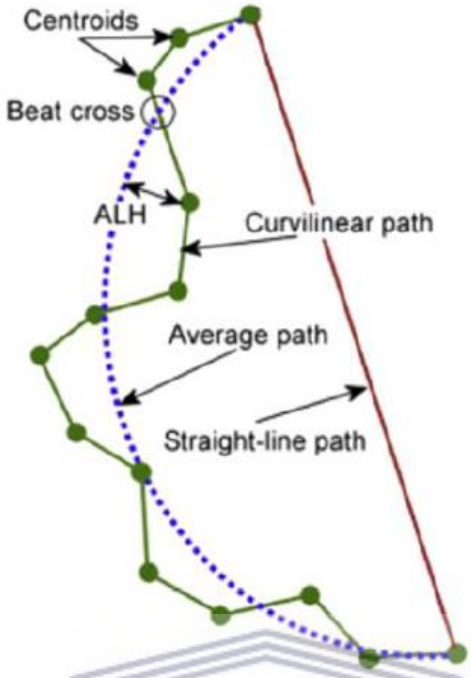


Figure 2.20 Schematic illustration of kinematic parameters determined by CASA during the assessment of sperm motion [Adopted from Krízková, et al. (2017)] ALH, the amplitude of lateral head displacement

Species-specific kinematic parameters are important and entail cut-off values, i.e. criteria to which sperm movement is analysed and compared (Mortimer, 2000). Cut-off values have not yet been established for honey bee sperm thus requires further research.

In addition, kinematic parameters can be used to determine sperm subpopulations, using sperm swimming speed (VCL) and linear progressiveness, resulting in the entire sperm population being divided into three subpopulations namely rapid-, medium- and non-progressive, while entire sperm populations are solely determined by swimming speed (VCL) (Valverde et al. 2019). Accordingly, many different qualitative permutations can be used to determine the quality of a sperm sample (Collins et al. 2004).

The establishment of parameter reference values to define semen quality, e.g. high and low viability, sperm concentration, sperm volume, sperm motility, and kinematics are currently lacking for honey bee sperm and require further research.

2.8 Factors affecting semen quality assessment

To obtain accurate, repeatable, and reliable results when determining parameters of sperm quality, all factors that can influence aspects of sperm collection, handling, preparation, and techniques applied, should be considered.

Semen collection

Semen collection and storage are mostly done for artificial insemination purposes in honey bee breeding programs. Efficient collection and storage can further enhance such programs, and also increase the quality of artificial reproduction (Collins, 2000; Yániz, et al., 2020).

Techniques for honey bee semen collection often involve dissection, to collect semen from either the spermatheca of the honey bee queen or the seminal vesicles of the drone (Cobey, et al., 2013; Yániz, et al., 2020). Additionally, a manual ejaculation technique, which involves the application of pressure on the drone abdomen and thorax, is used and semen is collected from the tip of the endophallus following eversion (Collins, 2005). Furthermore, when collecting semen using the latter method, semen preparation for quality assessment starts from the collection point, the endophallus tip (Collins, 2005) to avoid contamination of semen, an important influence of semen quality in terms of sperm behaviour and fertility. Sterile sperm collection and storage methods are crucial, to preserve semen quality and sperm integrity (Yániz, et al., 2020).

Decreasing vitality has been observed in bacteria-contaminated samples (Locke & Peng, 1993). Contamination during semen collection and incubation can further produce unreliable results (Yániz, et al., 2020). Proper sperm collection and preparation prevents contamination and unbearable, peculiar conditions such as over-dilution and the use of harsh media which might influence sperm behaviour and its survival ability (Locke & Peng, 1993; Yániz, et al., 2019; Yániz, et al., 2020; Halak, et al., 2020).

Given the sensitivity of drone sperm, semen quality and sperm behaviour are easily influenced by factors such as physiological media, chamber depth, incubation temperature, and deposition time and require standardization to produce reliable results (Yániz et al., 2018; Yániz, et al., 2019).

Physiological media

Physiological media are used to dilute and preserve sperm for *in vitro* quality assessment. When assessing semen characteristics, it is important to use the most suitable physiological media that matches each species semen characteristics (Verstegen et al., 2002). Moritz (1984) suggested that selecting a suitable, physiological media for sperm dilution and quality assessment will especially be beneficial for sperm quality assessment to enhance and also ensure success in artificial breeding programs (Moritz, 1984).

Different types of physiological media are used for honey bee drone sperm analysis, including different modifications of Tris buffer (Verma, 1978; Moritz, 1984), Hyes solution (Ruttner, 1976; Moritz, 1984), Saline (Mazeed & Mohanny, 2010) and Kiev buffered solution, of which the latter being the most commonly used (Collins, 2005). Kiev buffer consist of 2.43% trisodium citrate-2-hydrate, 0.3% D (+) glucose monohydrate, 0.21% sodium bicarbonate ($NaHCO_3$), 0.3% sulphanilamide and potassium chloride (KCl) in distilled water at pH 8.8 (Collins & Donoghue, 1999; Gençer & Kahya, 2011). The pH value of physiological media used for honey bee sperm dilution typically ranges between 7.2 – 8.8, merely to simulate the alkaline environment of the drone's seminal plasma (Gençer & Kahya, 2011; Wegener, et al., 2014).

It should furthermore be noted that honey bee sperm functionality can easily be influenced by physiological media, and therefore, diluents should be carefully chosen (Yániz, et al., 2019). Kiev buffered solution is the most common media used when determining sperm functionality (Locke & Peng, 1993; Taylor, et al., 2009; Wegener, et al., 2012; Ciereszko, et al., 2017). Honey bee sperm assessed in the original Kiev solution as described by Ruttner (1976), presented with signs of osmotic stress due to the low osmolarity in this media, hence a modified Kiev buffered solution was introduced at a later stage (Collins, 2005). The osmolarity of the modified Kiev buffered solution (384 mOsmol/L) is close to that of both honey bee semen (467 mOsmol/L) and seminal fluid (325 mOsmol/L) (Collins, 2005), and are therefore recommended for use when assessing honey bee semen quality.

Dilution

Dilution of semen with physiological media can affect honey bee sperm swimming patterns. Halak, et al. (2020) illustrated the effect of dilution on honey bee sperm, this can subsequently affect sperm motility (Yániz, et al., 2019; Halak, et al., 2020). Increasing dilution factors visibly change the swimming patterns of honey bee sperm; this is evident in the studies of Tofilski, et al. (2018) and Halak, et al. (2020). The former study illustrated the presence of circular groups of sperm in the spermatheca of a freshly mated queen, containing undiluted sperm (Tofilski, et al., 2018), similar to the circular swimming sperm in the pure, undiluted semen sample illustrated by Halak, et al. (2020). The latter study furthermore showed how sperm patterns became linear after 50% and 60% dilution (Figure 2.21).

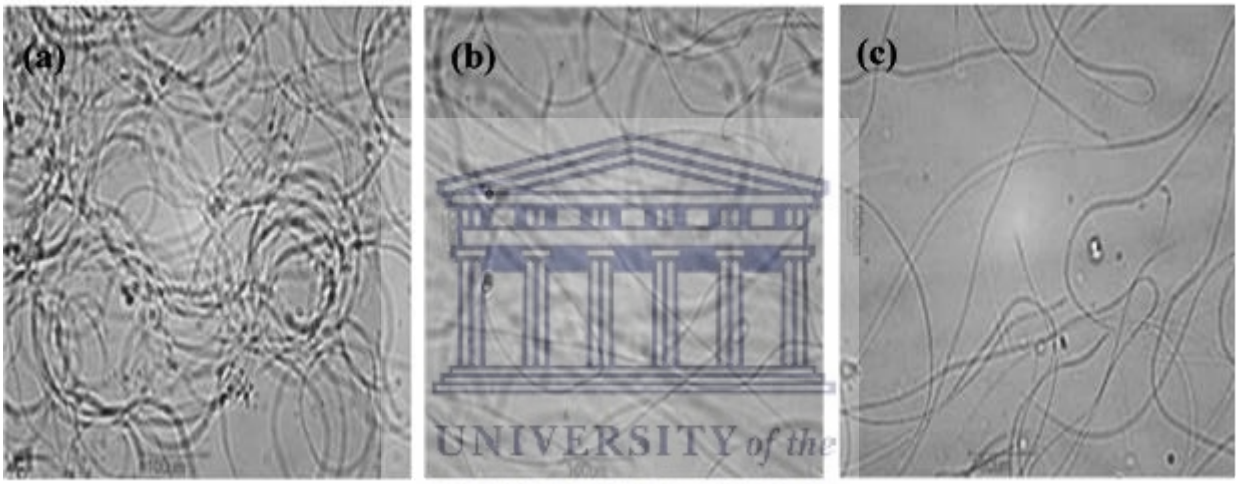


Figure 2.21 The effect of different saline dilutions on honey bee drone sperm. (a), undiluted semen; (b), 50% diluted semen; (c), 60% diluted semen [Adapted from Halak, et al. (2020)]

Since circular swimming sperm in honey bees is associated with good motility quality (Yániz, et al., 2020), over dilution, resulting in a linear sperm pattern, is thus likely to hamper sperm motility, and ultimately reduce fertilization potential. Studies investigating the effect of bull sperm storage on fertilization rates furthermore showed that diluted sperm presented with a reduced survival rate. In agreement with the above, Wilcox & Clark (1962) showed that the undiluted semen samples of chickens yielded higher fertilization rates compared to diluted semen. Therefore, given that sperm share similar behavioural traits across different species, careful consideration should be taken when deciding on a physiological media for honey bee semen analysis, to ensure that reliable results are obtained (Tofilski, et al., 2014; Halak, et al., 2019; Yániz, et al., 2019).

Chamber types and depths

The effect of different types of chambers and chamber depths on honey bee drone sperm behaviour has not been studied to the extent as in mammalian sperm (Yániz, et al., 2020). The depth and design of a chamber should be carefully considered, since it may influence sperm motility by restricting sperm displacement (Verstegen, et al., 2002).

The majority of previous studies investigating drone sperm motility often used slide coverslips (10 µL under a 22 x 22-mm coverslip) (Locke & Peng, 1993). However, Yániz, et al. (2019) has recently demonstrated that in comparison with mammalian sperm, honey bee drone sperm were more sensitive to chamber type and depth. The use of slide coverslips, (10 µL under a 22 x 22-mm coverslip), seemed to reduce the percentage of freely moving sperm. Furthermore, sperm analysed using a Leja 10 µm depth chamber, Leja 20 µm depth chamber as well as ISASD4C10 (10 µm deep), yielded a significantly higher percentage of motile sperm than glass cover slides and Makler chambers (10 µm deep) (Yániz, et al., 2019).

Sperm storage

Sperm storage following the prolonged storage periods of honey bee sperm in the reproductive organs of both drones (testes) and queen (spermatheca); it is expected of sperm quality to diminish as they age (Locke & Peng, 1993; Rhodes, et al., 2011; Stűrup, et al., 2013; Rousseau, et al., 2015). Decreased sperm quality with prolonged storage in drones or queens is owed to aging (Reinhardt, 2007), and the continued exposure to reactive oxygen species (Aitken & Krausz, 2001). Previous studies assessing the impact of semen storage in liquid and frozen states (Locke & Peng, 1993; Hopkins & Herr, 2010; Wegener, et al., 2012; Hopkins, et al., 2017), demonstrated that honey bee sperm can retain viability for prolonged periods *in vitro* (Collins, 2000).

Collins (2000), for example, stored semen in sealed capillary tubes for as long as six months and found that sample viability remained above 50%. Given that an average of three sperm cells is required for successful fertilization (Rubinsky, 2010), a sample presenting with 50% viability holds the potential to successfully fertilize numerous eggs. Collins (2000) reported that honey bee semen samples presenting with 45-65% vitality after 6 weeks of storage are still acceptable for fertilization.

Cryopreservation is a reversible process, used mainly to preserve genetic diversity, by ceasing sperm metabolism, this process has been used to preserve honey bee sperm, there are however harmful effects on sperm, such as crystallization and lipid peroxidation (Holt & Penfold, 2014). Alçay, et al.

(2019) investigated the effect of cryopreservation on honey bee semen characteristics and reported on decreasing motility measurements, reducing from a total motility percentage of 88.00 ± 2.73 % to 39.33 ± 3.71 %.

Literature further reports on the important role of drones in sperm preservation, suggesting that the contents of their seminal fluid contribute to the longevity of honey bee sperm (Collins, et al., 2006). Additionally, Gontarz, et al. (2016) also describe morphological defects which entail flipped, broken, double tails, and heads. However, these anomalies have only been observed after freezing in liquid nitrogen.

Incubation and deposition time

Vitality and motility parameters of honey bee sperm are prone to uncontrolled experimental conditions, such as incubation and deposition time therefore, it is important to standardize honey bee sperm quality assessment techniques, to ensure that reliable results are generated (Yániz, et al., 2018).

Yániz, et al. (2019) investigated the effect of incubation time, on drone sperm motility, and have shown the sensitive nature of honey bee sperm to incubation periods (Yániz, et al., 2019). Results obtained from the study of Yániz, et al. (2019) showed that sperm motility quality began to improve after 15 minutes. The percentage of motile and circular swimming sperm significantly increased after a 30-minute incubation temperature in the Leja10 chamber. In addition, it was further noted that circular swimming sperm were predominant in the samples when analysed after 60 minutes. In addition, incubation in the spermatheca over several hours was also proven to affect sperm motility and swimming patterns as demonstrated by Tofilski, et al. (2018) who reported on circular whirls of sperm following a 16-hour time-lapse in the spermatheca, speculating that densely packed sperm in the spermatheca arranges into whirls of sperm after a certain period in the queen. Previous studies also suggested that drone sperm requires incubation periods between 15 or 30 minutes, before semen analysis, to allow sperm to synchronize their swimming patterns (circular groups of sperm) and adapt to the peculiar surroundings (Locke & Peng, 1993; Wegener, et al., 2012; Ciereszko, et al., 2017).

2.9 Environmental factors affecting honey bee drone sperm quality

Spermatogenesis in honey bee drones is considered an extremely sensitive process, easily affected by internal and external environmental influences (Rhodes, et al., 2011; Brutscher, et al., 2019; Zhao, et al., 2021), and as spermatogenesis is completed in the drone pupae stage, sperm quality cannot be restored once compromised (Baer, 2005). Several internal and external environmental factors have

been shown to compromise sperm quality, for example, senescence, nutrition, colony infestations, and pesticide exposure are amongst the known factors contributing to poor sperm quality and ultimately colony losses (Czekońska, et al., 2013; Stűrup, et al., 2013; Brutscher, et al., 2019; Zhao, et al., 2021).

Drone age

Aging is an evident factor influencing both drone sperm production and maturation (Zhao, et al., 2021). The effect of drone age on sperm physiology is firstly visible during the sperm maturation process which occurs in drones aged between 6-14 days, during which semen composition changes (Zhao, et al., 2021).

This has been confirmed by numerous authors who noticed both morphological and physiological changes in the reproductive organs of aged honey bee drones (Bishop, 1920; Czekońska, et al., 2015). For example, the testes of aging drones decrease in size (Czekońska, et al., 2015), and semen volumes also decrease as drones age (Woyke and Jasinski, 1978). In addition, it has also been established that less sperm reaches the spermatheca of the queen when inseminated by older drones (Mazeed & Mohanny, 2010). The findings of Mazeed & Mohanny (2010) could be as a result of compositional changes in the ejaculates of older drones, given that as drones age the semen becomes darker of colour and presents with a higher viscosity (Hayashi & Satoh, 2019).

Furthermore, Locke & Peng (1993) has demonstrated that aging is associated with adverse effects on drone sperm vitality by demonstrating that four-week-old drones, and even older, had a significantly lower live sperm count when compared to younger, sexually mature drones ranging between two to three weeks old. These findings were furthermore substantiated by Stűrup, et al. (2013) who reported on a 50% decrease in sperm vitality of drones aged three weeks and older. On the contrary, Anderson (2004) and Rhodes, et al. (2011) found that drones of 35-days and older produced more sperm than younger drones aged between days 12 – 14. However, these authors have merely done a sperm count and omitted to determine the effect of age on sperm vitality, sperm motility, or sperm morphology. The effect of drone age on sperm quality, specifically on African subspecies are very limited.

Drone weight and size

Underdevelopment of drones is known to influence spermatogenesis (Szentgyörgyi, et al., 2016), and parameters such as the bodyweight of drones *per se* seem to affect semen volume (Yániz, et al., 2020). Schlüns, et al. (2003) for example compared sperm concentrations of differently sized drones, using wing length as size indicator, and have shown that the concentration of sperm produced is in

relation to drone body size, with fewer sperm numbers present in smaller sized drones when compared to those of normal size.

South African subspecies, such as Cape honey bee drones, are generally smaller in size than European subspecies, yet the concentration of sperm produced remains the same between subspecies (Buys, 1990). Honey bee queens have shown a preference for larger drones, however, given that sperm number is hardly affected by body weight or size, this might be due to their flight fitness advantage and not necessarily due to the quality or amount of semen provided during copulation (Schlüns, et al., 2003). Furthermore, smaller drones, especially those with asymmetrical wings were less successful than normal-sized drones when participating in mating flights (Jaffé & Moritz, 2010; Couvillon, et al., 2010). Additionally, drone size and wing asymmetry, is also affected by drone development, which in turn, can be affected by nutrition and genetics (Jaffé & Moritz, 2010; Rhodes, et al., 2011; Rousseau, et al., 2015).

Nutrition

Honey bee reproductive health partly depends on the correct nutritional intake (Zhao, et al., 2020). Pollen and nectar, particularly, are a primary source of nutrition, and important for the development of brood, larvae, and adult bees (Vaudo, et al., 2015). Pollen supplements drones with proteins, vitamins, minerals, and lipids. Malnourishment as a result of insufficient pollen consumption may result in morphological defects, unfit drones, and defective sperm development that can contribute to unsuccessful nuptial flights (Czekońska, et al., 2015). A pollen-poor diet during crucial lifecycle stages, specifically larval development, may result in the production of abnormally low semen volumes and may furthermore increase defective eversions of the drone endophallus during ejaculation (Czekońska, et al., 2015). Colonies supplemented with proteins furthermore presented with better semen quality and ultimately fertility, compared to colonies that did not receive additional supplementations (Rousseau & Giovenazzo, 2016). The correct nutritional intake is therefore of utmost importance during drone development, to ensure reproductive success, especially the quality of semen produced.

Temperature

Abnormal rearing temperatures have previously been reported as a noticeable factor to influence honey bee drone development and reproductive quality (Czekońska, et al., 2013, 2019).

Although drones reared at abnormally low temperatures (32°C) yielded semen with lower volumes, sperm vitality was seemingly higher (Czekońska, et al., 2013). Reproductive success and semen quality can furthermore be influenced by semen storage temperatures as well as incubation temperature during semen quality analysis (Zhao, et al., 2020). Although drones reared at lower temperatures presented with increased sperm viability percentages, exposing sperm at low temperatures *in vitro* yielded the opposite and showed that both abnormally high (40°C), and low incubation temperatures (9-10°C) were harmful, reducing the percentage of live sperm in a sample with 40% (Bienkowska, et al., 2011).

Season

Seasonal changes also affect drone reproductive development and sperm physiology (Rhodes, et al., 2011). Honey bee drones reared in the warmer seasons had a greater chance at survival (more than 4% reached the age of 36 days) compared to those reared in autumn (0% survival). The findings in this study furthermore highlighted the effect of seasonal changes on semen volume and sperm concentration, semen volumes were seemingly higher, and concentration lower, during spring (Rhodes, et al., 2011).

Differences in sperm morphometry at the beginning and end of a season have been described by Gontarz, et al. (2016) who has shown that sperm length reduced towards the seasonal end. Lengths of individual sperm components at the beginning and end of the season were as follows, the acrosome, 4.73 and 4.66 µm; nucleus, 4.78 and 4.69 µm; head, 9.43 – 9.35 µm); flagellum, 264.07 – 248.62 µm. As a result of decreased head and flagellum lengths, the total sperm length decreased from 273.50 µm at the beginning of a season, to 257.97 µm towards the end of the season (Gontarz, et al., 2016). However, such research is yet to be done on the sperm of African honey bee subspecies.

Diseases and parasite infestation

Disease, such as American Foul Brood, and parasitic infestations are amongst the environmental factors that adversely affect drone semen quality (Collins & Pettis, 2001; Peng, et al., 2015). Parasites such as *Varroa destructor* are one of the limiting factors of drone reproductive health and often lead to honey bee colony losses (Ramsey, et al., 2019). Although Collins & Pettis (2001) reported that the semen volumes of mite-infested drones were similar to unexposed drones, parasitized drones presented with a higher mortality rate, and a shortened lifespan, thus failing to reach the age of sexual maturity and therefore, unable to mate with a queen. While it is known that parasitic mite infestations account for the reduced lifespan of honey bee drones, preventing sexual maturation and reproduction,

the direct effect on semen functionality such as motility, and also vitality, remains unknown and warrants further investigation. Furthermore, African honey bee subspecies seem to be more resistant to diseases and pests (Strauss, et al., 2015); however, the effect of particularly a pest such as the *Varroa destructor* on drone sperm quality in African subspecies is not known.

Pesticides

Pesticides include, amongst others, insecticides (often used in plant protection programs to control and prevent disease) and acaricides, commonly used to treat *Varroa destructor* infestation. However, the use of pesticides is repeatedly reported as a factor underlying honey bee colony losses. Pesticides may result in reproductive toxicity and the increased use thereof in recent years, is adding more pressure on honey bee reproductive health and colony performance (Kairo, et al., 2017; Ciereszko, et al., 2017; Johnson, et al., 2013). Residues of pesticides used both outside and inside the hive were furthermore found on honey bee beeswax. (Fisher & Rangel, 2018). Therefore, these substances have lately received substantial attention due to the potential threats posed to the ecosystem, especially honey bees and their reproductive functionality, including the adverse effects on drone and queen reproductive health, a major cause of honey bee colony mortality (Williams, et al., 2015; Kairo, et al., 2017).

For example, the pesticide, fipronil, has been confirmed hazardous to honey bee reproduction (Kairo, et al., 2017). Although no direct lethal effects were reported, and semen volume remained unaffected, honey bee drone fertility was still affected as there was a decreasing correlation between sperm concentration, vitality, and fipronil exposure (Kairo, et al., 2017). In addition to these findings, drone sperm exposed to high concentrations of the neonicotinoid insecticide, imidacloprid, presented with significantly lower sperm motility speeds (Ciereszko, et al., 2017) and reduced sperm longevity (Chaimanee, et al., 2016). Studies investigating the effects of acaricides (miticides), (e.g. fluvalinate, coumaphos, fenpyroximate, amitraz, thymol, and oxalic acid) on honey bee sperm have produced controversial results, which could be attributed to the method and time of miticide application, which often excludes drone development periods. The adverse effects of miticides on honey bee drone and sperm physiology, specifically motility and vitality, have been reported by several authors (Collins & Pettis, 2001; Johnson, et al., 2013; Ciereszko, et al., 2017; Kairo, et al., 2017; Inouri-Iskounen, et al., 2020).

Contrary, Johnson, et al. (2013) reported that these miticides do not affect sperm longevity. However, their method used involved the topical application of miticides on adult drones, not including the possible effect of exposure during development.

Globally, risk assessments have now been implemented to evaluate the possible risks posed by these pesticides, as an attempt to address related concerns. Regulatory authorities are however aware of possible fertility impairment of exposed drones and queens, as well as anomalies in their offspring (Kairo, et al., 2017). Since honey bee reproduction is important for colony progression, health, and sustainability, investigating the impact of pesticide exposure on sperm physiology is essential (Luttik, et al., 2012; Medrzycki, et al., 2013).

Yet, the impact of pesticide exposure on honey bee reproduction, specifically the analysis of drone sperm motility parameters, cannot be readily assessed as a more sophisticated technique such as CASA are required (Al-Lawati, et al., 2009; Kairo, et al., 2017; Blacquiére, et al., 2012; Yániz, et al., 2020).

2.10 Adverse effects of miticide exposure on sperm quality: amitraz

The prevalence of mite infestations in honey bee hives has raised concerns amongst beekeepers on a global scale. Honey bee populations from many parts of the world are exposed to the ectoparasitic mite, *Varroa destructor* (Martin, et al., 2019). This includes South African honey bee populations (Allsopp, 2007); however, as previously mentioned, African honey bee species seem to be more resistant to these mites. These mites prefer a vital honey bee organ similar to the mammalian liver, called the fat body (Ramsey, et al., 2019). A dysfunctional fat body may result in a compromised immune system, resulting in a reduced life expectancy, finally contributing to the global health decline in honey bee populations (Ramsey, et al., 2019; Locke, 2016).

Miticides used to combat such infestations are used worldwide. and often result in prolonged beeswax contamination (Mullin, et al., 2010). The continuous use of miticides on honey bee hives to treat *Varroa destructor* infestations has resulted in mite resistance to synthetic miticides such as fluvalinate and coumaphos (Elzen & Westervelt, 2002; Pettis, et al., 2004). Therefore, products containing the active substance, amitraz, became the more popular choice for treatment (Chaimanee & Pettis, 2019) because it is an effective treatment against *Varroa destructor* and so far, has been shown to have little to no lethal side effects on honey bees (Johnson, et al., 2013).

Amitraz is an active substance of a widely used miticide Avipar®, commonly applied to regulate *Varroa destructor* infestations in honey bee colonies (Pirk, et al., 2014). Amitraz {N, N-[(methylimino) dimethylidyne] di-2, 4-xylidine} is a formamidine, which acts on the central nervous system of ectoparasites such as *Varroa destructor* by interacting with octopamine receptors which results in parasitic fatalities (Evans & Gee, 1980; Chaimanee & Pettis 2019). Treatment is parasite-

specific, but as miticides are placed inside colonies to treat mites, it does simultaneously expose eggs, larvae, and adult honey bees (Westcott & Winston, 1999). Although amitraz in its entirety does not accumulate in honey bee hives, its metabolized forms, 2,4-dimethylphenylformamind (DMF) and N-(2,4-dimethyl phenyl)-N'-methylformamidine (DPMF) have been detected on honey bees as well as beeswax (Korta, et al., 2001; Mullin, et al., 2010). Therefore, although literature reports on little to no lethal side-effects on honey bees, the ability of amitraz to interact with other in-hive compounds remains (Johnson, et al., 2013).

Furthermore, despite the effectiveness of amitraz thus far, greater dosages have been applied by beekeepers because of suspected resistance (Dahlgren, et al., 2012). Treatment with amitraz, exceeding the recommended dosage, can, however, be more toxic and have adverse effects on exposed honey bees and their reproductive health. Studies investigating this matter mostly focused on the effect of amitraz on worker bees (Rangel, et al., 2013; Garrido, et al., 2013), but it is also important to investigate the effect thereof on drone sperm quality owing to the importance of honey bee drone and queen reproductive health for colony growth and performance (Rangel, et al., 2013).

The effect of sublethal doses (LD = 0.916 µg / bee) of amitraz on sperm stored in the spermatheca of honey bees in Nebraska, Lincoln, has shown that sperm viability remained unaffected (Johnson, et al., 2013). This could however be due to the topical application of amitraz on the queen abdomen as well as in-hive exposure during honey bee adult stages, not considering the effects of in-hive, chronic exposure during the developmental stages of drones (Chaimanee & Pettis, 2019; Johnson, et al., 2015). Fisher & Rangel (2018) however, have demonstrated reduced sperm viability following in-hive amitraz exposure from contaminated beeswax during drone development. In this study, treatment was administered by means of spraying diluted amitraz directly on the hive frames. Carniolan honey bee drones exposed to miticide, Mitac (containing 2000 mg/L amitraz), during development presented with reduced body weight, concurrently hampering their flight activity (Shoukry, et al., 2013). Additionally, in Beijing, the chronic toxicity of amitraz has also been shown to affect honey bee survival, as larvae fed a diet containing a high concentration of amitraz (46 mg/L) (Dai et al., 2018).

Studies so far mainly investigated the effect of pesticides, including amitraz, on sperm viability, while sperm structure and motility should also be investigated (Yániz, et al., 2020). Furthermore, as honey bee drone spermatogenesis is completed already before emergence, the effect of prolonged amitraz exposure during development and drone reproductive health warrants further research.

2.11 Conclusion

From the literature, it is evident that drone fertility, particularly semen quality, is important to ensure colony health and sustainability. Drone infertility affects the productivity of the queen, rendering her incapable of fertilizing eggs and the inactivation of her reproductive system.

Owed to the relationship between semen quality and honey bee fertility, it can be hypothesized that sperm quality *per se*, can be a predictor of colony reproductive success and health. Comprehensive sperm quality analyses including sperm structural and functional parameters are therefore required, to identify sperm of good quality, which is important for further selection during artificial insemination. Methods used to assess sperm motility are either mainly manual or observational, or the less successful assessment of sperm motility using modern CASA systems.

Furthermore, there is also limited evidence regarding the role of sperm quality, particularly motility, and the effect of environmental factors thereon. For example, both *Varroa destructor* infestation and miticide application, in particular, have the potential to adversely affect drone fertility and more importantly sperm quality, which in turn has been directly linked to unsuccessful insemination, and subsequently in a dysfunctional queen.

It is, therefore, the aim of this study to establish accurate and less time-consuming methods to measure honey bee drone sperm quality routinely and to determine motility and kinematic parameters in honey bee drone sperm using a CASA system. Additionally, this study also aims to determine the effect of amitraz, an active substance of miticides, on sperm quality. Such data will provide a better understanding of honey bee sperm functionality, particularly motility and kinematics, and the effect of miticides thereon.

CHAPTER 3: MATERIALS AND METHODS

3.1 Introduction

In this study, standardized protocols were implemented to exclude the possible effect of deposition time and chamber depth on structural and functional parameters of honey bee sperm. Furthermore, a CASA system was used to ensure that the evaluation and analysis of sperm parameters remained unbiased, objective, and accurate.

3.2 Ethical approval

Research conducted on the Cape honey bee drones was ethically approved by the Animal Research Ethics Committee (AREC) of the University of the Western Cape (Registration number: 130416-019). The methodology used during sample collection was in agreement with the ethical guidelines of the University of the Western Cape AREC.

3.3 Husbandry

Cape honey bee drones were collected from colonies housed at the Agricultural Research Council – Plant Protection Research Institute (ARC-PPRI) Vredenburg campus in Stellenbosch, Western Cape, South Africa. Fynbos fields were the primary dietary source of these apiaries. Colonies were selected for drone collection based on colony health and the presence of adequate mature drones and a queen bee. The honey bee colonies were housed and maintained by the ARC-PPRI according to standard apicultural practices.

3.4 Cape honey bee drone collection

The drone collection period for this study took place during the approach of spring until the end of summer, from July to December 2020, which is the time when drones are frequently available. It should furthermore be noted that this study did not evaluate the impact of colony and seasonal variations on semen quality. A total of 50 sexually mature drones (Couvillon, et al., 2010) were randomly collected from ten healthy colonies twice a week during the mid-morning, 10-11 am, before their daily flights. Defecating drones were minimal at the time, but excluded from further laboratory analysis to prevent semen contamination.

3.5 Experimental design

The study consisted of two parts, firstly a baseline study to determine drone sperm motility parameters, and secondly, a dose-response study to determine the effect of different amitraz concentrations on drone sperm functional and structural characteristics. Both parts of the study entailed the assessment of sperm quality by performing various functional and structural tests. Figure 3.1 provides the experimental design of this study, and a detailed description of all methodology used to determine sperm quality parameters are explained in the remainder of chapter 3.

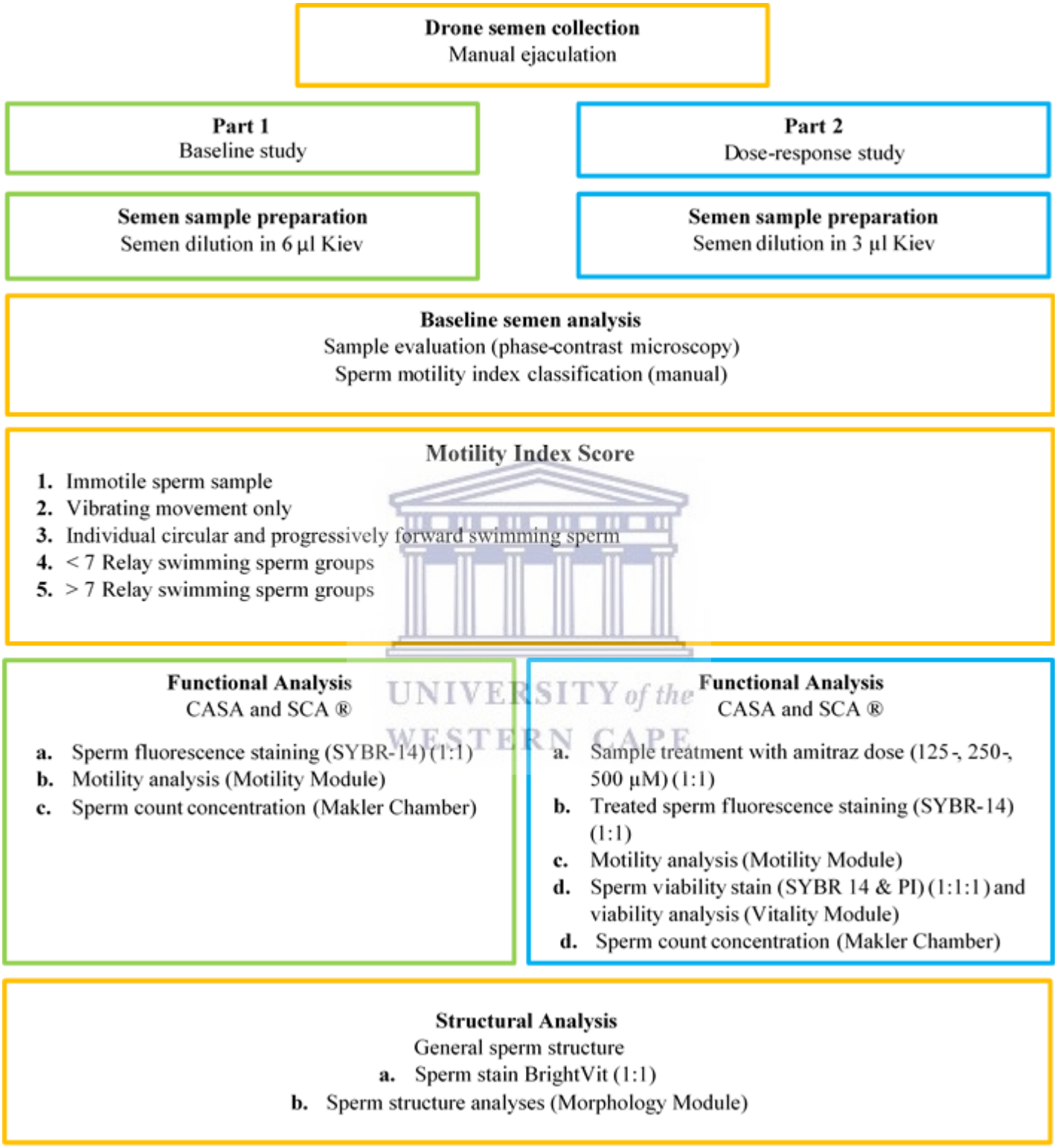


Figure 3.1 Experimental design

3.6 Semen collection

To eliminate the stressful effect of transportation and possible temperature changes, manual semen collection, occurred at the site of drone collection.

Honey bee drones were manually stimulated to ejaculate, using the method described by Collins (2005); this technique involved provoking the endophallus by carefully rubbing and bending the rigid thorax with your thumb and index finger until self-eversion occurred. The semen present on the endophallus tip was aspirated with a positive displacement pipette (1 µL), using sterile tips.

3.7 Sperm preparation

Once semen was collected, each sample was placed into a pre-warmed plastic micro-centrifuge Eppendorf tube (0.5 ml) (Sigma Aldrich, Germany) containing either 6 µL (for the first experiment, baseline study) - or 3 µL (for the second experiment, dose-response study) of modified Kiev buffer solution (pH 8.83) (Collins, 2005) respectively, for the baseline and dose-response experiments. The initial dilution with Kiev buffer solution for the dose-response study was less than the baseline study, given that further dilutions occurred when adding amitraz to the sample. Samples were then incubated at 37°C until analyses. These specific Kiev buffer solution volumes were used to preserve the original (*in vivo*) sperm motility and swimming behaviour of drone sperm as far as possible, which is often altered by over dilution (Halak, et al., 2019), and finally to ensure that there is sufficient sample to be separated into a control and a dose exposed sample for comparison in the amitraz experiment. Given the small volume of semen sample (range between 0.5 and 0.7 µL), a micro-centrifuge tube was used for storage to minimize the amount of air present, preventing the sample from drying out before analyses. Following semen sample collection procedures, semen samples were transported at 37ºC to the laboratory for further analyses and were analysed within one hour after collection (Yániz, et al., 2019).

In this study 51 drone semen samples were analysed for the baseline study and 102 samples for the dose-response study.

3.8 Baseline semen analyses

Sperm samples were firstly assessed using a Nikon Eclipse 50i Phase Contrast Microscope (IMP, Cape Town, South Africa) at 20X negative-phase objective, to eliminate mucus contaminated samples that could not be analysed.

3.8.1 Sperm motility classification

Sperm motility was classified by placing 1-2 µl of a diluted sample into a 4-chamber Leja slide (depth = 10 µm) (Leja Products B.V., Nieuw- Vennep, The Netherlands) using a micropipette and a sterile tip for each sample (n = 20). The Leja slide was then left undisturbed for 1-2 minutes at 37ºC, to allow the sperm to adapt to the new environment (e.g. temperature and chamber depth) (Inouri-Iskounen, et al., 2020).

For motility analyses, digital video recordings of samples were made using a 20X negative phase objective, and the Motility module of the CASA and SCA ®) (Microptic S.L., Barcelona, Spain) system, Version 6.5.0.44 using a Nikon Eclipse 50i Phase Contrast Microscope with a heated stage (37° C), and a mounted digital camera (Basler ace acA 1300-200uc colour USB 3.0) (Microptic S.L., Barcelona, Spain) (Figure 3.2). Configuration settings used for the CASA and SCA ® system were as follow: Frame rate = 50 frames/sec; Optics = pH + (positive phase contrast); Chamber = Leja 10; Species = Honey bee No Analyse.

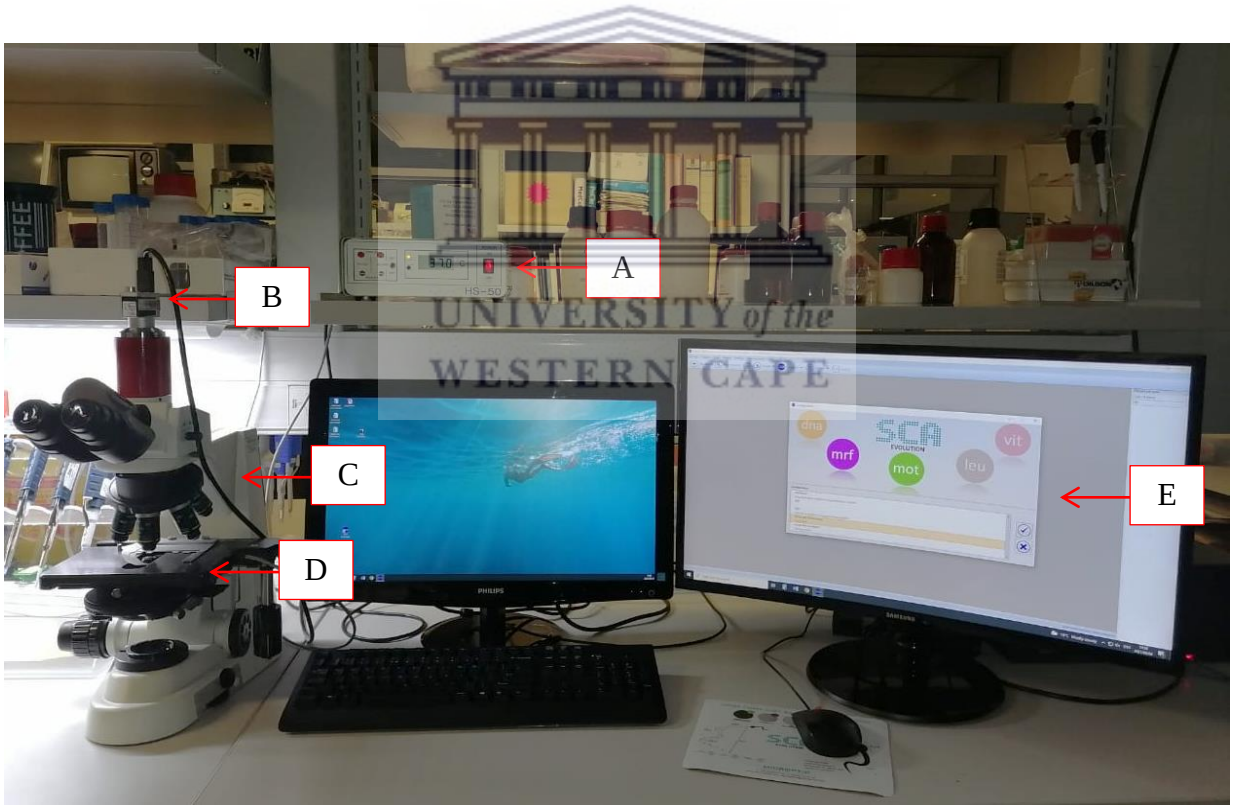


Figure 3. 2 Equipment used for analyses: conventional CASA system set up with a fully equipped phase microscope. (A) Heated stage control, (B) Digital camera (Basler ace acA 1300-200uc colour USB 3.0), (C) Nikon Eclipse 50i microscope, (D) Heated stage, (E) Computer with SCA ® software for CASA analysis

Sperm motility was firstly assessed with a manual semi-quantitative method using phase-contrast microscopy to classify sperm according to a five-point motility grade score (Motility index). Although there is a well-established motility classification system (Locke & Peng, 1993), there was a need for modification to take into account helical swimming groups of sperm, as described in previous studies (Tofilski et al. 2018; Yániz et al. 2020). The adjusted classification system in this study provides a more quantitative assessment of vigor sperm movement.

Motility grade scores were assigned to each sample as follow: 1 - no sperm movement present (immotile), 2 - vibrating sperm but no progressively forward swimming sperm, 3 - individual circular and progressively forward swimming sperm present, 4 - circular swimming groups, also called relay swimming sperm, consisting of less than 7 sperm per group and 5 – circular swimming groups (relay swimming), consisting of 7 or more sperm in a group (Table 3.1) (Figure 3.3). The latter two categories (Motility index scores 4 and 5) were established based on observation, sperm samples either distinctively presented with less than 7 circular swimming groups, or more than 7.

Table 3.1. Motility index for manual semi-quantitative classification of honey bee sperm

Motility index	Definition
1	Immotile sperm sample
2	Vibrating movement with no progressively forward-moving sperm
3	Individual circular and progressively forward-moving sperm
4	< 7 Relay swimming sperm groups
5	>7 Relay swimming sperm groups

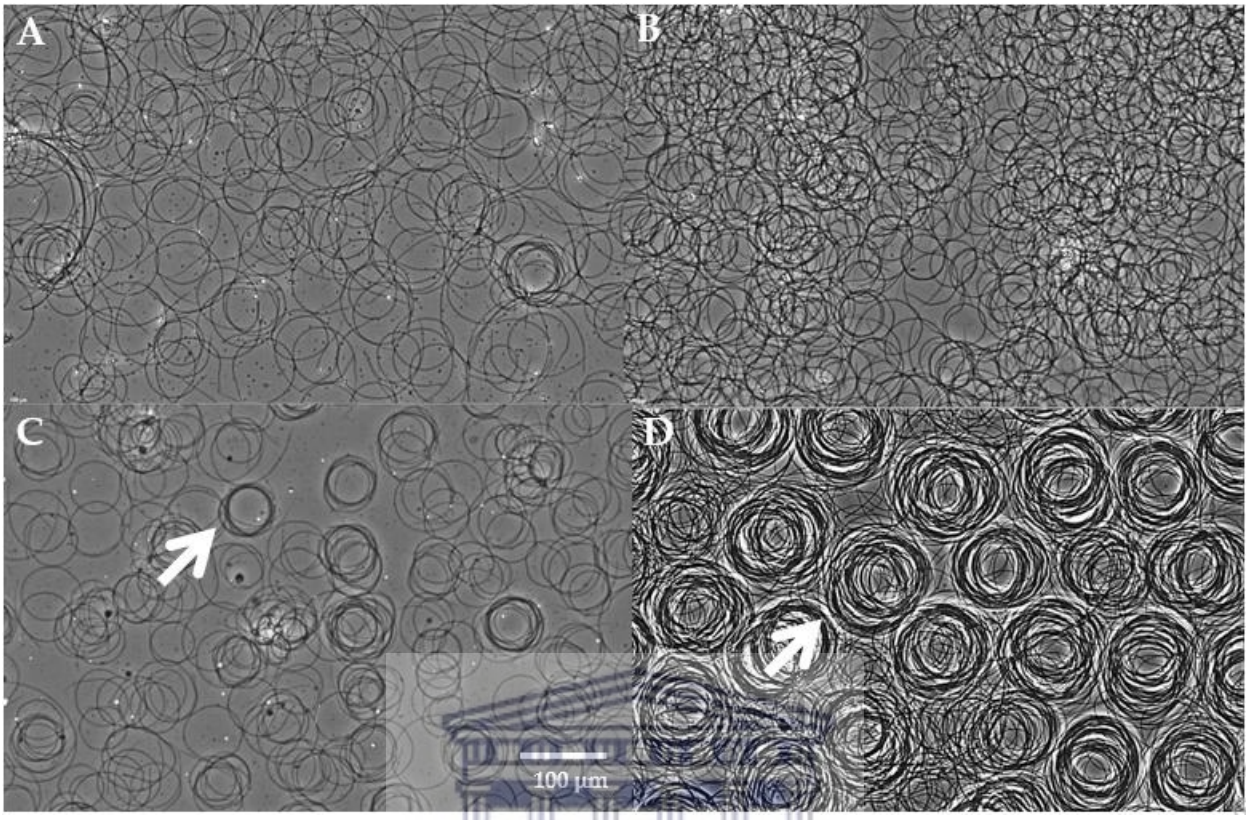


Figure 3. 3 Semi-quantitative manual motility index classifications using phase-contrast microscopy. (A) Motility index 2, (B) Motility index 3, (C) Motility index 4, (D) Motility index 5. The white arrows in figures C and D indicate relay swimming sperm, a collective term used to refer to many sperm swimming together in a group. Scale A-D = 100 µm

Once a motility grade score was assigned, semen samples were prepared according to the individual protocols for the structural and functional analysis for both baseline and dose-response studies.

3.9 Computer-aided sperm analysis (automated and qualitative)

For the routine assessment of drone sperm functional and structural quality (motility and vitality), a fluorescence method was developed to use a CASA system on honey bee sperm. The possible impact of fluorochromes on sperm functionality and behaviour was validated with a preliminary study, which involved the manual motility assessment of unstained sperm samples, using image analysis and the manual, hand-tracking of sperm heads for every frame per second (Murray, 2019 unpublished). For both methods, sperm swimming behaviour was similar.

3.9.1 Sperm functional analysis

Baseline-study

Tests to analyse some functional sperm characteristics were all automated using CASA, SCA ® in combination with a newly developed fluorescence microscopy technique. For the fluorescence technique, an SYBR-14 (L-7011, LTC Tech, Fairland, South Africa) stock solution was prepared by making a 50 times dilution of SYBR-14 dye (100 µL of a 1 mM solution) in Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich, Merck, South Africa), and stored at -20ºC as per product instructions). A working solution was further made by making a 5 times dilution of SYBR-14 stock solution in Kiev buffer solution. The working solution was freshly made on the day of analysis.

For the staining of samples, a 1:1 ratio of the diluted semen sample and SYBR-14 working solution was then incubated at 37˚C for 10-minute (this specific incubation time allowed for the sufficient staining of sperm, whereas incubation times of less than 10 minutes did not allow for sufficiently stained sperm).

Dose-response study (amitraz)

Three different concentrations of amitraz, 125 µM, 250 µM, and 500 µM were used for the dose-response study. These concentrations were determined based on a preliminary experiment using different amitraz concentrations. Each semen sample analysed, was split into equal parts, one part was treated with one of the three concentrations and one part was used as the control. Amitraz dosages were randomly assigned to samples to avoid bias.

The amitraz concentrations were made as follows, firstly an amitraz stock solution was made up by dissolving 0.07335g amitraz powder (Sigma-Aldrich, Merck, South Africa) in 1000 µL DMSO (amitraz concentration = 0.25 M), and secondly, different amitraz concentrations were made by diluting the stock solution in different volumes of Kiev buffer solution (Table 3.2). The stock solution was always stored at 4ºC and amitraz concentrations were freshly made on the day of analysis. Initially, four different concentrations of amitraz working solutions (125 µM, 250 µM, 500 µM, and 1000 µM) were made by diluting the stock solution in different volumes of Kiev buffer solution. Of these concentrations, only 125 µM, 250 µM, and 500 µM were used, based on the high motility observed at 125 µM and low motility at 500 µM. The majority of sperm were immotile at 1000 µM resulting in CASA being unable to analyse these samples, therefore this dosage was

excluded from the study. Semen samples were then treated with one of the three concentrations of amitraz working solutions in a 1:1 ratio (Sample: amitraz).

Table 3.2 Individual dilutions used for each amitraz concentration, and final dosage of *in vitro* amitraz after dilution in Kiev buffer solution

Working solution recipe	In vitro concentration (dosage)
1 µL Amitraz stock solution in 1000 µL Kiev = 250 µM	125 µM
1 µL Amitraz stock solution in 500 µL Kiev = 500 µM	250 µM
1 µL Amitraz stock solution in 250 µL Kiev = 1000 µM	500 µM

To determine sperm motility of amitraz exposed samples, using CASA, the control for each amitraz concentration was stained with SYBR-14 working solution (as described for the baseline study) and simultaneously, the other part of the sample treated with an amitraz concentration, was incubated with the SYBR-14 working solution (1 µL amitraz treated sample: 1 µL SYBR-14), to eliminate motility deterioration as a result of prolonged time-lapse.

Sperm motility analysis

For both studies, sperm motility parameters such as percentage total motility and kinematic parameters (VCL, VSL, VAP, STR, LIN, ALH, WOB, and DNC) were determined by CASA, SCA ®. Motility cut-off values used to determine honey bee drone sperm motility kinematic parameters were as follow: Rapid (µm/s) >75, Slow-Medium (µm/s) = 50, Static (µm/s) <25. These settings furthermore allowed for the identification of honey bee sperm subpopulations.

For the baseline study, 4 µL of each sample, stained with SYBR-14 (section 3.9), was transferred onto a frosted glass microscopic slide, and covered with a coverslip (chamber depth = 8, 3 µm as determined by CASA, SCA ®). Samples were allowed 1-2 minutes for adjustment before analysis. A detailed motility assessment was done using the Motility module of the CASA, SCA ® system, in combination with a Nikon Eclipse 50i Fluorescence Microscope and a Basler aCA 1920 -155uc fluorescence digital camera (Figure 3.4).

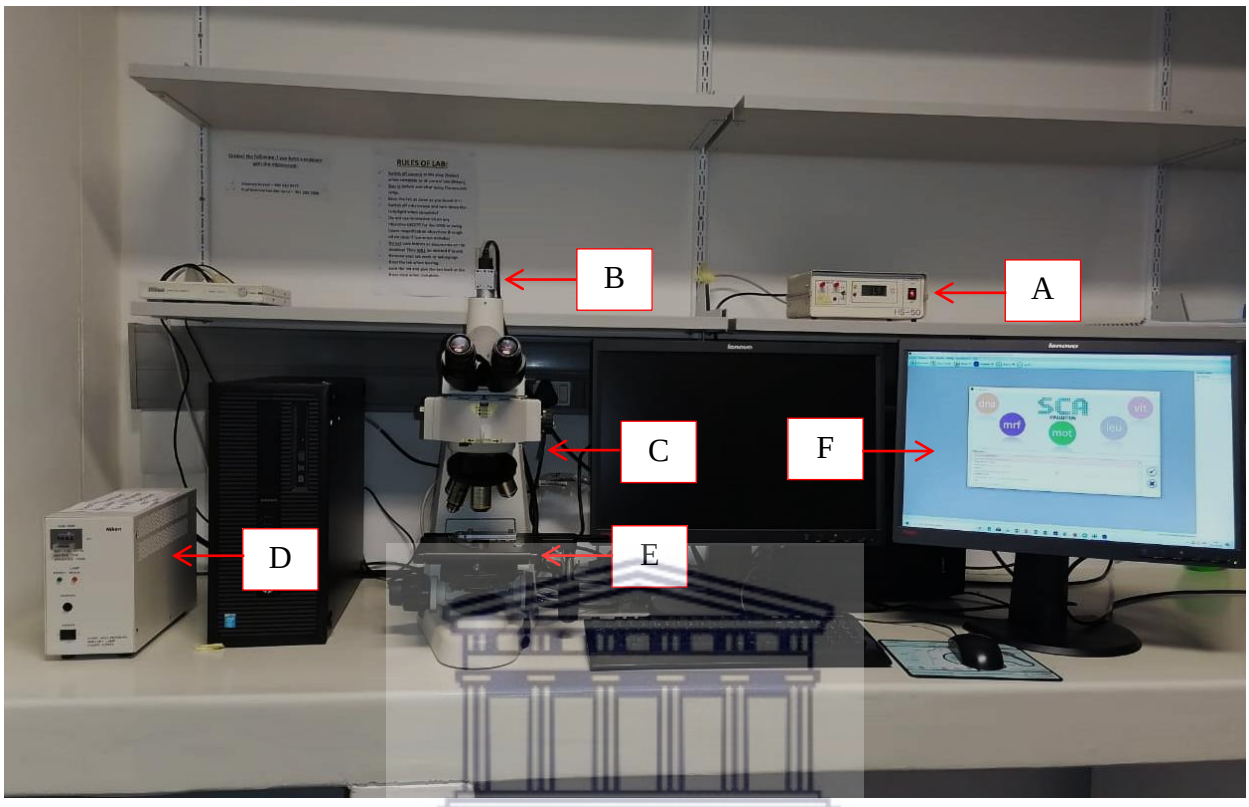


Figure 3.4 Fluorescence CASA system set up with a fully equipped phase microscope. (A) Heated stage control, (B) Digital fluorescence camera (Basler aCA 1920 -155uc), (C) Nikon Eclipse 50i Fluorescence Microscope, (D) Fluorescence light source, (E) Heated stage, (F) Computer with SCA ® software for CASA analysis

For motility analyses, configuration settings of the CASA and SCA ® system were as follow: Frame rate = 50 images/sec; Optics = Fluorescence microscopy; Chamber = Glass cover slide; Species = Invertebrate. A minimum of 100 spermatozoa per sample was analysed using a 40X fluorescence lens and FITC filter (B-2A Nikon: ex450-490; DM 505; BA 520).

3.9.2 Sperm structural analysis

Sperm morphology analysis

Sperm morphology analyses were done solely on amitraz treated samples versus their respective controls. A Nigrosin-eosin dye-exclusion assay, BrightVit (Delfran, Johannesburg, South Africa), generally used to determine vitality, was used in this study to differentiate between the different

sperm components. This stain consists of eosin which penetrates the compromised membrane, staining the cell pink while viable cells remain white. Secondly, it contains Nigrosin which functions as a purple background, providing contrast between the sperm cell and its background.

Honey bee drone sperm samples were stained in a 1:1 ratio (stain: diluted sample) for 15 minutes and incubated at 37 °C. After incubation, a smear was made by placing a 2 µL drop on a microscopic slide where after it was left to dry. On the following day, a coverslip was mounted, once dried, using DPX mounting medium (Sigma-Aldrich, Merck, Cape Town, South Africa). The staining procedure is illustrated in figure 3.5 below.

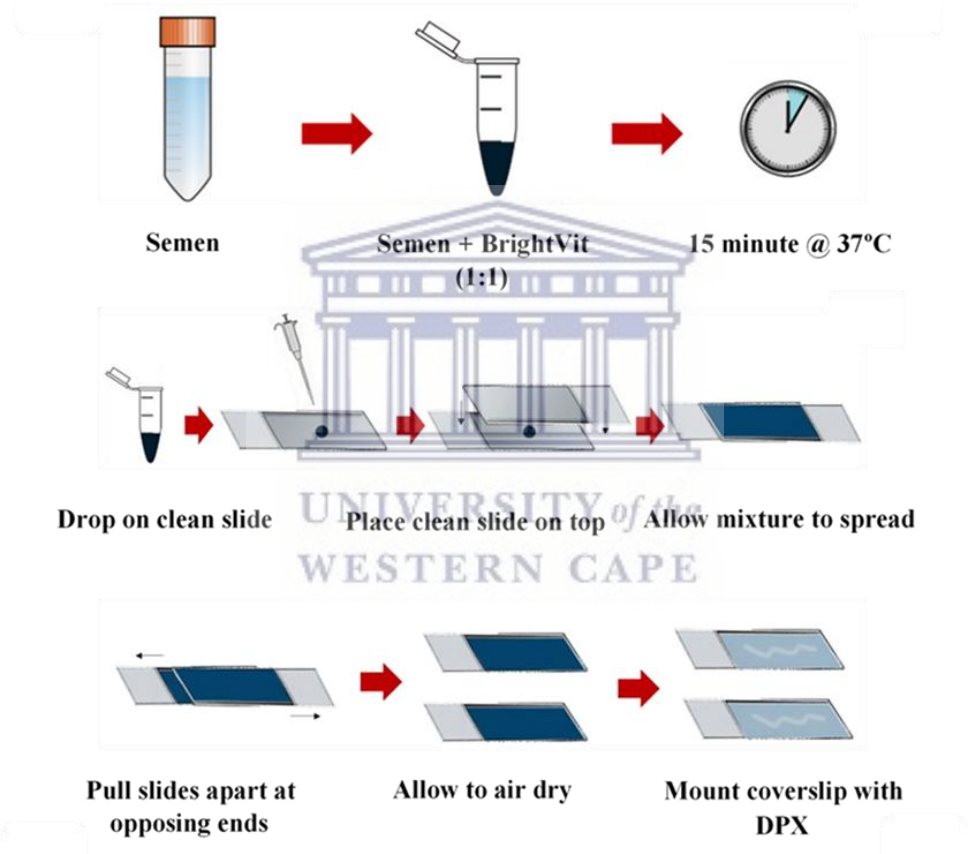


Figure 3. 5 BrightVit staining procedure for honey bee drone sperm morphology analysis [Adapted from Microptic (2021)]

Once mounted, the slide was viewed under 20X objective to measure total sperm length and tail and lastly at 100X objective to measure the sperm head and mitochondria using bright field optics on a Nikon Eclipse 50i microscope (IMP, South Africa), with an attached digital camera (Basler ace acA 1300-200uc colour USB 3.0; Microptic S.L., Barcelona, Spain). Only dead sperm components could

be measured using the measuring tool in CASA, SCA ® since live sperm is white and components are indistinguishable. Sperm components measured included the head, mitochondria, and tail (Figure 3.6).

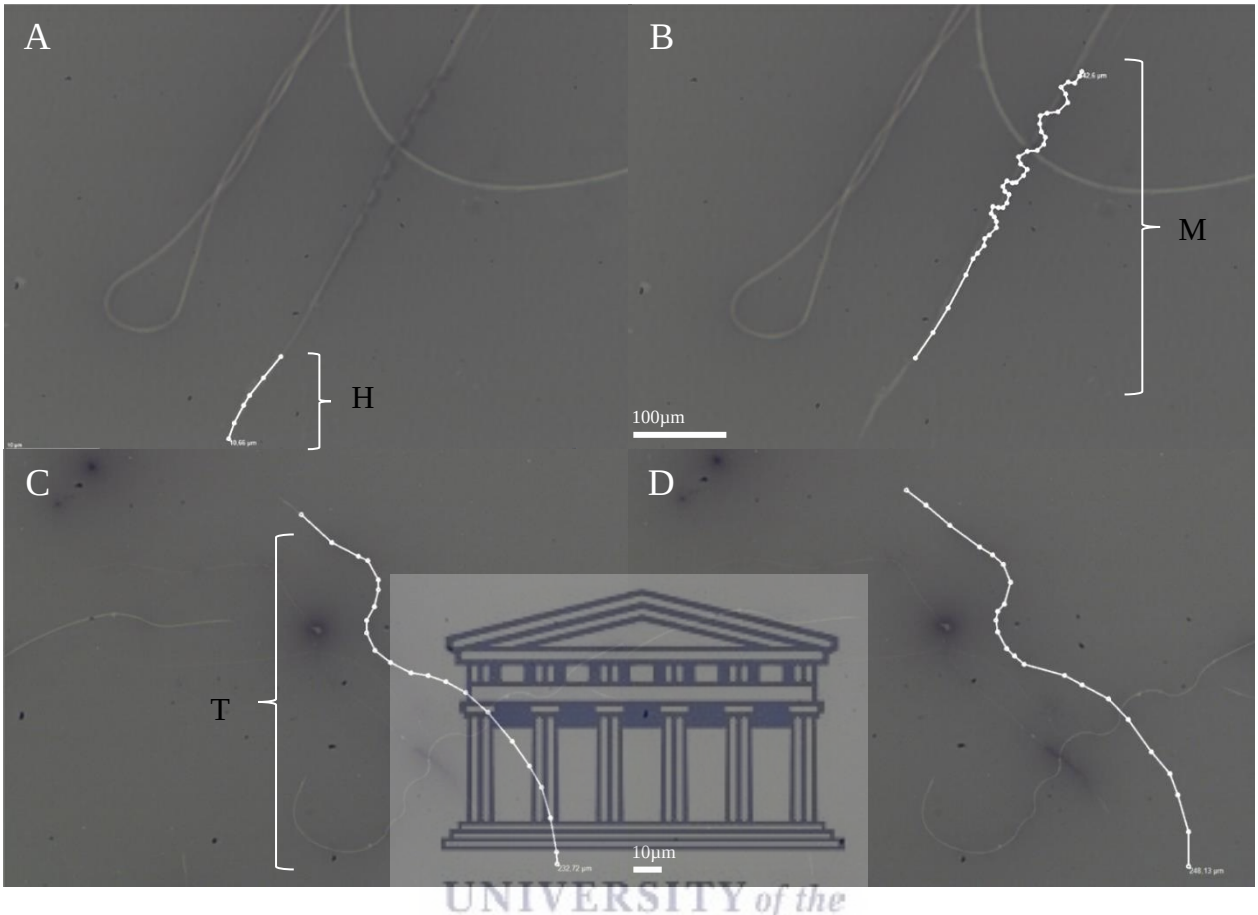


Figure 3. 6 Illustration of component measurement using the motility module of CASA and SCA ®. (A) Head length measurement (100X objective), (B) Mitochondria length measurement (100X objective), (C) Tail length measurement (20X objective), (D) Total sperm length measurement (20X objective). H, head; M, Mitochondria; T, tail. Scale A-B = 100 µm and C-D = 10 µm

Sperm vitality analysis

Based on the results from a preliminary study, the average percentage motility for honey bee drone samples in the baseline study was generally above 85% (Murray, 2019 unpublished), therefore viability analysis was performed solely on samples treated with different concentrations of amitraz in the dose-response study. It should also be noted that sperm viability could not be assessed on the same semen samples used for sperm motility assessment, as a result of the small semen volumes produced by honey bee drones.

However, to assess the effect of amitraz concentrations on sperm viability a dead/live staining kit (SYBR14 and propidium iodide) was used. As described above, samples treated with different amitraz concentrations were stained with SYBR14 for motility analyses; where after 0.5 µL of the counterstain Propidium iodide (PI) (L-7011, LTC Tech, Fairland, South Africa) was added to 2 µL SYBR14 stained sample, and further incubated for 5 minutes at 37°C.

This fluorescence-based assay allows for the evaluation of live (green) and dead (red) cells. Compromised plasma membranes are highly permeable, allowing PI to penetrate the dead sperm cell, changing its colour to red, while intact cells remain green (Gamer et al., 1995). An automated viability analysis using the vitality module of the CASA, SCA ® system was used to determine an accurate percentage of live and dead spermatozoa. Sperm cells stained with SYBR-14 and PI are illustrated in Figure 3.7.

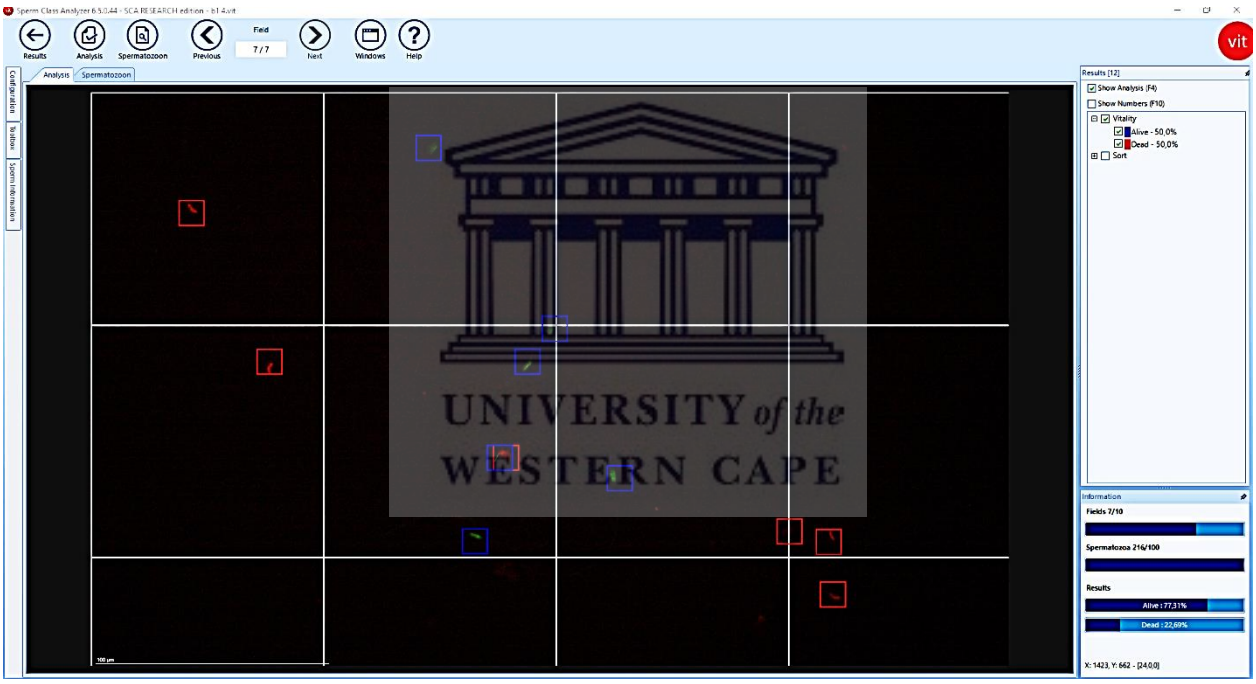


Figure 3. 7 Sperm cells stained with SYBR-14 and PI, recognized with CASA, SCA® (red = dead; green = live sperm)

3.9.3 Sperm Concentration

Honey bee drone sperm concentration was done on fluorescence-stained sperm, using the recordings made for motility analysis. A Makler ® chamber, obtained from the toolbox setting in the CASA, SCA ® Motility module, was superimposed on the captured images (Figure 3.8). A total of 10 chambers were counted per sample to determine concentration. The equation used to calculate the

sperm concentration is presented in figure 3.9, and was modified accordingly to determine honey bee sperm concentration in million sperm/µl.

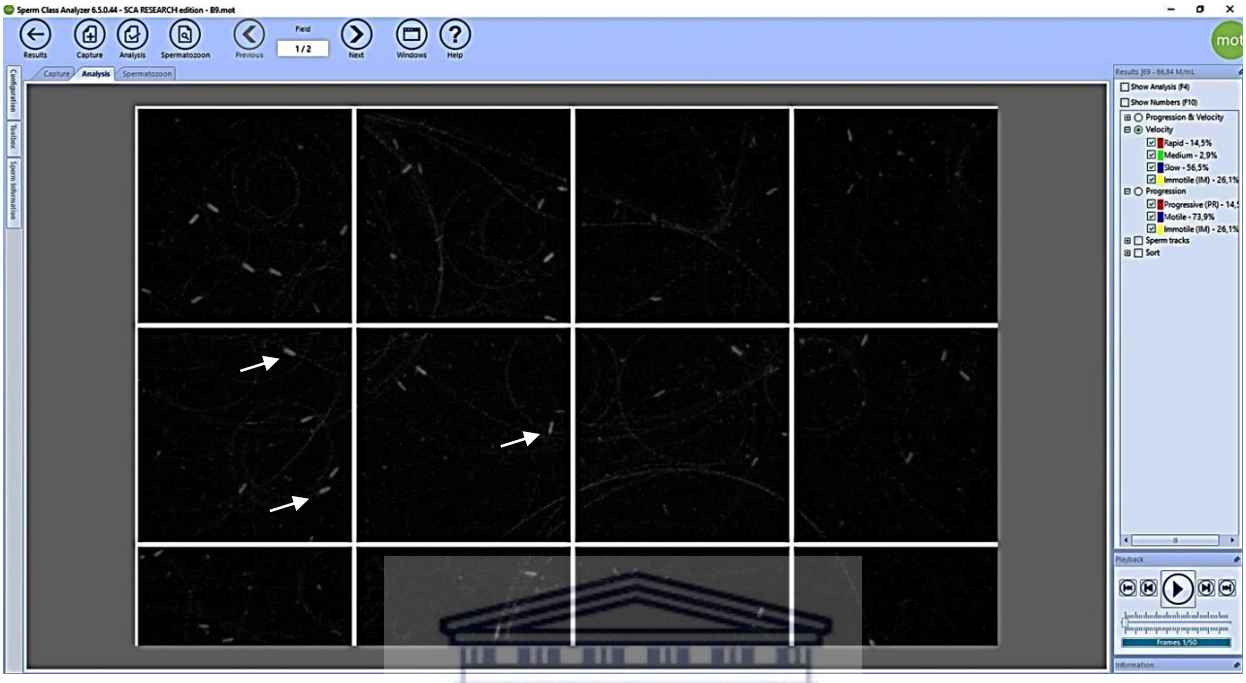


Figure 3. 8 Imprinted Makler ® chamber in CASA, SCA®, used to determine sperm concentration. White arrows indicate sperm heads

$$Concentration\ (Mil/\mu L) = \frac{SpermCount \times Dilution\ factor}{1000}$$

Figure 3. 9 Equation used to calculate honey bee drone sperm concentration [WHO (2010)]

3.10 Statistical analysis

Data was saved using CASA, SCA ®, and exported into an excel sheet, whereafter it was imported to MedCalc software (version 19.5.3, Mariakerke, Belgium) for further statistical analysis. A Levene's test for normality of distribution was used to determine data distribution. For normally distributed data (Levene's test $p > 0.05$) a one-way analysis of variance (ANOVA) test was performed. Results reporting on 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. Where significant differences ($P<0.05$) were found, variances were illustrated using graphs. For data that were not normally distributed (Levene's test $p < 0.05$) a non-parametric, Kruskal-Wallis test was

performed. Additionally, receiver operating curve (ROC) graphs were constructed to determine cut-off values for motility functional characteristics which aided in identifying sperm of good quality.

CHAPTER 4: RESULTS

4.1 Introduction

This study involved the automated assessment of sperm functional parameters (motility and kinematics) under normal (baseline study), and miticide (amitraz) exposed conditions (dose-response study), and the assessment of sperm structural parameters (sperm components and vitality) of amitraz-exposed sperm. The purpose of the baseline study was to establish a quantitative and qualitative method for drone sperm quality measurement. Firstly, to establish a fast and effective semi-quantitative method for motility classification with a manual motility index classification system developed for honey bee drone sperm, and secondly using a CASA system to routinely determine drone sperm motility and kinematic parameters. Chapter 4 will provide a description, and illustration, of the statistically analysed results, obtained. Statistically analysed data are expressed as mean and standard deviation (mean ±SD) (also presented by error bars on figures) unless stated otherwise. Results obtained from the baseline study will be discussed first, followed by the results obtained from the dose-response study.

4.2 Baseline study

4.2.1 Baseline semen and sperm characteristics

Cape honey bee drone semen volumes ranged between 0.4 – 0.7 µL, while sperm concentrations ranged between 1.10 x 10^6 – 16.5 x 10^6 million per µl. It should be noted that owing to the small amount of semen produced by Cape honey bee drones (volumes less than 1 µL), it was not possible at that stage to establish a method to measure the exact semen volume per sample routinely, and therefore the volumes of randomly selected semen samples, by weighing micro Eppendorf tubes (0.5 mL) with and without semen, can only be provided, and reported as a range, instead of a mean and standard deviation. Furthermore, due to the complexity of insect sperm, CASA, SCA ® did not detect all the sperm heads in a sample, and sperm concentration was determined manually by using the Makler chamber function of CASA and SCA ®, as indicated in Chapter 3 (Materials and Methods), to obtain the concentration range.

Baseline sperm characteristics of the Cape honey bee drone are presented in Table 4.1 and Figure 4.1, illustrating the comparison between the manual motility index scores and the automated total motility percentages for each CASA-determined motility percentage category.

The mean and standard deviation of the motility index score was 4.11 ± 1.11, and indicated that the majority of samples contained less than seven relay swimming sperm groups presenting with high mean total motility percentages (85.5 ± 17.2 %). Furthermore, mean values for both motility index scores and total motility percentage differed significantly between the > 20-59 % motility category and > 80 and 60-79 % motility categories (P = 0.01) (H-value = 7.82). Mean values for both motility index scores (2.83 ± 0.41), and total motility percentage (43.0 ± 6.33 %) in the > 20–59 % motility category were almost half of the mean values presented in the > 80% motility category (motility index score = 4.32 ± 1.04; total motility percentage = 91.9 ± 5.34 %).

The manual motility index scores corresponded with the total motility percentages determined by CASA and SCA ®. Motility index scores (4.32 ± 1.04;4.14 ± 1.22; 2.83 ± 0.41) and total motility percentages (91.9 ± 5.34 %; 72.9 ± 6.71 %; 43.0 ± 6.33 %) both decreased significantly along with motility percentage categories (> 80%; > 60-79 %; > 20–59 %) determined by CASA and SCA ® (P < 0.001) (F-ratio = 2.09).

Table 4.1 Baseline sperm characteristics of Cape honey bee drones (mean ± SD) for respective motility categories

	Mean	**>80 % motility**	**>60-79% motility**	**>20-59% motility**	**P-value**	**F-ratio/ H-value**
Sample size (n)	51	38	7	6		
Motility Index	4.11 ± 1.11	4.32 ± 1.04[a]	4.14 ± 1.22[b]	2.83 ± 0.41[ab]	0.01	7.82
Total Motility (%)	85.5 ± 17.2	91.9 ± 5.34[ab]	72.9 ± 6.71[ac]	43.0 ± 6.33[bc]	<0.001	2.09

Parametric data are reported as mean and standard deviation (SD). Means with the same letters in the same row differed significantly (one-way ANOVA, P < 0.05). The test statistics are reported as the F-ratio for an ANOVA and the H-value where a Kruskal-Wallis test was performed.

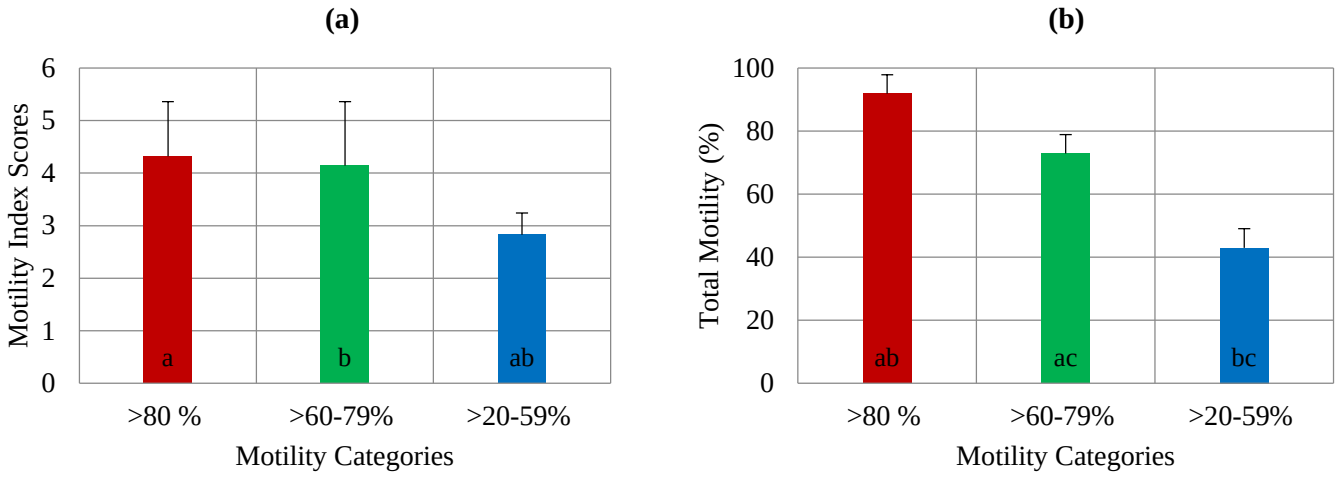


Figure 4.1 Comparison of (a) mean motility index scores *vs* percentage motility categories and (b) total motility percentage *vs* percentage motility categories (mean ± SD). Error bars indicate the positive standard deviations only. Bars with the same alphabetical letters differ significantly. Please note the scale difference.

4.2.2 Sperm functional characteristics

Motility and kinematic parameters

The baseline study kinematic parameters for honey bee sperm, according to the three motility percentage categories, are presented in Table 4.2. The majority of the kinematic parameter values in the > 80% motility category, except for ALH (1.78 ± 0.40 µm/s) (P = 0.51) (F-ratio = 0.69) and DNC (185 ± 79.7 $\mu m^2/s$) (P = 0.12) (F-ratio = 2.22), were significantly higher, compared to the other motility categories.

Furthermore, the percentage motility and kinematic parameter measurements corresponded as the values of the velocity parameters, VCL, VSL, and VAP, significantly decreased as percentage motility decreased (also illustrated in Figure 4.2).

Interestingly, STR only significantly differed between the > 80% (73.8 ± 4.39 µm/s) and > 60-79% (68.6 ± 4.19 µm/s) categories (P = 0.01) (F-ratio = 4.94). Significant differences, similar to those of the velocity parameters above, were observed amongst the motility categories for LIN (P = 0.001) (F-ratio = 8.49) and WOB (P = <0.001) (F-ratio = 14). Values were significantly higher in the >80% motility category for both LIN (61.2 ± 5.66 %) and WOB (79.7 ± 4.62 %), versus the > 60-79% (LIN = 52.7 ± 7.44 % and

WOB = 70.1 ± 8.58 %) and > 20-59% (LIN = 54.2 ± 5.63 % and WOB = 70.5 ± 6.89 %) motility categories, with the latter two presenting no significant differences between each other.

As for BCF, a trend similar to that of STR was seen, with significant differences observed only between the >80% (16.3 ± 1.39%) and > 60-79% (13.7 ± 1.69%) motility categories (P = <0.001) (F-ratio = 11.6).

Table 4.2 Baseline honey bee drone sperm kinematic parameter measurements (mean ± SD) across three different motility percentage categories

Parameters	Mean	> 80 % motility	> 60-79% motility	> 20-59% motility	P-value	F-ratio
Sample (n)	51	38	7	6		
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 alphabetical letters in the same row differed significantly (one-way ANOVA, P < 0.05). 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.

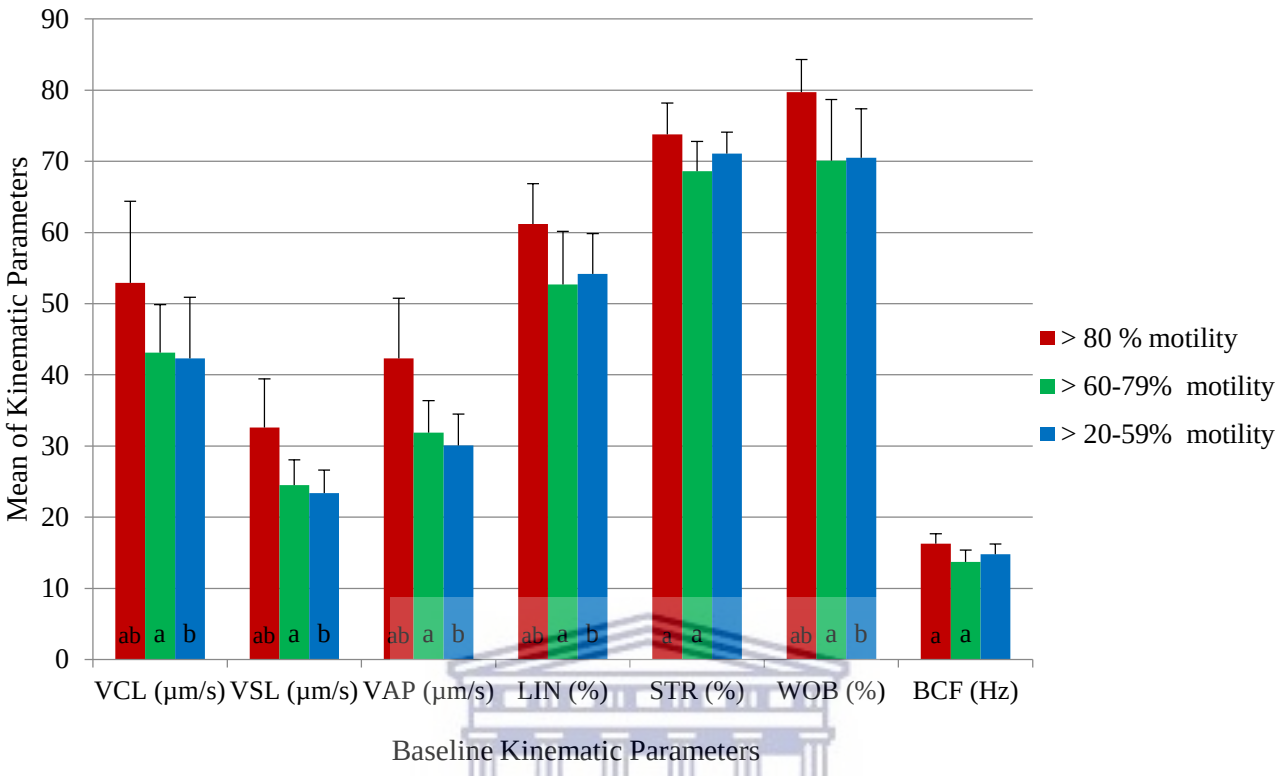


Figure 4.2 Cape honey bee sperm kinematic parameter measurements (mean ± SD) for different motility categories. VCL, Curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; DNC, dance. Error bars indicates the positive standard deviations only.

Cut-off values for sperm subpopulations

Cut-off values for drone sperm velocity parameters were determined for the three motility groupings using ROC curve analyses. The statistically analysed baseline kinematic parameter values, VCL (Sensitivity = 100 %; Specificity = 100 %), VSL (Sensitivity = 76.2 %; Specificity = 86.7 %), VAP (Sensitivity = 85.7 %; Specificity = 90.0 %), and ALH (Sensitivity = 81.0 %; Specificity = 66.7 %) yielded sensitivity and specificity values greater than 60%; values obtained from these parameters were thus used as restrictions (Table 4.3). Sensitivity and specificity values of less than 60% were furthermore disregarded due to the lower certainty based on the statistics at hand, and therefore not used as cut-off criteria. Data meeting all the above-mentioned requirements were used as restriction criteria and cut-off values for motility and kinematic analyses. However, for this study, using only VCL to distinguish

between sperm sub-populations was sufficient. Cut-off values used to distinguish between honey bee drone sperm subpopulations, rapid-, medium- and non-progressive are presented in Table 4.3, and ROC curves used to determine the cut-off values are additionally illustrated in Figure 4.3.

Table 4.3 Cape honey bee sperm motility restrictions as obtained from ROC analyses (mean ± SD)

Kinematic Parameters	Restrictions
VCL (µm/s)	>49.85
VSL (µm/s)	>31.09
VAP (µm/s)	>39.88
ALH (µm)	>1.74

VCL, Curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; ALH, amplitude of lateral head displacement.

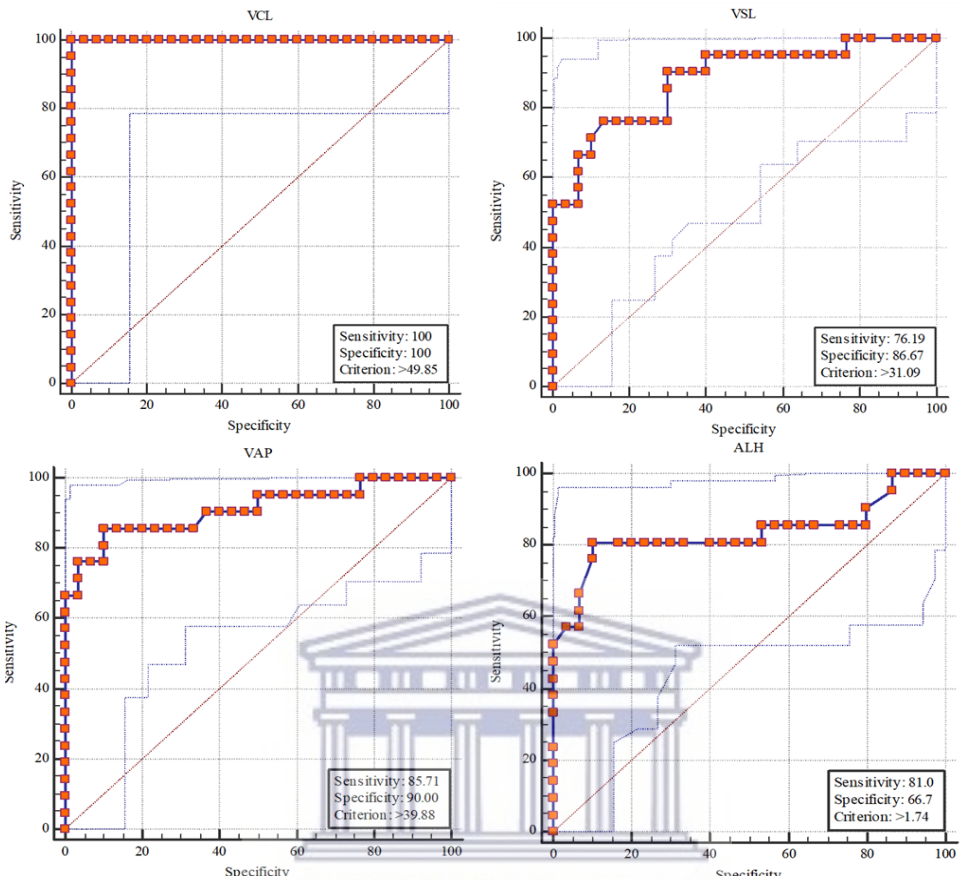


Figure 4.3 ROC curve analysis to determine cut-off values for assessing sperm kinematic parameters. VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; ALH, the amplitude of lateral head displacement.

Cut-off values obtained from the ROC curves were applied and settings for the Motility module in CASA, SCA ® were adjusted accordingly. Adjusted settings for the Motility module were as follows: Medium-Rapid > 75 µm/s, Slow = 50 µm/s and Static < 25 µm/s. The adjusted settings allowed CASA, SCA ® to identify total motility for different sperm subpopulations (progressive, rapid, medium, slow), based on sperm swimming speeds. From this point forward, data presented for sub-populations and swimming speeds were obtained by using the adjusted values in the CASA, SCA ® Motility module and are indicated accordingly as “adjusted”.

Motility parameters for subpopulations and swimming speeds across motility categories

Table 4.4 presents the percentage of progressive swimming sperm in a semen sample and the percentage of progressive swimming subpopulations (i.e. rapid-, medium- and non-progressive) for each motility percentage category. The subpopulations are based on both swimming speed (VCL) and progressivity. Additionally, the percentage of different swimming speeds (rapid, medium, and slow) in a semen sample (based solely on VCL), for the motility categories are also presented. Figure 4.4 illustrates significant differences between the percentage rapid-, medium- and non-progressive sperm in a semen sample, while Figure 4.5 illustrates significant differences between the percentage sperm population swimming speeds (rapid, medium, and slow).

The mean percentage of progressive swimming sperm for a semen sample was 27.5 ± 15.5 %. Furthermore, the percentage of progressive swimming sperm significantly decreased from the >80% motility category (32.6 ± 13.9 %) to the lower motility categories, >60-79% (17.5 ± 8.44 %) and >20-59% (6.37 ± 2.75 %) ($P < 0.001$) (H-value = 19.8).

Mean percentages for the different swimming speeds (rapid, medium, and slow) in semen samples were indicating that majority of sperm had slow swimming speeds (rapid (10.3 ± 8.66 %), medium (24.6 ± 11.7 %), and slow (48.6 ± 13.9 %)). As for the subpopulations, the same tendency was seen for the swimming speeds decreasing across the motility categories. When comparing the percentage of swimming speeds across the different motility categories, rapid swimming sperm seemed to decrease from the higher to the lower motility categories, and only significantly so, between the >80% (12.4 ± 8.92 %) and >20-59% (2.03 ± 1.88 %) categories ($P < 0.001$) (H-value = 13.5), while the percentage medium swimming sperm decreased significantly ($P < 0.001$) (F-ratio = 25.3) from the >80% (29.2 ± 8.91 %) to >60-79% (16.2 ± 7.27 %) and >20-59% (5.50 ± 3.14 %) motility categories. Furthermore, there were significantly more slow swimming sperm in the >20-59% motility category (35.5 ± 6.27 %) compared to the other motility categories (>80% = 50.3 ± 14.6 % and >60-79% = 50.5 ± 7.62 %) ($P = 0.04$) (H-value = 6.63), as one would have expected.

Table 4.4 Baseline motility measurements of Cape honey bee drone sperm (using adjusted cut-offs) (mean ± SD)

Parameters	Mean	> 80 % motility	> 60-79% motility	> 20-59% motility	P-value	F-ratio/ H-value
Sample (n)	51	38	7	6		
TP (%)	27.5 ± 15.5	32.6 ± 13.9[ab]	17.5 ± 8.44[a]	6.37 ± 2.75[b]	<0.001	19.8
RP (%)	5.18 ± 5.02	6.23 ± 5.31[a]	3.05 ± 2.40	0.98 ± 0.97[a]	0.003	11.9
MP (%)	22.3 ± 11.6	26.4 ± 9.80[ab]	14.4 ± 7.02[a]	5.39 ± 2.37[b]	<0.001	20.8
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	13.5
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	6.63

Parametric data are presented as mean and standard deviation (SD). [a,b]Means with the same alphabetical letters in the same row differed significantly. The test statistics are reported as the F-ratio for an ANOVA and the H-value where a Kruskal-Wallis test was performed. TP, Total progressivity; RP, Rapid progressive; MP, Medium progressive; NP, Non-progressive. (TP % = RP % + MP %).

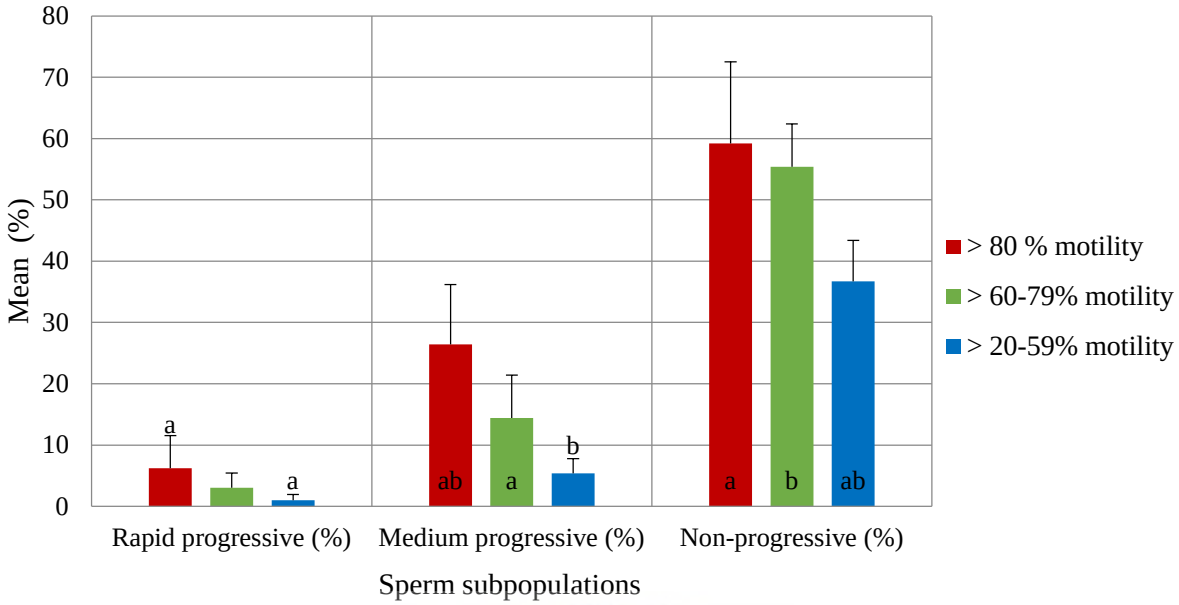


Figure 4.4 Progressive motility subpopulations of Cape honey bee drone sperm across three total motility percentage categories. Bars with the same alphabetical letters differed significantly. Error bars indicate the positive standard deviations only.

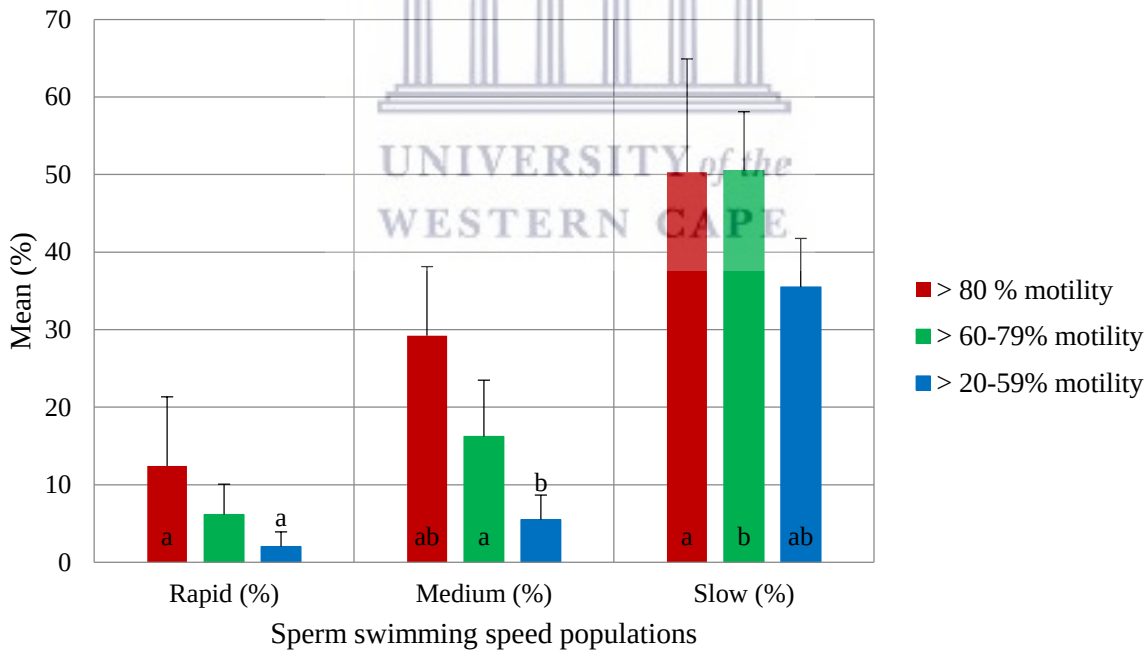


Figure 4.5 Sperm motility speeds of Cape honey bee drone sperm amongst total motility percentage categories. Bars with the same alphabetical letters differed significantly. Error bars indicate the positive standard deviations only.

Kinematic parameters for sperm subpopulations

Table 4.5 and Figure 4.6 present the complete baseline results of honey bee drone kinematic parameters for sperm subpopulations. Data obtained from the adjusted CASA cut-off values were used to determine kinematic parameters for the sperm subpopulations. Hence, values for sperm sub-populations appear much higher when compared to the mean values of the kinematic parameters of the entire sperm populations as obtained in the baseline study. Significant differences were observed between subpopulations for all baseline kinematic parameters.

Mean values for velocity parameters (VCL, VSL, VAP), significantly decreased from rapid- to non-progressive, VCL (99.1 ± 23.0 µm/s, 73.3 ± 15.5 µm/s and 38.0 ± 6.00 µm/s) ($P < 0.001$) (H-value = 119), VSL (69.8 ± 13.7 µm/s, 44.9 ± 4.61 µm/s and 21.6 ± 4.26 µm/s) ($P < 0.001$) (H-value = 127) and VAP (78.9 ± 15.0 µm/s, 57.8 ± 4.69 µm/s and 29.4 ± 5.98 µm/s) ($P < 0.001$) (H-value = 127). Although not significantly so, values of all other kinematic parameters, LIN, STR, ALH and DNC appeared to decrease from rapid- to non-progressive, except for WOB and BCF. The mean values for WOB (83.7 ± 7.25 %) and BCF (17.1 ± 2.07 Hz) was significantly higher (WOB, $P<0.001$; H-value = 10.6 and BCF, $P = 0.00004$; H-value = 20.4) in the medium progressive subpopulation, compared to the rapid- and non-progressive subpopulations.

Table 4.5 Kinematic parameters for Cape honey bee sperm subpopulations (rapid, medium, non-progressive) (using adjusted cut-offs) (mean ± SD)

Parameters	Rapid progressive	Medium progressive	Non progressive	P-Value	F-ratio/ H-value
Sample (n)	51	51	51		
VCL (µm/s)	99.1 ± 23.0^{ab}	73.3 ± 15.5^{ac}	38.0 ± 6.00^{bc}	<0.001	119
VSL (µm/s)	69.8 ± 13.7^{ab}	44.9 ± 4.61^{ac}	21.6 ± 4.26^{bc}	<0.001	127
VAP (µm/s)	78.9 ± 15.0^{ab}	57.8 ± 4.69^{ac}	29.4 ± 5.98^{bc}	<0.001	127
LIN (%)	71.7 ± 13.7^{ab}	67.8 ± 10.2^{a}	54.7 ± 7.35^{b}	<0.001	35.1
STR (%)	86.5 ± 12.7^{ab}	78.7 ± 6.65^{ac}	69.2 ± 5.38^{bc}	<0.001	49.1
WOB (%)	80.8 ± 14.6^{ab}	83.7 ± 7.25^{a}	74.4 ± 7.69^{b}	<0.001	10.6
ALH (µm/s)	3.11 ± 1.25^{ab}	2.32 ± 0.73^{ac}	1.53 ± 0.23^{bc}	<0.001	73.6
BCF (Hz)	15.7 ± 3.74^{a}	17.1 ± 2.07^{ab}	15.3 ± 2.01^{b}	0.00004	20.4
DNC (µm^2/s)	662 ± 399^{ab}	357 ± 232^{ac}	117 ± 34.9^{bc}	<0.001	108

Parametric data are reported as mean and standard deviation (SD). ^{a,b,c}Means with the same letters in the same row differed significantly as obtained from ANOVA. The test statistics are reported as the F-ratio for an ANOVA and the H-value where a Kruskal-Wallis test was performed. ALH, amplitude of lateral head displacement; BCF, beat cross frequency; DNC, dance; LIN, linearity; STR, straightness; VAP, average path velocity; VCL, Curvilinear velocity; VSL, straight-line velocity; WOB, wobble.

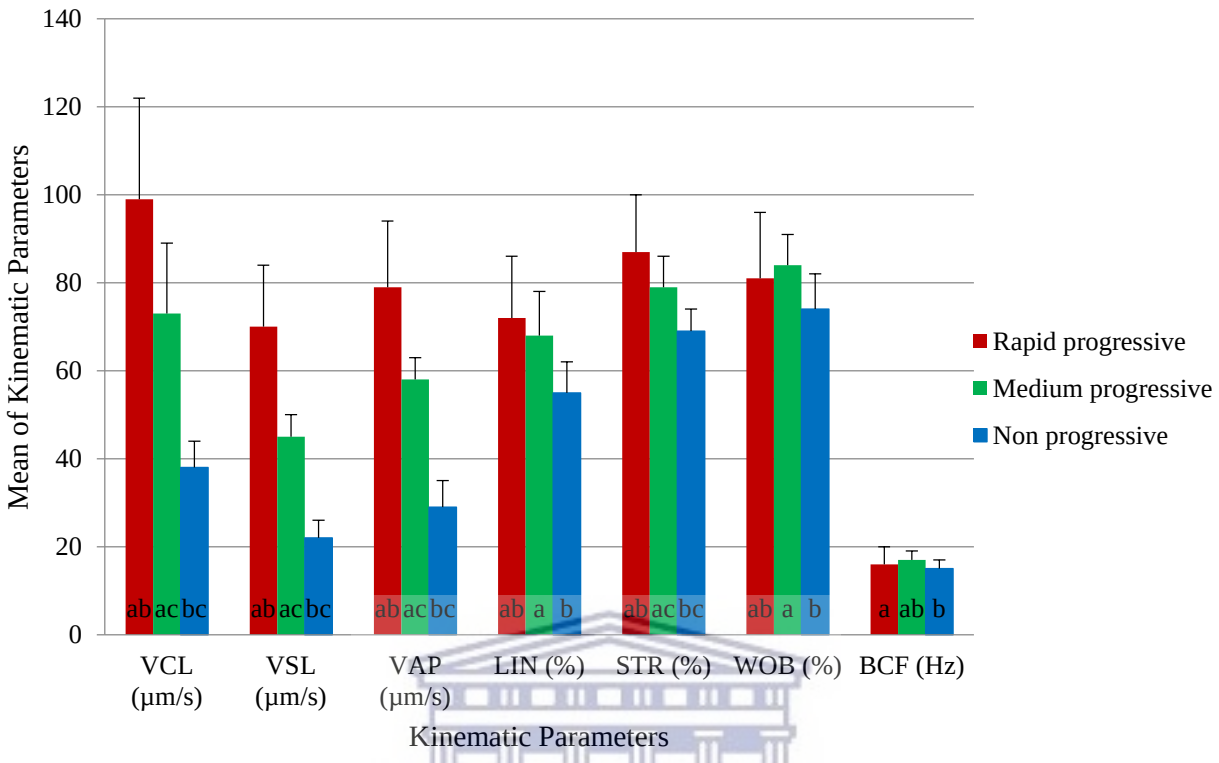


Figure 4.6 Kinematic parameters of honey bee drone sperm subpopulations (rapid-, medium-, non-progressive) (mean ± SD). Error bars indicate the positive standard deviations only. Bars with the same alphabetical letters differed significantly. BCF, beat cross frequency; LIN, linearity; STR, straightness; VAP, average path velocity; VCL, Curvilinear velocity; VSL, straight-line velocity; WOB, wobble.

The actual sperm tracks, constructed by CASA and SCA®, for the different sperm subpopulations are additionally illustrated in Figure 4.7.

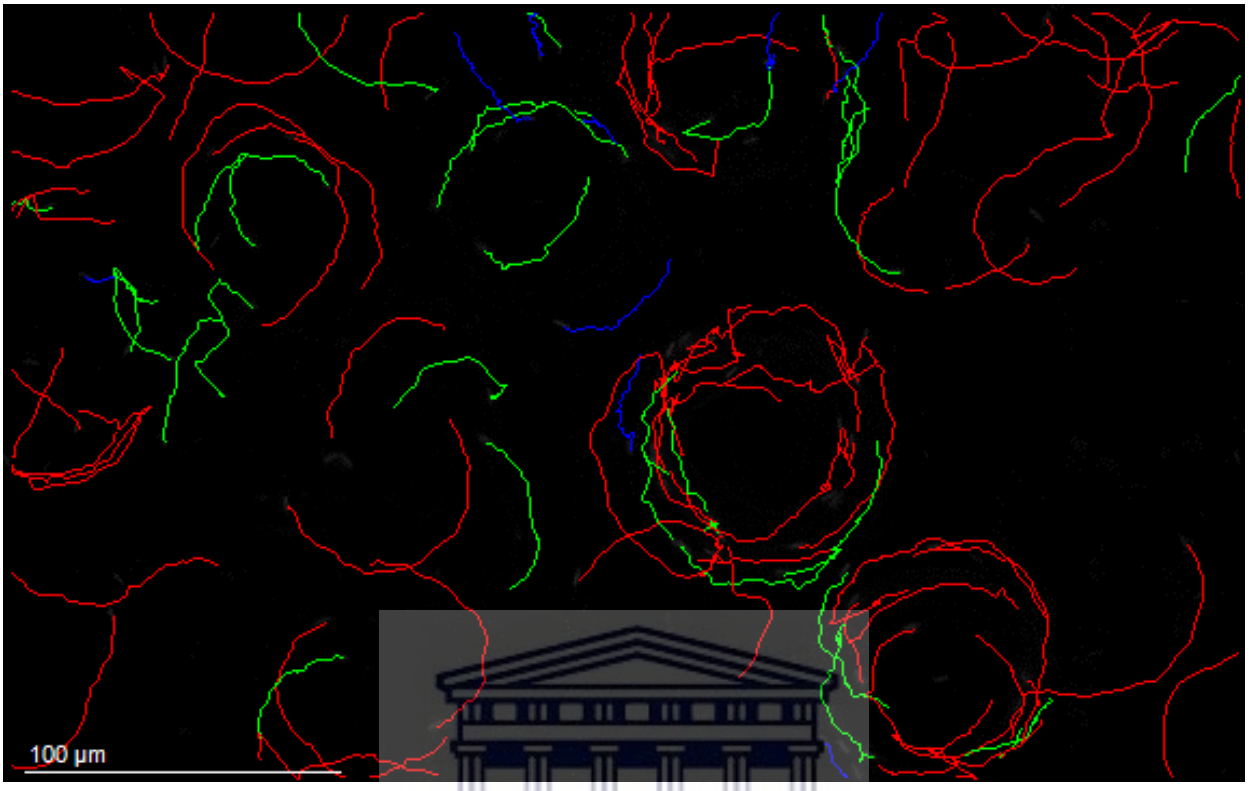
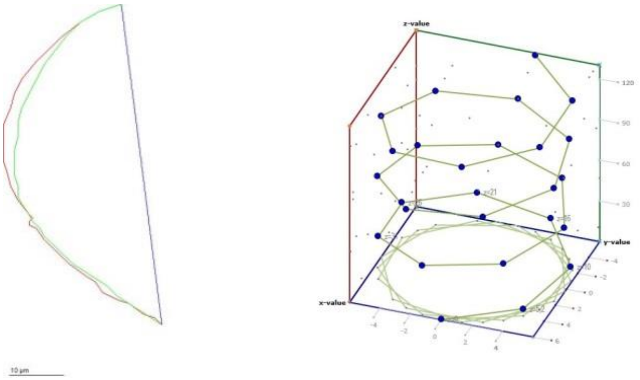


Figure 4. 7 Honey bee drone sperm motility tracks of subpopulations analysed by CASA, SCA ® (red tracks = rapid progressive swimming sperm; green tracks = medium progressive swimming sperm; blue tracks = non-progressive swimming sperm).

Furthermore, Figure 4.8 presents randomly selected 2D sperm tracks of individual sperm from the subpopulations, i.e. rapid-, medium- and non-progressive, as constructed by CASA and SCA ® as well as corresponding 3D tracks, constructed in Microsoft Word. The 2D sperm tracks illustrate the kinematic parameters VSL (blue), VCL (red), and VAP (green), using the X and Y coordinates for every 50th of a second. The 3D reconstructions on the right of each 2D track are built by indirectly determining the Z-axis, with the assumption that sperm swim in a spherical helix.

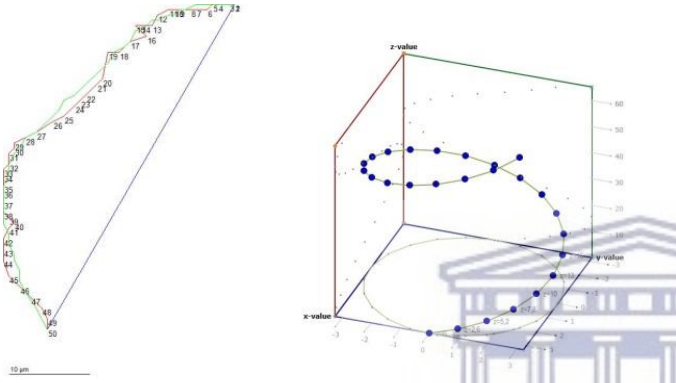


Velocity: Rapid Progressive

VCL: 130, 78 µm/s

VSL: 94.10 µm/s

VAP: 125.14 µm/s

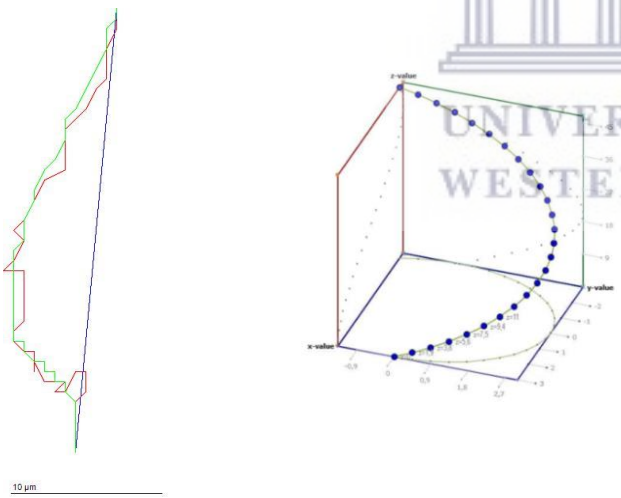


Velocity: Medium Progressive

VCL: 65.90 µm/s

VSL: 47.84 µm/s

VAP: 60.04 µm/s



Velocity: Non Progressive

VCL: 47.17 µm/s

VSL: 30.26 µm/s

VAP: 35.55 µm/s

Figure 4.8 Left – Individual, randomly selected sperm motility tracks for rapid, medium, and non-progressive swimming sperm constructed using CASA and SCA ® based on X and Y coordinates (blue tracks = VSL; red tracks = VCL; green tracks = VAP) Right - 3D reconstructions of individual sperm tracks. Scale = 10 µm.

Kinematic parameters for the entire sperm population

Kinematic parameter measurements for the entire sperm population were grouped into rapid, medium, and slow swimming sperm, as determined from the adjusted CASA cut-off values. These swimming categories refer to sperm swimming speed and are entirely based on VCL (µm/s). Means for the different sperm swimming speeds are presented in Table 4.6. Significant differences between swimming speeds (rapid, medium, and slow) were found across all kinematic parameters ($P < 0.001$) (H-value = 135). The values for velocity parameters (VCL, VSL and VAP) significantly (with almost 50 % each time), decreased from rapid (VCL = 185 µm/s (± 34.5), VSL = 118 µm/s (± 21.4), and VAP = 139 µm/s (± 21.9)), to medium (VCL = 62.5 µm/s (± 5.52), VSL = 44.6 µm/s (± 7.05), and VAP = 53.6 µm/s (± 5.76), to slow (VCL = 30.9 µm/s (± 4.12), VSL = 20.3 µm/s (± 4.75), and VAP = 24.9 µm/s (± 4.28) swimming sperm. The changes in velocity parameters for the three different swimming speeds are illustrated in Figure 4.9.

Furthermore, when compared to rapid and slow sperm populations, medium swimming sperm had significantly higher values for LIN (70.5 ± 5.89 %) (F-ratio = 17.4), STR (80.1 ± 3.41 %) (H-value = 31.9), WOB (84.9 ± 4.13 %) (F-ratio = 44.0), and BCF (F-ratio = 88.2) ($P < 0.001$). In contrast, ALH (H-value = 128) and DNC (H-value = 135) seemed to significantly decrease from rapid (ALH = 3.13 µm/s (± 0.51), DNC = 1214 $\mu m^2/s$ (± 424)) ,to medium (ALH = 1.63 µm/s (± 0.34), DNC = 232 $\mu m^2/s$ (± 213)) and slow swimming sperm (ALH = 0.88 µm/s (± 0.18) (H-value = 128), DNC = 243 $\mu m^2/s$ (± 892) (H-value = 135)) ($P < 0.001$).

Table 4.6 Kinematic parameters for sperm swimming speed classes (rapid, medium, slow) (using adjusted cut-offs) (mean ± SD)

Parameters	Rapid	Medium	Slow	P-Value	F-ratio/ H-value
Sample (n)	51	51	51		
VCL (µm/s)	185 ± 34.5^{ab}	62.5 ± 5.52^{ac}	30.9 ± 4.12^{bc}	< 0,001	135
VSL (µm/s)	118 ± 21.4^{ab}	44.6 ± 7.05^{ac}	20.3 ± 4.75^{bc}	< 0,001	135
VAP (µm/s)	139 ± 21.9^{ab}	53.6 ± 5.76^{ac}	24.9 ± 4.28^{bc}	< 0,001	135
LIN (%)	63.9 ± 6.79^{a}	70.5 ± 5.89 ^{ab}	63.4 ± 7.43^{b}	< 0,001	17.4
STR (%)	76.8 ± 3.89^{a}	80.1 ± 3.41^{ab}	75.0 ± 5.26^{b}	< 0,001	31.9
WOB (%)	76.4 ± 4.56^{a}	84.9 ± 4.13^{ab}	78.6 ± 5.49^{b}	< 0,001	44.0
ALH (µm/s)	3.13 ± 0.51^{ab}	1.63 ± 0.34^{ac}	0.88 ± 0.18^{bc}	< 0,001	128
BCF (Hz)	9.09 ± 2.36^{a}	15.1 ± 2.85^{ab}	10.0 ± 2.05^{b}	< 0,001	88.2
DNC (µm^2/s)	1214 ± 424^{ab}	232 ± 213^{ac}	243 ± 892^{bc}	< 0,001	135

Parametric data are reported as mean and standard deviation (SD). ^{a,b,c}Means with the same letters in the same row differed significantly as obtained from ANOVA. The test statistics are reported as the F-ratio for an ANOVA and the H-value where a non-parametric Kruskal-Wallis test was performed. 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.

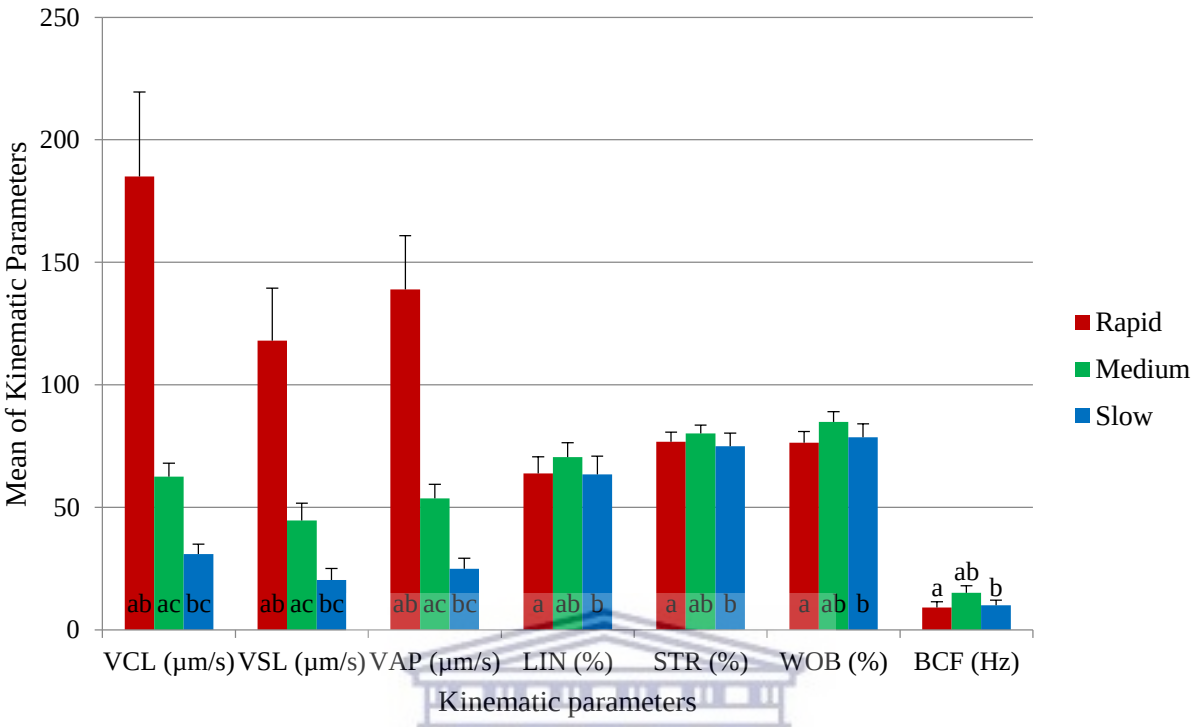


Figure 4.9 Kinematic parameters of the honey bee drone sperm population swimming speeds (rapid-, medium-, slow) (mean ± SD). Error bars indicate the positive standard deviations only. Bars with the same alphabetical letters differed significantly. BCF, beat cross frequency; LIN, linearity; STR, straightness; VAP, average path velocity; VCL, Curvilinear velocity; VSL, straight-line velocity; WOB, wobble.

4.3 Dose-response study (amitraz)

4.3.1 Baseline semen and sperm characteristics

Table 4.7 reports on baseline sperm characteristics of Cape honey bee drone sperm exposed to different concentrations of amitraz. For this study, CASA could only analyse 13 samples that were exposed to 125 µM amitraz. The mean motility index score was 4.20 (± 0.92) and the mean total motility percentage was 68.07 % (± 27.18). No significant differences were observed for motility index scores (F-ratio = 0.68) (P = 0.67) or the mean total motility (F-ratio = 2.09) (P = 0.11), between the control and the three amitraz dosages. Furthermore, compared with the control, both the 125 µM and 500 µM concentrations had a lower total motility %, but not significantly so.

Table 4.7 Baseline characteristics of Cape honey bee sperm exposed to different amitraz concentrations (125 µM, 250 µM, 500 µM) (mean ± SD)

Motility Parameters	Control	125 µM Amitraz	250 µM Amitraz	500 µM Amitraz	P-value	F-ratio
Sample (n)	51	13	18	20		
Motility index	4.20 ± 0.92	4.08 ± 0.95	4.44 ± 0.86	4.05 ± 0.95	0.67	0.68
Total motility (%)	68.07 ± 27.18	75.02 ± 19.80	56.95 ± 29.58	55.46 ± 30.63	0.11	2.09

Parametric data are presented as mean and standard deviation (SD) as obtained from ANOVA.

4.3.2 Effect of amitraz on sperm functional characteristics

Kinematic parameters

Honey bee sperm motility and kinematic parameters were determined at 50 frames per second, by CASA and SCA ®, with cut-off values, as used in the baseline study: slow < 50 > medium < 70 > rapid. These motility settings furthermore allowed for the establishment of sperm subpopulations.

The effects of different amitraz concentrations on honey bee drone sperm kinematic parameters are presented in Table 4.8 and Figure 4.10. None of the kinematic parameters were significantly affected by the different amitraz concentrations. While the mean velocity kinematic values of sperm exposed to the 125 µM amitraz concentration were slightly higher than the control samples' values. However, increased concentrations of amitraz (250 µM and 500 µM) seemed to have some adverse effects on the majority of the motility kinematic parameters, especially the velocity parameters. Velocity parameters showed the tendency to slightly decrease with increasing amitraz exposure (VCL – 125 µM = 68.11 ± 19.25, 250 µM = 58.08 ± 16.21 and 500 µM = 57.45 ± 18.70 µm/s; VSL – 125 µM = 41.89 ± 9.68, 250 µM = 36.15 ± 9.45 and 500 µM 34.57 ± 9.81 µm/s and VAP – 125 µM = 55.52 ± 15.31, 4, 250 µM = 6.99 ± 13.72 and 500 µM = 45.68 ± 15.05 µm/s).

Parameters such as ALH, BCF, and DNC had similar total means throughout the different concentrations.

The trends seen in Table 4.8 are further illustrated in Figure 4.10.

Table 4.8 The effect of amitraz concentrations (125 µM, 250 µM, 500 µM) on Cape honey bee sperm kinematic parameters (using adjusted cut-offs) (mean ± SD)

Parameters	Control	125 µM Amitraz	250 µM Amitraz	500 µM Amitraz	P-value	F-ratio
Sample (n)	51	13	18	20		
VCL (µm/s)	65.26 ± 21.99	68.11 ± 19.25	58.08 ± 16.21	57.45 ± 18.70	0.261	0.138
VSL (µm/s)	39.67 ± 11.91	41.89 ± 9.68	36.15 ± 9.45	34.57 ± 9.81	0.159	1.765
VAP (µm/s)	51.86 ± 17.43	55.52 ± 15.31	46.99 ± 13.72	45.68 ± 15.05	0.244	1.411
LIN (%)	61.63 ± 7.83	62.3 ± 5.63	62.98 ± 8.29	61.44 ±7.08	0.908	0.182
STR (%)	75.05 ± 5.77	74.69 ± 4.56	75.3 ± 6.79	74.92 ± 4.84	0.993	0.031
WOB (%)	79.07 ± 6.91	81.35 ± 4.47	80.32 ± 7.22	76.26 ± 15.61	0.388	1.018
ALH (µm/s)	2.15 ± 0.66	2.28 ± 0.61	1.94 ± 0.52	1.96 ± 0.59	0.302	1.233
BCF (Hz)	16.83 ±2.11	16.69 ± 2.17	17.49 ± 1.78	17.87 ± 2.06	0.194	1.6
DNC ($\mu m^2/s$)	308 ± 201	331 ± 174	241 ± 144	246 ± 155	0.302	1.233

Parametric data are reported as mean and standard deviation (SD). 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.

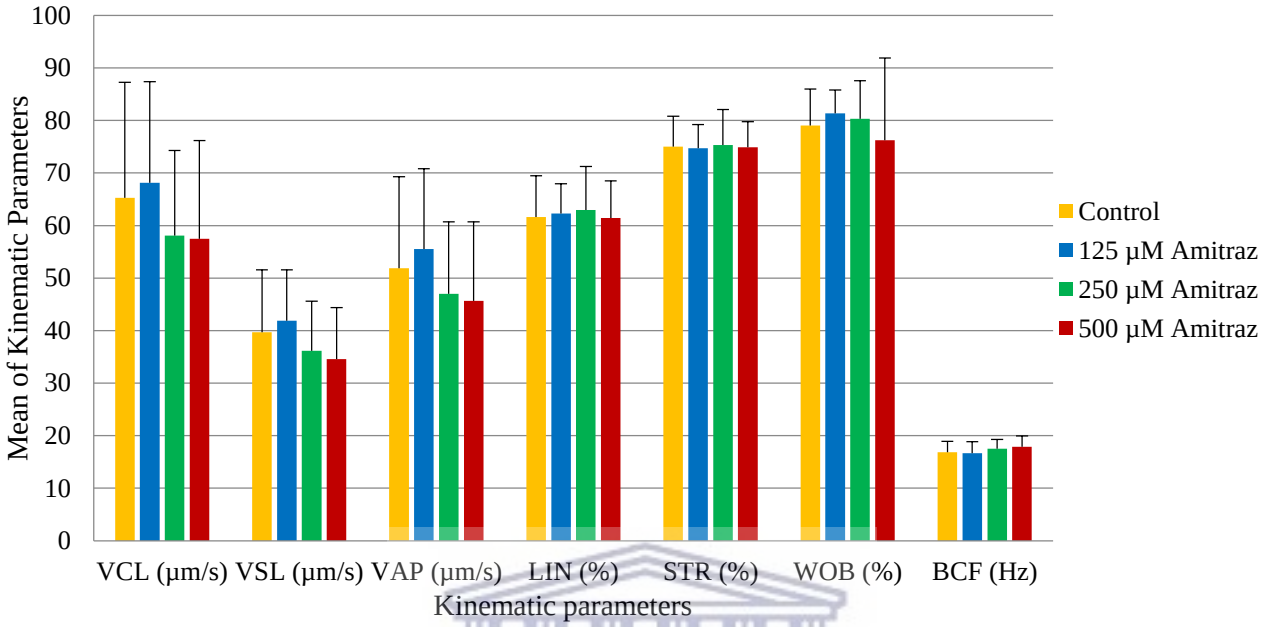


Figure 4.10 Effect of amitraz concentrations on Cape honey bee sperm kinematic parameters (mean ± SD). Error bars indicate the positive standard deviations only. BCF, beat cross frequency; LIN, linearity; STR, straightness; VAP, average path velocity; VCL, Curvilinear velocity; VSL, straight-line velocity; WOB, wobble.

Motility parameters for sperm subpopulations and swimming speeds

Table 4.9 presents the percentage Cape honey bee drone sperm motility for progressive swimming sperm, subpopulations (rapid-, medium- and non-progressive sperm), and swimming speeds (rapid, medium, slow) in a semen sample exposed to three different amitraz concentrations. The mean percentage total progressive motility under normal conditions (control group) was 39.3 ± 28.4 %, and as seen in values for the velocity kinematic parameters, sperm exposed to 125 µM amitraz had a slightly higher value for all progressive motility categories than the control samples, although not significantly so. Furthermore, total progressive motility seemed to decrease with increased amitraz exposure, (125 µM = 45.7 ± 27.9 %, 250 µM = 28.9 ± 25.7 % and 500 µM = 28.1 ± 26.4 %). The same tendency was seen in the subpopulations of motility parameters, i.e. percentage rapid progressive (125 µM = 14.4 ± 12.2 %, 250 µM = 8.37 ± 9.91 % and 500 µM = 7.92 ± 9.83 %), medium progressive (125 µM = 31.3 ± 16.4 %, 250 µM = 20.4 ± 16.6 % and 500 µM = 20.2 ± 17.2 %) and lastly, non-progressive sperm (125 µM = 45.7 ± 27.9 %, 250 µM =

28.9 ± 25.7 % and 500 µM = 28.1 ± 26.4 %). The results of the respective sperm swimming speeds also showed a similar, dose-response trend, but no significant differences amongst the control groups (rapid = 25.9 ± 26.9 %; medium = 19.2 ± 11.1 % and slow = 23.2 ± 13.9 %) and those exposed to amitraz. The percentage of each swimming speed decreased with increased amitraz concentrations, rapid- (125 µM = 31.0 ± 28.3 %, 250 µM = 16.8 ± 21.2 % and 500 µM = 17.7 ± 22.7 %), medium- (125 µM = 21.7 ± 7.36 %, 250 µM = 17.0 ± 12.6 % and 500 µM = 15.6 ± 12.2 %) and slow swimming sperm (125 µM = 22.3 ± 13.3 %, 250 µM = 23.2 ± 12.5 % and 500 µM = 22.1 ± 12.3 %), however, not significantly so.

Table 4.9 The effect of amitraz concentrations (125 µM, 250 µM, 500 µM) on Cape honey bee sperm subpopulations and swimming speeds (using adjusted cut-offs) (mean ± SD)

Parameters	Control	125 µM Amitraz	250 µM Amitraz	500 µM Amitraz	P-value	F-ratio
Sample (n)	51	13	18	20		
TP (%)	39.3 ± 28.4	45.7 ± 27.9	28.9 ± 25.7	28.1 ± 26.4	0.163	1.745
RP (%)	13.3 ± 13.6	14.4 ± 12.2	8.37 ± 9.91	7.92 ± 9.83	0.2	1.576
MP (%)	26.0 ± 16.0	31.3 ± 16.4	20.4 ± 16.6	20.2 ± 17.2	0.164	1.74
NP (%)	28.8 ± 15.3	29.2 ± 13.6	28.1 ± 14.1	27.4 ± 13.6	0.981	0.6
Rapid (%)	25.9 ± 26.9	31.0 ± 28.3	16.8 ± 21.2	17.7 ± 22.7	0.283	1.287
Medium (%)	19.2 ± 11.1	21.7 ± 7.36	17.0 ± 12.6	15.6 ± 12.2	0.421	0.946
Slow (%)	23.2 ± 13.9	22.3 ± 13.3	23.2 ± 12.5	22.1 ± 12.3	0.989	0.405

Parametric data is presented as mean and standard deviation (SD) as obtained from ANOVA. TP, Total progressivity; RP, Rapid progressive; MP, Medium progressive; NP, Non-progressive. (TP % = RP % + MP %).

Kinematic parameters for sperm subpopulations

The effect of different amitraz concentrations on kinematic parameters for drone sperm subpopulations (rapid-, medium- and non-progressive) are presented in Table 4.10. Increasing concentrations of amitraz do not have any significant effect on total sperm motility. However, although not significantly so, kinematics such as velocity parameters (VCL, VSL, and VAP) tended to decrease with increasing concentrations of amitraz.

Table 4.10 Effect of amitraz doses (125 µM, 250 µM, 500 µM) on Cape honey bee sperm subpopulations (rapid, medium, and non-progressive) kinematic parameters (using adjusted cut-offs) (mean ± SD)

	Control	125 µM Amitraz	250 µM Amitraz	500 µM Amitraz	P-value	F-ratio/ H-value
Sample (n)	**51**	**13**	**18**	**20**		
Rapid Progressive						
VCL (µm/s)	90.3 ± 28.9	97.6 ± 6.94	91.4 ± 23.7	72.9 ± 44.2	0.39	2.90
VSL (µm/s)	67.3 ± 21.4	73.2 ± 7.12	69.3 ± 18.7	55.3 ± 33.4	0.7	1.41
VAP (µm/s)	75.7 ± 23.9	82.2 ± 7.39	77.6 ± 20.9	62.6 ± 37.8	0.74	1.26
LIN (%)	69.5 ± 21.6	75.6 ± 7.39	72.1 ± 18.8	57.4 ± 34.3	0.59	1.89
STR (%)	81.6 ± 24.2	88.5 ± 2.56	84.1 ± 21.2	65.9 ± 39.1	0.29	3.71
WOB (%)	78.2 ± 23.8	85.3 ± 6.79	80.7 ± 20.9	64.9 ± 38.7	0.68	1.25
ALH (µm/s)	2.56 ± 0.97	2.78 ± 0.57	2.68 ± 0.83	2.12 ± 1.31	0.64	1.67
BCF (Hz)	14.6 ± 5.27	15.8 ± 1.76	13.9 ± 4.24	11.2 ± 6.86	0.32	3.53
DNC (µm2/s)	509 ± 222	547 ± 137	523 ± 181	414 ± 269	0.27	1.34
Medium Progressive						
VCL (µm/s)	77.8 ± 18.3	77.9 ± 14.4	68.3 ± 20.6	72.8 ± 15.6	0.86	1.49
VSL (µm/s)	45.9 ± 5.01	44.2 ± 3.53	42.1 ± 11.1	42.4 ± 4.74	0.06	2.51
VAP (µm/s)	61.6 ± 10.0	62.9 ± 10.2	56.8 ± 15.9	58.1 ± 10.3	0.29	0.29
LIN (%)	64.5 ± 12.3	61.7 ± 9.85	62.8 ± 18.3	63.3 ± 11.2	0.89	0.2
STR (%)	75.7 ± 9.99	73.1 ± 9.03	72.4 ± 19.6	74.8 ± 9.18	0.73	0.43
WOB (%)	83.3 ± 7.66	82.7 ± 5.77	80.5 ± 20.9	82.3 ± 8.19	0.84	0.28
ALH (µm/s)	2.53 ± 0.82	2.60 ± 0.63	2.19 ± 0.77	2.39 ± 0.75	0.39	1.01
BCF (Hz)	16.9 ± 2.66	16.3 ± 2.23	16.2 ± 4.49	18.3 ± 3.48	0.15	1.82
DNC (µm2/s)	421 ± 257	421 ± 179	329 ± 165	369 ± 198	0.44	0.91
Non Progressive						
VCL (µm/s)	43.3 ± 6.19	46.2 ± 6.71	42.5 ± 6.36	41.9 ± 6.53	0.23	1.26
VSL (µm/s)	23.7 ± 4.46	27.1 ± 2.15	24.5 ± 3.95	23.8 ± 3.68	0.05	2.65
VAP (µm/s)	32.6 ± 5.97	36.3 ± 5.27	32.9 ± 5.34	32.3 ± 5.58	0.18	1.67
LIN (%)	55.2 ± 10.2	57.4 ± 7.27	58.5 ± 9.56	57.8 ± 7.93	0.5	0.79
STR (%)	70.4 ± 7.99	70.9 ± 6.32	72.2 ± 7.79	72.7 ± 5.59	0.62	0.6
WOB (%)	72.9 ± 11.4	78.7 ± 6.14	76.9 ± 7.42	76.4 ± 6.07	0.13	1.92
ALH (µm/s)	*	*	*	*	*	*
BCF (Hz)	*	*	*	*	*	*
DNC ($\mu m^2/s$)	*	*	*	*	*	*

Parametric data are reported as mean and standard deviation (SD). The asterisk (*) indicates no data obtained. The test statistics are reported as the F-ratio for an ANOVA and the H-value where a non-parametric Kruskal-Wallis test was performed.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.

For the majority of the kinematic parameters of the subpopulations (rapid-, medium- and non-progressive), no significant differences were observed between the different amitraz concentrations and controls. A declining trend was however noted, across all subpopulations, with further increasing concentrations of amitraz for the respective velocity parameters (VCL – 125 µM = 97.6 ± 6.94, 250 µM = 91.4 ± 23.7 and 500 µM = 72.9 ± 44.2 µm/s; VSL – 125 µM = 73.2 ± 7.12, 250 µM = 69.3 ± 18.7 and 500 µM = 55.3 ± 33.4 µm/s; VAP – 125 µM = 82.2 ± 7.39, 250 µM = 77.6 ± 20.9, 500 µM = 62.6 ± 37.8 µm/s). A similar trend was seen in parameters LIN, STR and WOB, where data obtained seemed to decline with an increased concentration amitraz, or non-progressive sperm, VSL was the only parameter that significantly decreased in a dose response manner with increased amitraz concentrations ($p = 0.05$), (VSL – 125 µM = 27.1 ± 2.15, 250 µM = 24.5 ± 3.95 and 500 µM = 23.8 ± 3.68 µm/s). No data on ALH, BCF or DNC were obtained.

Kinematic parameters for swimming speeds

Table 4.11 presents the results of kinematic parameters of swimming speeds (rapid, medium, and slow swimming sperm) for different amitraz concentrations. No significant differences between the kinematic parameters of the different swimming speeds (rapid, medium, and slow), and respective amitraz concentrations were observed. For rapid swimming sperm, however, there seemed to be negative associations between increasing amitraz concentrations and LIN, STR, and DNC.

Table 4.11 Effect of amitraz concentrations (125 µM, 250 µM, 500 µM) on kinematic parameters for swimming speeds (rapid, medium, slow) (using adjusted cut-offs) (mean ± SD)

	Control	500 µM Amitraz	250 µM Amitraz	125 µM Amitraz	P-value	F-ratio/ H-value
Sample (n)	**51**	**13**	**18**	**20**		
			Rapid			
VCL (µm/s)	168 ± 39.2	160 ± 32.1	160 ± 41.3	166 ± 33.9	0.80	0.33
VSL (µm/s)	108 ± 22.3	99.3 ± 21.6	117 ± 48.2	105 ± 22.2	0.56	2.07
VAP (µm/s)	127 ± 23.3	121 ± 22.5	129 ± 2.82	127 ± 22.1	0.77	0.46
LIN (%)	64.7 ± 4.99	62.7 ± 8.85	65.0 ± 4.38	66.5 ± 6.11	0.22	1.49
STR (%)	78.5 ± 8.83	79.6 ± 3.88	76.9 ± 3.70	77.0 ± 2.88	0.27	3.89
WOB (%)	77.1 ± 5.98	77.3 ± 6.58	79.0 ± 4.43	77.9 ± 5.71	0.69	0.50
ALH (µm/s)	3.05 ± 0.35	2.93 ± 0.29	3.29 ± 1.32	2.96 ± 0.24	0.52	2.26
BCF (Hz)	9.56 ± 2.06	9.78 ± 2.41	9.28 ± 3.22	9.74 ± 1.98	0.95	0.11
DNC (µm2/s)	1038 ± 311	941 ± 232	1075 ± 574	986 ± 234	0.64	0.57
			Medium			
VCL (µm/s)	60.6 ± 3.77	61.0 ± 1.90	58.8 ± 6.26	61.7 ± 1.81	0.32	3.50
VSL (µm/s)	44.0 ± 4.84	41.0 ± 5.61	44.4 ± 7.39	43.9 ± 3.28	0.22	4.48
VAP (µm/s)	52.7 ± 7.23	49.9 ± 5.23	50.6 ± 4.34	52.5 ± 2.84	0.41	2.87
LIN (%)	71.4 ± 6.48	67.6 ± 8.85	70.0 ± 8.44	72.5 ± 6.01	0.32	3.53
STR (%)	81.0 ± 7.00	79.5 ± 3.88	81.1 ± 5.40	81.9 ± 2.59	0.33	3.47
WOB (%)	82.9 ± 4.39	82.2 ± 8.02	83.2 ± 7.03	83.6 ± 4.83	0.90	0.20
ALH (µm/s)	1.44 ± 0.29	1.49 ± 0.25	1.44 ± 0.36	1.59 ± 0.12	0.39	1.00
BCF (Hz)	13.4 ± 2.58	14.8 ± 2.89	13.8 ± 2.84	14.5 ± 1.67	0.20	1.59
DNC (µm2/s)	175 ± 36.0	181 ± 34.1	171 ± 52.0	195 ± 12.2	0.29	1.26
			Slow			
VCL (µm/s)	30.8 ± 10.9	36.0 ± 1.96	35.1 ± 4.98	37.4 ± 1.18	0.14	5.41
VSL (µm/s)	23.79 ± 5.19	22.3 ± 3.48	25.2 ± 5.21	24.5 ± 2.49	0.27	1.31
VAP (µm/s)	30.9 ± 10.9	27.8 ± 3.00	29.0 ± 2.82	29.7 ± 1.90	0.25	4.09
LIN (%)	63.3 ± 9.81	60.4 ±7.63	63.3 ± 8.59	65.4 ± 6.76	0.44	0.90
STR (%)	74.8 ± 12.0	75.5 ± 5.97	76.7 ± 6.24	77.9 ± 3.71	0.68	1.52
WOB (%)	75.9 ± 9.66	75.9 ± 5.92	78.1 ± 5.68	77.5 ± 5.54	0.75	1.22
ALH (µm/s)	0.93 ± 0.20	1.02 ± 0.16	0.96 ± 0.25	0.97 ± 0.16	0.45	0.89
BCF (Hz)	12.1 ± 3.24	14.0 ± 2.97	12.7 ± 3.35	12.5 ± 3.13	0.18	1.68
DNC ($\mu m^2/s$)	67.3 ± 15.4	73.00 ± 12.8	66.7 ± 21.1	72.6 ± 11.0	0.40	1.00

Parametric data are reported as mean and standard deviation (SD). The test statistics are reported as the F-ratio was reported for an ANOVA and the H-value where a Kruskal-Wallis test was performed.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.

4.3.3 Effect of amitraz doses on sperm structural characteristics

Vitality

Figure 4.11 illustrates live and dead honey bee sperm as stained with SYBR14 (green) and counterstain, PI (red).

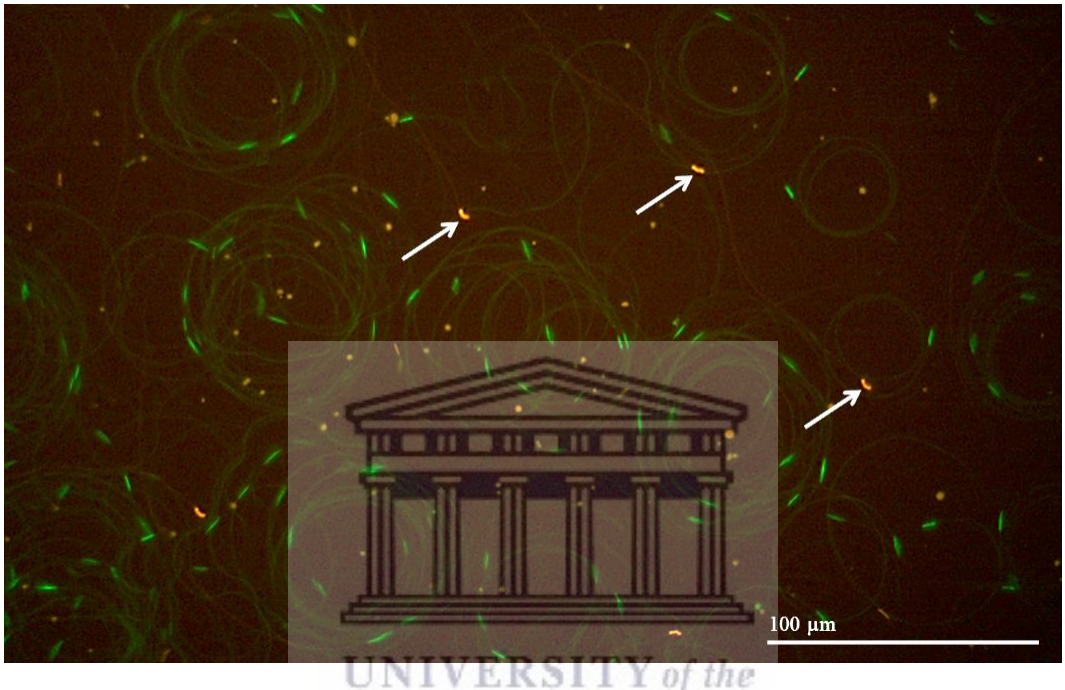


Figure 4.11 Sperm cells stained with SYBR14 and PI for vitality analysis (red = dead;green = live)

Table 4.12 and Figure 4.12 present the effect of amitraz concentrations on honey bee drone sperm vitality. Increased amitraz concentrations negatively affect vitality. The percentage vitality for honey bee drone sperm was significantly higher (P = 0.01) in the control group (79.4 ± 13.7 %) versus the 250 µM and 500 µM amitraz concentrations (125 µM = 68.8 ± 21.2, 250 µM = 58.3 ± 26.7 and 500µM = 56.3 ± 20.4 %).

Table 4.12 The effect of amitraz concentrations (125 µM, 250 µM, 500 µM) on honey bee sperm vitality

	Control	125 µM Amitraz	250 µM Amitraz	500 µM Amitraz	P-value	F-ratio
Sample size (n)	51	13	18	20		
Vitality (%)	79.37 ± 13.75[ab]	68.82 ± 21.16	58.30 ± 26.70[a]	56.27 ± 20.35[b]	0.04	5.60

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.

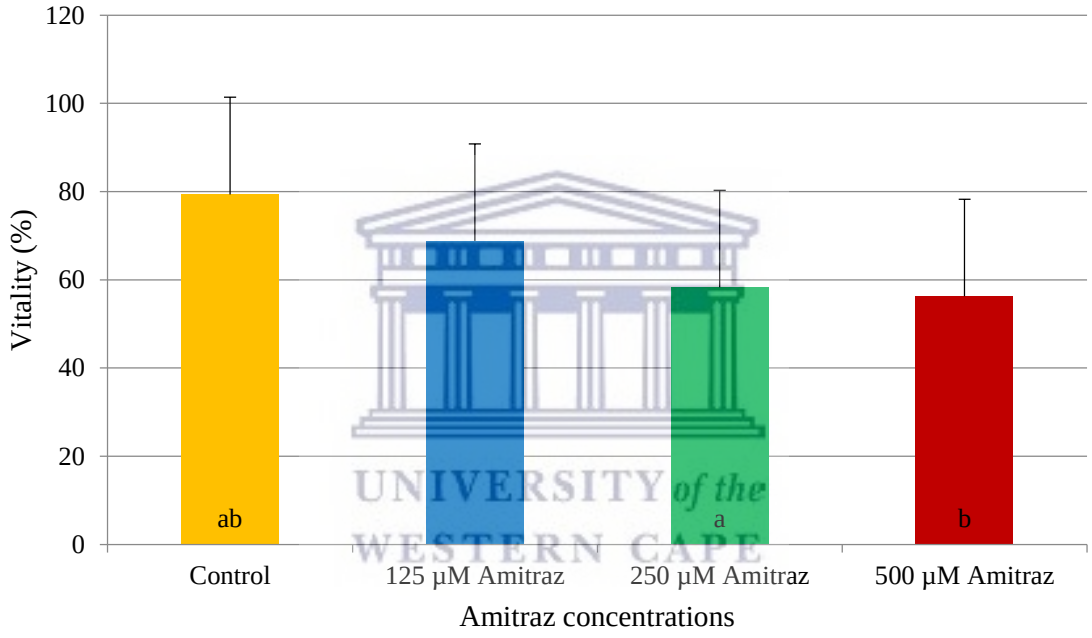


Figure 4. 12 Bar graph illustrating the effect of amitraz concentrations (125 µM, 250 µM, 500 µM) on Cape honey bee sperm vitality. Error bars indicate the positive standard deviations only. Bars with the same letter indicate significant differences between them.

General sperm morphology

The measurements of different honey bee drone sperm components of dead sperm as obtained from BrightVit stained sperm are presented in Table 4.13. The mean length of honey bee drone sperm components in the control group was 8.92 ± 1.15 µm (head), 209 ± 10.7 µm (tail), and 217 ± 10.7 µm (total length).

No significant differences were observed between control and amitraz concentrations for the main sperm components, i.e. head, tail, and total sperm lengths. A unique structure, only visible in dead stained sperm, was observed. The structures appear to be mitochondrial derivatives arranged in a helical fashion around the axoneme. Measurements of mitochondrial structures significantly differed between the control and amitraz concentrations (P = 0.01). Lengths of the 125 µM and 500 µM exposed sperm decreased significantly compared with the control samples.

Table 4. 13 Effect of amitraz doses (125 µM, 250 µM, 500 µM) on sperm component measurements of Cape honey bee drone sperm (mean ± SD) (n = 51)

	Control	125 µM Amitraz	250 µM Amitraz	500 µM Amitraz	P-value	F-ratio/ H-value
Head (µm)	8.92 ± 1.15	8.98 ± 0.95	9.23 ± 1.30	9.50 ± 0.89	0.10	2.95
Tail (µm)	209 ± 10.7	206 ± 12.1	208 ± 9.56	204 ± 12.2	0.23	1.47
Total length (µm)	217 ± 10.7	215 ± 12.3	217 ± 9.32	214 ± 12.2	0.33	3.06
Mitochondrial structure (µm)	39.7 ± 3.86^{ab}	38.9 ± 4.54^{a}	37.2 ± 3.43^{c}	37.9 ± 2.86^{bc}	0.01	4.46

Parametric data are presented as mean and standard deviation (SD). ^{a,b,c}Means with the same alphabetical letter in the same row differed significantly. The test statistics are reported as the F-ratio for an ANOVA and the H-value where a Kruskal-Wallis test was performed.

It furthermore showed how the mitochondrial structure separates from the rest of the sperm cell when the cell is no longer viable. Figure 4.13 shows a viable sperm cell, in a circular position, *vs*. a non-viable sperm cell, and Figure 4.14 further illustrates the separation of the mitochondrial derivative and the non-viable sperm cell.

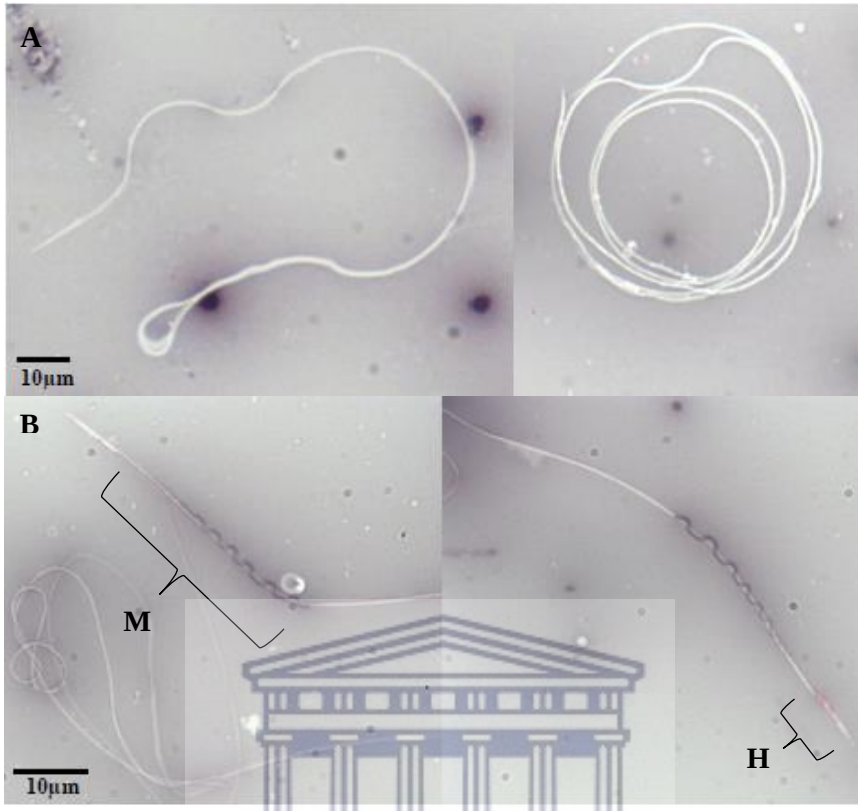


Figure 4.13 BrightVit stained sperm (Bright-field micrograph taken using 60X objective) (a) White (live sperm) vs. (b) Pink stained, non-viable sperm (Bright-field micrograph taken using 100X objective). H, head; MS, mitochondrial structure; T, tail.

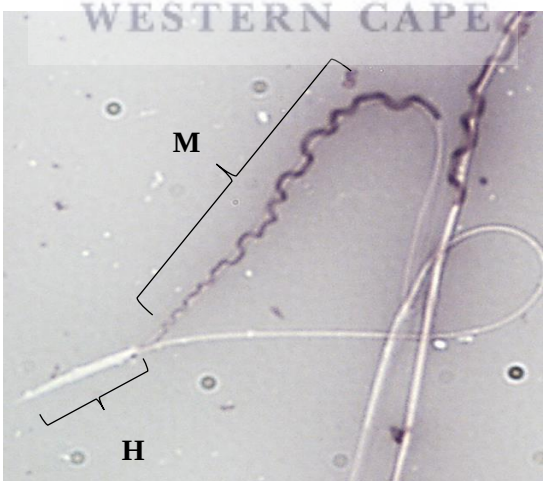


Figure 4.14 Honey bee drone sperm stained with BrightVit (100X objective) illustrating mitochondrial separation from non-viable sperm. H, sperm head; M, Mitochondria.

CHAPTER 5: DISCUSSION AND CONCLUSION

5.1 Introduction

This study aimed to evaluate sperm quality of the Cape honey bee, especially sperm motility, using both a semi-quantitative and qualitative method, and to determine the effect of amitraz on sperm quality parameters (structural and functional). Honey bee drone semen quality is important owing to its overall effect on queen quality, and colony performance (Ciereszko et al. 2017; Yániz et al. 2020). Drone semen and sperm quality, to date, have mainly been described in terms of semen volume, sperm concentration, and vitality, while the role of sperm motility received less attention in determining sperm quality (Yániz, et al., 2020). Besides drone sperm motility that is required for sperm to migrate in the female reproductive tract following copulation and for fertilization of eggs, it is suggested that sperm motility and swimming patterns, particularly in the spermatheca is of benefit for fertilization. Furthermore, as part of environmental factors, the effect of miticides (such as active ingredient amitraz) on sperm viability is inconclusive.

In this study 51 Cape honey bee drone semen samples were analysed for a baseline study, and 102 drone semen samples were analysed for a dose-response study.

This study aimed to achieve the following objectives:

- To establish a technique to assess honey bee drone sperm motility by determining sperm motility and kinematics using a fluorescence method and a CASA system and compare with a manual motility index score
- To determine and compare the *in vitro* effect of three amitraz concentrations on sperm functional characteristics, i.e., motility parameters and motility kinematic parameters using a CASA system.
- To determine and compare the *in vitro* effect of three amitraz concentrations on sperm structural characteristics including dimensions of sperm components using the BrightVit staining method
- To determine and compare the *in vitro* effect of three amitraz concentrations on sperm viability using dead/live staining methods, SYBR14 and propidium iodide, and BrightVit.

For the remainder of Chapter 5 the results, as presented in Chapter 4, will be discussed to achieve the aim and objectives of this study outlined above. The discussion will first focus on results obtained from the baseline study, including the basic semen characteristics, and comparisons between different methods used to determine total sperm motility percentage, followed by the comparison of parameters among different motility categories. Secondly, the results obtained from the dose-response study will be discussed, including the comparisons between exposed and unexposed samples. Lastly, possible limitations of the study as well as recommendations for future research will be provided.

5.2 Honey bee drone baseline study

5.2.1 Standard semen characteristics

Semen volume and sperm concentration

Semen volumes obtained from the Cape honey bee drones in this study ranged between 0.4-0.7 µL per sample, which is less compared to previously reported mean semen volumes for this species (1.15 µL per sample) (Buys 1990). Semen volumes from this study were also less compared to the mean semen volumes of subspecies of south-east Australian (0.72 – 1.12 µL/sample) (Rhodes, et al., 2011). However, the semen volumes of the Cape honey bee drone are in agreement with those of the European subspecies, *A.m Linnaeus* , (0.4 – 2.4 µL/sample) (Rousseau, et al., 2015). Discrepancies between the findings of this study and other studies might be due to the lack of suitable equipment in this study to measure semen volume more accurately, such as a precision syringe, which is commonly used in honey bee drone semen research (Yániz, et al., 2020). Furthermore, variations between semen volumes obtained from different subspecies might also be owed to biological differences such as drone size (Schlüns, et al., 2003), which was not assessed in this study.

Honey bee drone semen volume is made up of sperm, and seminal fluid which mainly contributes to the maintenance of sperm longevity, and furthermore sperm competition inside the reproductive tract of the queen bee (Brutscher, et al., 2019). Semen volume is an indicator of sperm concentration and further contributes to the successful insemination of honey bee queens (Mackensen, 1964). Both components of semen, i.e. seminal fluid and sperm are related to queen fertility and colony performance (Pettis, et al., 2016; Brutscher, et al., 2019). Seminal fluid contributes to sperm longevity, especially during storage in the spermatheca of the queen, and to the post-copulation changes that occur in the queen (Brutscher, et

al., 2019; Jasper, et al., 2020). Sperm on the other hand is required to fertilize eggs, to give rise to worker, and queen bees that are in turn important for the whole colony (Johannsmeier, 2001).

Honey bee drone sperm concentration is one of the most commonly assessed parameters, and furthermore an important indicator of semen quality, given that sperm numbers contribute to fertilization success (Baer, et al., 2006; Yániz, et al., 2020). In this study, the CASA system could not detect and capture all the sperm present in a sample owing to complex swimming patterns of honey bee sperm in samples with minimal dilution. To rectify this, a manual sperm count was performed using a Makler counting chamber, and therefore only a sperm concentration range (1.10×10^6 – 16.5×10^6 million per µL) was determined, instead of a mean value. Average sperm concentrations for honey bee drones usually range between 2 – 9 million sperm per µL in other subspecies (Yániz, et al., 2020). Taken into account that Cape honey bee semen volumes in this study were less than the average 1 uL of semen volume per sample, its sperm concentrations are in agreement with values reported for other honey bee species.

It should be noted that honey bee sperm concentration is highly variable, and several factors can contribute to variations in semen volume and sperm concentration, such as drone age, genetics, and size (Schlüns, et al., 2003; Czekońska, et al., 2013). For example, sperm concentrations reach a peak in the seminal vesicles of drones aged between days 7 to 9, whereas the sperm concentrations obtained from the ejaculates of sexually matured drones (day 12 and older) are usually lower (1.5 – 7.3 million per sample) compared to the numbers in seminal vesicles (Mackensen, 1964). Indeed, the sources of sperm collection and assessment methods (Buys, 1990; Rhodes, et al., 2011; Rousseau, et al., 2015) may also affect semen volume and subsequently sperm concentration. For example, in Cape honey bee drones, Buys (1990) measured semen volume and sperm concentration from the spermathecal contents of the queen, while Rhodes, et al. (2011) and Rousseau, et al. (2015) determined semen volume and sperm concentration from freshly, and manually ejaculated European drones (different *Apis mellifera* species from South East Australia, and Italy).

Although the exact ratio of seminal fluid *versus* sperm concentration in the honey bee is unknown, it is likely to vary among different species, and it further seems that semen volume *per se* is not necessarily always determining sperm concentration in some species.

5.2.2 Sperm functional characteristics

Manual motility index and CASA total motility percentage

Results obtained from the baseline study have shown that motility of a drone semen sample, as assigned by a motility index score, taking both sperm movement and swimming patterns into account, is comparable with its motility percentage as determined by a CASA system. Cape honey bee semen samples in this study generally had a high motility index score of 4.11, indicative of the presence of at least seven groups of relay swimming sperm in a semen sample, which furthermore corresponded with a high total sperm motility percentage (85.5 %) as determined by CASA. These findings are in agreement with North-African honey bee drone (*Apis mellifera intermissa*) sperm total motility percentages of ± 80% determined by a CASA system (Inouri-Iskounen, et al., 2020). Furthermore, in this study, both the manual motility index scores and total motility percentages (CASA) significantly decreased alongside decreasing (determined using CASA) three motility percentage categories (>80%; 60-79%, and <25% motility).

Findings from this study further highlighted the relationship between sperm swimming patterns and motility, particularly as seen for circular, relay swimming groups of sperm demonstrating high sperm motility. These results are in agreement with the findings of Inouri-Iskounen (2020), illustrating that the number of circular swimming sperm is continually present in samples with high motility percentages and index scores. Furthermore, for other insect species, such as the desert ant motility of sperm increased in the presence of sperm-sperm interaction, i.e. sperm swimming together in groups presented increased motility values compared to isolated singular swimming sperm (Pearcy, et al.,2014),

Motility grade scores, based on the percentage of motile sperm in a semen sample, and the manual count of motile sperm, have so far been the sole indicators of sperm motility quality in honey bees (Yániz, et al., 2020). Recent studies typically report on the percentage of motile honey bee sperm present in a sample, as it is considered an important motility parameter that is readily identifiable, and gives an immediate impression of sperm functionality and overall quality (Yániz, et al., 2019).

The phase-contrast methods used to determine honey bee sperm motility, however, do not quantify actual sperm velocity (Inouri-Iskounen, et al., 2020). Furthermore, gross sperm motility does include sperm swimming patterns, which have been previously reported as an indicator of sperm quality (Yániz, et al., 2020). Although Locke & Peng (1993) previously established a motility classification system: for this

study, there was a need for modification thereof to take into consideration particularly swimming patterns of sperm (e.g. helical swimming groups of sperm), as reported on in recent literature (Tofilski, et al., 2018; Yániz, et al., 2020).

Additionally, Tofilski et al. (2018) has suggested that the presence of whirls of circular swimming sperm close to the spermathecal wall, at the entrance of the spermathecal duct, are most likely the ones to enter the duct first, and migrate to the egg for fertilization, thus assuming that there is a relation between circular swimming groups of sperm and motility quality. The association between swimming patterns and sperm motility has further been supported by Wegener et al. (2017) who demonstrated that motility increased with circular swimming sperm, and suggested that sperm of good quality are utilized during the start of oviposition (Wegener, et al., 2017). The circular swimming pattern of insect sperm is also owed to a long sperm tail, suggested to prevent entanglement during storage and also as releasing mechanism of individual sperm for fertilization (Al-Lawati, et al., 2009). Therefore, groups of sperm swimming together are assumed to be associated with good motility quality and having an advantage in sperm competition before egg fertilization (Al-Lawati, et al., 2009; Wegener, et al., 2012).

This study further report for the first time on successful analyses of honey bee drone sperm motility using a fluorescent method and CASA and SCA system. In-depth sperm motility assessments in humans and other animal species are routinely done using a CASA system, which entails the assessment of motility percentage and kinematic parameters, furthermore differentiating between the averages of entire sperm populations as well as its respective subpopulations (Maree & Van der Horst, 2013). However, such analyses are lacking for honey bee drone sperm. Using CASA to assess sperm motility allow for an objective report on motility quality, simultaneously also increasing the reproducibility of sperm measurements (Maree & Van der Horst, 2013). A system such as CASA does, however, require a prominent sperm head as a point of reference, therefore, tracking honey bee sperm behaviour with CASA could, until recently, not be used successfully to determine sperm motility and kinematic parameters (Lu, et al., 2014; Yániz, et al., 2020). To overcome the problem of an indistinguishable head and tail, as present in honey bee sperm, a fluorescence-based CASA and SCA ® technique was developed, to allow CASA to detect, and track the honey bee sperm head, and accurately measure motility, which allowed for the standardization of drone sperm velocity parameters and other important kinematic parameters.

Motility and kinematic parameters

Kinematic parameters for the three motility percentage categories (determined by CASA), as expected, have shown that sperm with the highest motility percentage (>80% motility category) (groups of sperm swimming in a circular or relay fashion) had the highest values for velocity parameters.

Compared to the results obtained for velocity parameters in this study (VCL = 50.3 µm/s, VSL = 30.4 µm/s and VAP = 39.5 µm/s), using CASA, manually-determined drone sperm swimming speeds (VCL range = 19 – 32 µm/s) (Al-Lawati, et al., 2009), and CASA-determined kinematic parameter values, using conventional phase-contrast microscopy (VCL = 21 um/s, VSL = 8.19 µm/s, VAP = 13.98 um/s) (Inouri-Iskounen, et al. 2020), were much lower. However, results in this study correspond with findings from a preliminary study, using only phase-contrast microscopy and the manual tracking of a sperm head (VCL = 56.81 µm/s and VSL = 42.73 µm/s) (Murray, 2019 unpublished). It should further be noted that Inouri-Iskounen, et al. (2020) assessed drone sperm motility using CASA and phase-contrast microscopy, the low values obtained in their study could thus be ascribed to CASA not being able to detect and track sperm heads accurately. Discrepancies in results from both manual and CASA studies could be attributed to the techniques used, sample size, and CASA analysis settings (Al-Lawati, et al., 2009; Inouri-Iskounen, et al., 2020). In addition, it should be noted that the accurate tracking of honey bee sperm, especially when using CASA, requires a fluorescence technique, as demonstrated in this study.

The significance of velocity parameters, VCL, VSL, and VAP, is owed to their direct association with sperm migration through the female genital tract (Robayo, et al., 2008). VCL describes the swimming speed of a sperm cell, and it also directly correlates with in-vitro fertilization (IVF) success in other animals and the rate at which it occurs (Holt, et al., 1994). Sperm movement characteristics, such as VCL, are therefore a prerequisite for successful capacitation during fertilization; high values for velocity parameters are considered predictors of sperm fertilization success rates. However, the fertilization success rates of a sperm cell are not only dependent on velocity, but also rely on parameters such as ALH, given its association with cervical mucus penetration (Mortimer, et al., 1995).

As for the velocity parameters, markers of sperm progressivity, i.e. LIN, STR, and WOB were the highest in semen samples that had a total motility percentage greater than 80%, the same tendency was seen for BCF, a parameter referring to the vigor of flagellar beating, contributing to sperm progressiveness (Bompart, et al., 2018). This study further established motility cut-off values for Cape honey bee drone sperm, using ROC curve analysis based on the kinematic parameter values (VCL, VSL, VAP, and ALH)

that significantly differed among the three motility percentage categories. The cut-off values determined (VCL > 49.85 µm/s, VSL > 31.09 µm/s, VAP > 39.88 µm/s and ALH > 1.74 µm/s) could be applied to accurately determine other motility parameters, such as progressivity and swimming speeds. Establishing species-specific cut-off values is important to correctly determine motility parameters, kinematic parameters and identify motility subpopulations, thus allowing for a detailed report on sperm kinematic parameters, which subsequently describes fertility potential and also the true effect of treatment and environmental factors on individual sperm cells (Maree & Van der Horst, 2013). To our knowledge, this study is the only study thus far to determine and make use of such velocity cut-off values for honey bees, and insects. The only similar study done on honey bee sperm by Inouri-Iskounen, et al. (2020), did not report on the use of cut off values.

For the first time, this study reports on the progressivity and swimming speed of honey bee sperm. Sperm progressiveness was firstly measured as total progressivity, describing the total amount of sperm that moved progressively forward, which was low (27.5 %). This is understandable because, as in this study, circular groups of swimming sperm are more prevalent in the honey bee than progressively forward swimming sperm (Tofilski, et al., 2018; Yániz, et al., 2020). However, the >80% motility percentage category had the highest percentage of progressive swimming sperm, illustrating the relationship between total motility and progressivity, but also indicating that although honey bee sperm swims in a circular fashion it is still progressive. Furthermore, it should be taken into account, that as illustrated by Rothschild (1955), honey bee sperm, after swimming in a coiled or circular fashion, uncoil to become forward (snake-like) swimming sperm, which is believed in moths to result in sperm penetration of the egg for fertilization (Xu & Wang, 2011). In mammals, progressive motility is an important indicator of the sperm cell's ability to penetrate, and navigate through harsh conditions such as cervical mucus, to ultimately reach an oocyte (Mortimer & Mortimer, 2013).

Regarding the swimming speeds (rapid, medium, slow) of honey bee sperm, there was a larger population of slow swimming sperm (48.6 %), compared to the amount of rapid swimming sperm (10.3%) in this study, however, the >80% motility category still had the most rapid swimming sperm compared to the other categories. A possible explanation for the large percentage of slow swimming sperm is that since the more sperm are motile, the more sperm there may be per distribution, and in this case clearly the more rapid, but also because more sperm is alive, there is still a large proportion slow motile and even among the faster sperm still non-progressive sperm. There is very little to almost no literature available on detailed qualitative sperm motility in insects. It has only been in the last two years that there have been

newer approaches such as CASA, to study sperm motility sub-populations. Thus, swimming speed corresponded with motility. Furthermore, to determine kinematic parameters of the entire sperm population, kinematics was determined for the three swimming speeds. In this study velocity parameters (VCL, VSL, and VAP) as well as ALH for rapid swimming sperm were significantly higher, compared to medium and rapid swimming sperm. Interestingly, values tended to decrease by almost 50% from medium to slow swimming sperm.

Determining mean kinematic parameter values and total progressivity for the entire sperm population alone is not sufficient to accurately describe semen quality, specifically sperm motility. Establishing subpopulations in a sperm population is also recommended, given that the kinematic parameter values of a subpopulation are highly sensitive to external factors such as treatment, which is often overlooked when assessing only the mean values of a sperm population (Maree & Van der Horst, 2013). Therefore, this study determined subpopulations of progressivity (rapid, medium, and non-progressive), which reflects individual sperm progressivity. As for total progressivity, the >80% motility category had the highest percentage of the rapid, medium, and non-progressive sperm. Furthermore, kinematic parameters were determined of the subpopulations, to identify the kinematic values of individual sperm. Kinematic results of these subpopulations confirmed that rapid progressive swimming sperm have the highest kinematic parameter values, except BCF. When comparing the kinematic parameter values with those obtained for the entire sperm population (for the three swimming speeds), values for the entire sperm population were seemingly lower than the kinematics of sperm subpopulations, which allowed for a clear distinction between rapid, medium, and slow swimming sperm populations. The low mean values obtained from the kinematic parameters for the entire sperm population could be attributed to the presence of a larger population of slow swimming sperm (48.6 %), compared to the amount of rapid swimming sperm (10.3%) present, therefore not reporting on the true mean values of each sperm cell.

Determining subpopulations in honey bees will be especially beneficial to evaluate the true effect of harmful conditions such as pesticide exposure on drone semen quality and sperm functionality. Furthermore, assessing drone sperm motility using CASA assist in the accurate determination of firstly, cut-off values of honey bee species using VCL, and secondly identifying the subpopulations of an entire sperm population (Maree & Van der Horst, 2013; Prag, 2017).

5.3 Effect of amitraz on honey bee drone sperm quality

5.3.1 Sperm functionality parameters

Manual Motility index and CASA total motility percentage

The average motility index scores of sperm samples exposed to different concentrations of amitraz remained unaffected; high averages were observed throughout the increasing exposure of miticide doses.

Current literature only reports on the effect of insecticides on honey bee drone sperm functionality, specifically motility quality, and as of yet there are no other studies reporting on the effect of miticide treatment on drone sperm motility quality. Regarding the effect of insecticides on honey bee sperm, Inouri-Iskounen, et al. (2020), have shown that intensifying *in vitro* imidacloprid exposure resulted in decreased motility index scores (Control - motility score = 4,10 µM imidacloprid = 2 and 25 µM imidacloprid = 1).

The mean motility percentage of controls (CASA) obtained from the dose-response study was generally lower (68.07 % motility) than those of the baseline results. However, these findings were still in agreement with the results of Ciereszko, et al. (2017) who reported on drone semen motility percentages ranging between 69 – 79%. Furthermore, total motility percentages (CASA) seemed to decrease as the concentration of *in vitro* exposure was intensified from 125 µM to 500 µM. For the insecticide imidacloprid the number of motile sperm significantly decreased in semen samples exposed to higher concentrations of imidacloprid (Control = 78.5 ± 4.47%, 1 µM = 33.07 ± 7.37%, 10 µM = 24 ± 6.67%, and 25 µM = 8.46 ± 3.11%). Finally, variances in motility results obtained for the baseline and control groups in the dose-response experiment may be as a result of seasonal changes. The baseline study was conducted at the onset of the warmer season, while the dose-response study was completed towards the end of the season, and environmental factors are known to have an impact on drone sperm quality (Rhodes, et al., 2011).

Motility and kinematic parameters

As for motility, compared to the controls, no significant effects of amitraz doses were found on kinematic parameter values. However, sperm in the control group were slightly higher when compared to the sperm groups exposed to different amitraz concentrations. There was furthermore a tendency of velocity parameters to decrease as the concentration amitraz increased from 125 µM to 500 µM, Inouri-Iskounen,

et al. (2020) reported that kinematic parameter values decrease in a dose-response manner when exposed to intensifying concentrations of imidacloprid, and they also observed the lowest values in the treatment group where the highest concentration of imidacloprid was administered (e.g. VSL – Control = 8.19 ± 0.16 µm/s vs. 25 µM = 2.28 ± 0.23 µm/s; VCL – Control = 21 ± 0.32 µm/s vs. 25 µM = 17.66 ± 0.6 µm/s).

This study reports for the first time on the effect of miticide doses on honey bee sperm subpopulations. No significant differences were found between the control and the different concentrations of amitraz for each of the sperm subpopulations. Different concentrations of amitraz also did not significantly affect sperm swimming speed.

Owing to the limited reports on honey bee drone sperm motility and kinematic parameters, the findings from both the baseline and dose-response study could only be compared to each other.

Kinematic parameters for sperm subpopulations

5.3.2 Sperm structural characteristics

Vitality

Vitality results in this study were obtained using CASA and SCA®.

In this study, amitraz, an active ingredient in well-known miticides, negatively affected drone sperm vitality. The control group demonstrated a significantly higher vitality percentage (79.4 %) compared to amitraz concentration of 125 µM (68.8 %), 250 µM (58.3 %) and 500 µM (56.3 %). It is thus clear that the percentage of live sperm decreased in a dose-response manner. Results from this study are in agreement with Fisher & Rangel (2018) showing that residues of amitraz on beeswax significantly decreased drone sperm vitality (Control group = 99.2 % versus amitraz-treated group = 80.1 %). It should be noted that the amitraz in the study by Fisher & Rangel (2018), administered diluted amitraz (147 µM) to beeswax in hives during drone development. The results also showed that only drones exposed during the developmental phase were affected, which is further in agreement with the findings of Dai, et al. (2018), also reporting that the developmental rate of bees fed with an amitraz contaminated diet (157 µM) during the larval stage was significantly slower compared to unexposed bees. These findings confirm that pesticide exposure during drone development, negatively affects drone development (Dai, et al., 2018) and reproductive health, specifically semen quality (Fisher & Rangel, 2018).

Contrary to the reports of the latter study, Johnson, et al. (2013) reported that drone sperm vitality percentages remained unaffected following the topical, thoracic exposure of amitraz (3.12×10^{-6} µM per bee), however, they furthermore report on a high variability in sperm vitality amongst drones but only provide a mean vitality percentage (81.1 %) of all semen samples tested, therefore not discriminating between untreated and treated samples.

In this study, amitraz was applied *in vitro* to adult drone sperm and reflects an acute exposure effect, not reflecting a chronic exposure as per usual treatments in hives over a few weeks as in the majority of studies. The significantly low vitality percentages obtained could furthermore be as a result of the concentrations of amitraz used, which is considerably higher than what is normally administered by other studies.

As in the current study, Chaimanee & Pettis (2019) also determined the effect of amitraz on drone sperm after ejaculation but stored in the spermatheca. However, in their study, chronic exposure to sublethal amitraz dosages (8.53, 42.66, 85.32, and 170.65 µM) did not affect the vitality of sperm. The percentage vitality ranged from 26-42 %, which is unusually low for honey bee sperm, and much lower than the values obtained in the control group of our study. Furthermore, Straub, et al. (2016) further reported that neonicotinoid insecticides (thiamethoxam and clothianidin) have no significant impact on sperm quantity, however, vitality significantly decreased from 92% (control group) to 83.5 % after feeding the larvae with insecticide contaminated pollen.

Vitality results obtained in studies differed and seems to be affected by the amitraz dose and method of application used. In the case of in-hive treatments, it is difficult to determine the final concentration of the substance that will penetrate and affect the sperm in the drone's reproductive tract.

Besides a visible colour difference in this study, dead sperm cells furthermore presented a slightly curved and swollen sperm head, lying in a linear position. The structure of live sperm cells remained unaltered, in a circular swimming position.

For the baseline study, the vitality of samples was not determined because CASA and SCA ® generally yielded total motility percentages greater than 90 %, and since the percentage of viable cells must exceed the number of motile cells present in a sample, this is a clear indication of highly viable sperm (WHO, 2010; van der Horst, 2014). Furthermore, our findings are supported by vitality percentages reported for honey bees, varying between 81.1 and 98.1 % (Gençer & Kahya, 2011; Johnson, et al., 2015).

General sperm morphology

BrightVit, a Nigrosin and Eosin stain, generally used as a dead/live stain have been used in this study to differentiate between honey bee sperm components. Although Gontarz, et al. (2016) recommends silver nitrate to distinguish between sperm components, an eosin and gentian violet stain can also be used to identify individual sperm components. In this study, live sperm cell heads are stained white and the heads of dead cells stained pink, distinguishing between the sperm head and tail. However, interestingly, only in dead linear sperm, a prominent wrinkled purple-stained structure attached to the end of the sperm head and arranged around the proximal part of the tail in a helical fashion, or lying next to the tail, was observed. This structure is similar to what was described and illustrated by Lodesani, et al. (2004) as double tails, illustrated using propidium iodide, a red counterstain used to identify dead sperm. We suggest that it rather resembles mitochondria that are spiralling around the axoneme in the tail region as shown for other insect species (Lensky, et al., 1979). Lensky et al (1979) assessed the honey bee sperm ultrastructure using negative staining and electron microscopy and noted that in dead honey bee sperm there is a separation of the two mitochondrial derivatives that became non-viable. The same mitochondrial structure was noted in a preliminary study on Cape honey bee drone sperm (Murray, 2019 unpublished). Such a structure has not been described by studies investigating the ultrastructure of honey bees, and to confirm our results, other staining methods should be used to identify honey bee mitochondrial arrangement e.g. MitoTracker.

Morphometry measurements

Morphometric measurements determined in this study included the total sperm length, as well as its components (sperm head, tail, and mitochondrial derivatives). Mean lengths of sperm components obtained in control samples in this study, was as follows, head region = 8.92 µm, tail = 209 µm, and total sperm length =217µm, of which the head and tail lengths were slightly shorter compared to component measurements obtained from other subspecies such as the Carniolan honey bee drones (Gontarz, et al. 2016). The total sperm length of Cape honey bee drone sperm was shorter, compared to previously reported honey bee total sperm lengths varying from 241 µm to 273.50 µm (Phillips, 1970; Gençer & Kahya, 2011; Gontarz, et al., 2016).

Our samples for the dose-response study were mainly collected near the end of the season which also could have resulted in shorter sperm tail lengths, and subsequently shorter total lengths, as indicated by Gontarz, et al. (2016).

This study reported for the first time the effect of *in vitro* amitraz exposure on honey bee drone sperm morphology. Results have shown that amitraz exposure does not seem to affect the sperm components such as the head, tail, and total sperm lengths. Interestingly, although not significantly, sperm head lengths differed from the control group, and seemed to increase concurrently with amitraz concentrations from 125 µM to 500 µM. Futures studies using a stain such as silver nitrate to determine more detailed sperm components, such as the acrosome, acrosomal complex, and nucleus, will be able to determine the effect on these components that could possibly result in an increased head length.

Furthermore, the total sperm length in this study only showed a tendency to decrease with increasing amitraz concentrations. Sperm length, especially in insects plays a vital role in reproductive success given that elongated sperm have a higher chance of fertilizing an egg since it is favoured in sperm competition (Simmons & Kotiaho, 2002). The exact role of sperm length and its relationship with functionality and fertilization success in honey bees, however, remains indefinite, but it is often associated with sperm functionality and reproductive success (Snook & Karr, 1998). It has also been suggested that longer honey bee sperm may be a selected trait associated with increased longevity, and long-term survival in the spermatheca, allowing better energy production, but also increasing its success to fertilize eggs (Slater et al., 2021).

The mitochondrial structure length was the only component that presented a significant difference between the control and amitraz concentrations, decreasing with increased amitraz concentrations. The mitochondria regulate different reproductive functions that vary across species (Zhang, et al., 2015), however, in insects, it may play a different role, such as that it is required for sperm elongation (Werner & Simmons, 2008), which is beneficial for both motility and sperm competition given that sexual selection favours sperm length (Simmons & Kotiaho,2002). Thus, if the mitochondria reduce in size with amitraz treatment, it may also affect sperm structure and functionality.

Although not significantly so, honey bee sperm components seemed to be affected by amitraz in a dose-response manner; the *in vitro* exposure of amitraz seems to alter sperm structure and therefore also functionality, i.e. the differences in sperm components lengths such as the sperm head and zone of overlap, and also sperm vitality. These changes ultimately affect honey bee fertility, since compromised

functionality will hamper sperm longevity in the spermatheca and also migration through the spermathecal duct.

In general, the normality of sperm structure and its different components (head, midpiece, and tail) are an important indicator of fertility potential, as it relates to sperm functionality (motility), and vitality (Maree, 2011). Morphology is routinely assessed during semen analysis in mammals; however, this parameter is often overlooked in the assessment of honey bee semen (Yániz, et al., 2020). Reported morphological anomalies, of honey bee drone sperm structure, include occasional defective tail regions, such as flipped tails, broken tails, double tails, and double heads (Lodesani, et al., 2004; Paynter, et al., 2014; Gontarz, et al., 2016).

5.4 Limitations and recommendations

- This study determined semen volume using a weighing method and could only provide a semen volume range instead of an average. Consequently, the use of an accurate method or equipment to measure Cape honey bee drone semen volumes should be investigated for future studies.
- Due to the complexity of honey bee drone sperm swimming patterns, CASA and SCA ® could not locate and track all sperm cells in a semen sample. For that reason, the sperm concentrations obtained from CASA were inaccurate, and a sperm concentration range had to be obtained by manual sperm count. Therefore, to accurately measure drone sperm concentrations in future work using CASA, the motility module settings in SCA ® should be optimized.
- Results obtained for motility and kinematic parameters in this study were slightly different between the baseline study and controls of the amitraz dose-response study. As the samples for the baseline study were collected at the beginning of the season, the majority of samples for the dose-response study were collected nearing the end of the season and could therefore have affected the results. Future studies should therefore avoid such differences in results by collecting samples during the same month either at the beginning or end of a season, or statistically determine if differences occur between seasons.
- Large standard deviations or variations were obtained in some of the results which could be attributed to other biological factors such as colony and seasonal variations that were not referenced in this study, and should be included in further work. In addition, differences between colonies were not assessed and should be taken into account in future studies.

- For some of the results obtained in the dose-response study, no significant differences were found by using an ANOVA. In the light of small sample sizes, correlations and t-tests could be included, and should be applied for future analyses.

- This study only entails a general description of honey bee drone morphology and made use of only one stain to determine drone morphology. Furthermore, reports on morphometric values of drone sperm components, were not correlated with sperm motility and kinematic parameters. For example, Hook & Fisher, (2020) suggests looking at the ratios between the lengths of sperm components and then relating it to sperm function. Since most literature only briefly describes drone sperm morphology, this serves as an opportunity for further research on drone sperm structure and its relation to sperm function, especially its ability to fertilize an egg.
- Lastly, although the *in vitro* exposure of amitraz, does harmfully affect sperm vitality, direct exposure is not how sperm are normally introduced to miticides. The in-hive treatment, using the correct, prescribed dosages as per package inserts, is therefore recommended as this will yield true results on how drone sperm is affected when exposed to miticides during both its developmental- and adult phase.

5.5. Conclusion

In conclusion, this study has contributed to the understanding of honey bee semen quality, particularly sperm motility and kinematics, as well as the measurement thereof. The study further improved knowledge regarding the effect of amitraz on adult drone sperm and supports the current knowledge on the effect thereof on honey bee sperm vitality. The study has further provided a baseline for the future assessment of sperm quality, specific functionality.

Results obtained from this study and the discussion has highlighted the following aspects:

- Given the structural nature of honey bee sperm, routine semen quality assessment using CASA can only be done in combination with fluorescence microscopy.
- Current literature requires further investigation on the composition of seminal fluid and sperm numbers in a semen sample, and how it relates to reproductive success.
- Honey bee drone sperm presents interchangeable swimming patterns which potentially relate to sperm progressiveness and velocity.
- Motility index scores and kinematic parameters seemed to decrease with the percentage total motility of a semen sample.

- Honey bee drone sperm motility and vitality, under normal conditions, are seemingly always high, often exceeding 85%.
- Exposure to high concentrations of amitraz had no significant effect on sperm motility.
- The acute toxicity of amitraz on honey bee sperm was seen in the significantly decreasing percentage of viable sperm in treatment groups with high amitraz concentrations.
- Staining with nigrosin/eosin exposed disintegrating mitochondria, in dead sperm.
- Amitraz did not affect the morphometric measurements of the sperm head or tail, however, a significant decrease was seen in the length of the mitochondrial structure.

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ADDENDA

ADDENDUM A: ETHICAL APPROVAL

UNIVERSITY of the WESTERN CAPE

50 YEARS of hope, action & knowledge

01 October 2020

Dr C Kotze
Medical Biosciences
Faculty of Natural Science

Ethics Reference Number: AR 17/5/3

Project Title: A focus on black soldier flies (Hermetia Illucents) and honey bees.

Approval Period: 13 February 2020 – 13 February 2023

I hereby certify that the Animal Research Ethics Committee of the University of the Western Cape approved and ethics of the above mentioned research project.

This approval is subject to submitting an annual progress report by 30 November for the duration of your project and subsequent approval of the report. If the project do not require a renewal, a final project report must be submitted no later than two months after completing the study.

Any amendments, extension or other modifications to the protocol must be submitted to the Ethics Committee for approval.

The Committee must be informed of any serious adverse event and/or termination of the study.

Prof TK Monsees, PhD
Chairperson: Animal Research Ethics Committee (AREC)
University of the Western Cape

Director: Research Development
University of the Western Cape
Private Bag X 17
Bellville 7535
Republic of South Africa
Tel: +27 21 959 4111
Email: research-ethics@uwc.ac.za

NHREC Registration Number: BMREC-130416-050

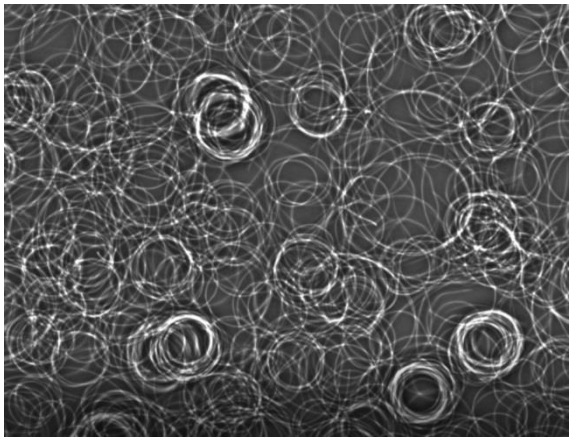
ADDENDUM B: NON-PEER REVIEWED PUBLICATION (MICROPTIC BLOG)

A major breakthrough: Fully automated Computer Aided Sperm Motility Analysis of African bee sperm!

We alluded in an earlier blog to the importance of bees as keystone species globally and provided some background on the reproduction of the African bee and our research on bee sperm. Sperm motility in all animals including insects is one of the most important parameters for measuring sperm quality. It is relatively easy to develop a manual scale for evaluating different grades of sperm motility but these have been shown to be subjective and prone to many errors. Accordingly, also in bee sperm and insect sperm at large there is a need to be able to quantify sperm motility using quantitative and objective methods such as CASA. The applications are numerous ranging from simple sperm quality assessment to toxicology to sperm competition.

However, despite many efforts by many groups, to our knowledge no one has succeeded/published analysing bee sperm using fully automated CASA. The closest method used was a semi-quantitative CASA method (Murray, 2019). Why is it so difficult to measure CASA of bee and other insect sperm? There are three major challenges: the sperm tail is extremely long and in the bee about 225 µm compared to vertebrates where it ranges from about 40 to 75 µm; secondly the tails of many sperm are often heavily coiled in a helix and this complicates analysis; thirdly the head width is about the same as the tail width (so head centroid analysis impossible using traditional CASA by means of phase contrast methodology).

The typical swimming patterns of bee sperm is either in a helical form of single or combined sperm and what can be regarded as more progressive sperm (Fig. 1)



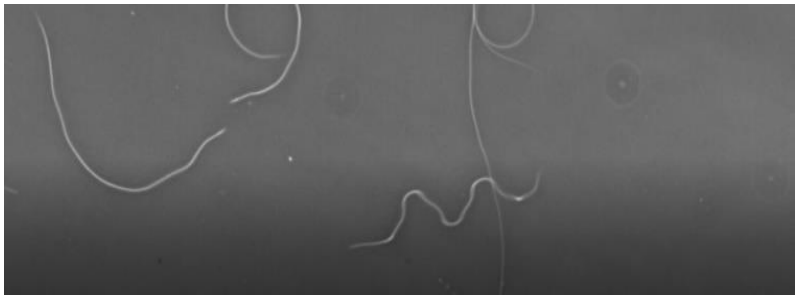


Fig. 1: On left single and combined helical swimming patterns and on right more progressive swimming sperm of African bee as viewed with negative phase contrast microscopy.

Modern CASA systems such as SCA (Microptic Sl, Barcelona) provides an option in the Motility Module to analyse sperm in the fluorescence mode. This method has been extensively used with great success in more sophisticated Andrology laboratories for human semen samples containing a lot of background particles (noise) as well as in the domestic animal market dealing with frozen-thawed bull sperm containing egg yolk particles. Accordingly, this methodology provides quantitative CASA analysis without interference of background particles since only the sperm heads fluoresce. Therefore, accurate sperm concentration and sperm motility can be measured.

There are fortunately fluorescence methods which involve vitality stains such as Sybr-14 which can be adapted for bee sperm. Using the FITC filter of a fluorescence microscope, the sperm head of live and motile bee sperm can be viewed (Fig. 2.).

Fig.2: Fluorescence microscopy of African bee sperm visualizing particularly the short sperm head (8 to 9 µm long) using Sybr-14 (Adapted from Murray, 2019).

All of this sounds very promising (head centroid movement can be performed) but there are three major hurdles to overcome. The width of the sperm head of insects is less than 1 µm and even if there are many sperm the fluorescence signal emanating from the sperm heads is very weak (low). Most of the current cameras used for CASA simply cannot analyse motile bee sperm heads at even minimal acceptable frame rates such as 25 fps since the fluorescence signal is very low (almost no bright enough image on screen). Yes, sperm head fluorescence (exposure) is sufficient to visualize and analyse at 1 to 5 fps but in terms of motility is of no value.

How have we solved the problem and made the breakthrough? It is a combination of many technical aspects of sperm concentration, medium used, concentration of Sybr 14, exposure time and many other aspects which will be published in the near future (Covid allowing). However, one of the most important aspects is that we used one of the new range Basler cameras specifically developed for fluorescence microscopy. Using one of these advanced cameras it was possible to perform motility of bee sperm at frame rates between 40 and 50 fps in fluorescence mode. Fig. 3 shows the sperm heads of motile sperm in grey level fluorescence and Fig. 4 the subsequent automatic analysis during about 0.5 to 1 second.

Fig. 3: SCA CASA analysis: no tracks

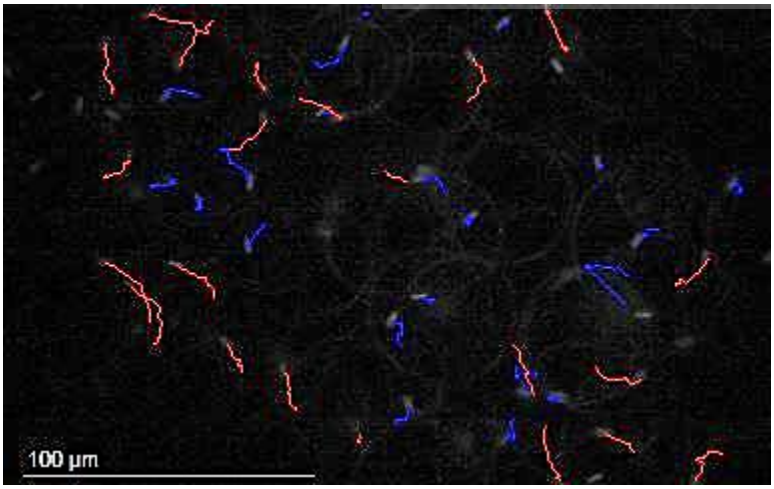


Fig. 4: SCA CASA analysis: showing sperm tracks of sperm in Fig. 3. Red tracks rapid and progressive sperm and blue slow non-progressive sperm

What are the motility percentages and kinematic parameters for bee sperm in the commonly used standard Kiev medium? The data is currently prepared for a peer-reviewed journal and the detailed base-line data can accordingly not be presented here. However, in more holistic terms the percentage progressive sperm motility ranged from approximately 20 to 60% and the Curvilinear Velocity (VCL) was low and ranged from about 20 to 90 µm/s. Improvement is required to make this a standard CASA technique for bee sperm but hopefully also for those insects with motile sperm.

Reference: Murray, JF (2019). The developmental effect of maturity and unusual apiary rearing conditions on Cape honey bee drone spermatozoa. Honours B.Sc. dissertation, Department of Medical Bioscience, University of the Western Cape, Bellville, South Africa

By:

Prof Gerhard van der Horst (PhD, PhD, Senior consultant to Microptic)

Ms Janice Murray (Hons. BSc) (MSc student at UWC – bee sperm research)

Dr Retha Kotze, (PhD). Project leader on Insect sperm, Comparative Spermatology Laboratory (UWC), Bellville, South Africa

ADDENDUM C: RESEARCH PRESENTATION (ANNUAL STUDENT SYMPOSIUM IN SCIENCE)

Masters research outcomes will be presented on the 28th of October at The Annual Student Symposium in Science, hosted by the North-West University.

Die kwantitatiewe en kwalitatiewe evaluering van spermgehalte in die Kaapse heuningby en die *in vitro* effek van varroa-beheer gifstowwe op spermfunksionaliteit en struktuur

JF Murray, R Kotze, G van der Horst, M Allsopp

Departement van Mediese Biowetenskap, Universiteit van die Weskaapland

3567716@myuwc.ac.za

Afgehandel

MSc Mediese Biowetenskap

Die heuningby is 'n hoeksteenspesie en suksesvolle voortplanting daarvan is noodsaaklik. Die spermgehalte (bepaal deur strukturele en funksionele eienkappe) van die mannetjiesby speel 'n belangrike rol in voortplantingsukses. Die mannetjiesby is uiters sensitief, spermgehalte word dus maklik beïnvloed deur omgewingsfaktore, soos varroa miete, en die blootstelling aan bestrydende gifstowwe. Die toepassing van moderne tegnieke, vir in diepte bepaling van sperm funksionaliteit is tans beperk. Verder, ontbreek navorsing oor die spermgehalte van die Suid-Afrikaanse Kaapse heuningby, (*Apis mellifera capensis*). Die doel van hierdie studie was dus om 'n metode vas te stel om spermgehalte te bepaal met behulp van 'n gerekenariseerde-semen analise stelsel, asook om die *in vitro* effek van gifstowwe op spermkwaliteit vas te stel. Hierdie studie lewer vir die eerste keer 'n akkurate verslag op sperm funksionaliteit (beweeglikheid en kinematiese parameters), met behulp van fluoresensiemikroskopie en 'n gerekenariseerde-stelsel, en rapporteer verder op die nadelige impak van gifstowwe op spermgehalte.