# MARINE INVERTEBRATE SPERM: ASSESSMENT OF SPERM QUALITY USING COMPUTER-AIDED SPERM ANALYSIS

*by*


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## Doctor of Philosophy


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# DECLARATION

I, **Monique Bennett**, declare that "**Marine Invertebrate Sperm: Assessment of Sperm Quality Using Computer-Aided Sperm Analysis**" is my original work and that all the sources I have used or cited have been indicated and acknowledged by means of complete references.

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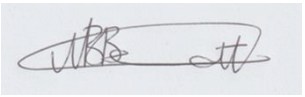
| **Monique Bennett** | : |  |
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# DEDICATION

*To my family*

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# ABSTRACT

The Southern African marine ecosystems are dominated by a variety of marine broadcast spawners. In this study, five species of marine invertebrates, namely *Parechinus angulosus* (Echinoidea), *Choromytilus meridionalis* (Bivalvia), *Crassostrea gigas* (Bivalvia), *Donax serra* (Bivalvia) and *Haliotis midae* (Gastropoda) were investigated. The sperm morphology and base-line data were gathered concerning the sperm concentration, motility and sperm kinematic parameters using computer-aided-sperm analysis (CASA). Sperm morphology and sperm motility play an important role in determining sperm quality as they relate to fertilization success. More specifically, head length and total sperm length have been used to find associations with swimming speed for best rates of fertilization. The implementation of CASA allows detailed quantification of the nature of the sperm swimming track, percentage motility groupings and detailed kinematics for rapid-, medium-and slow-swimming sperm subpopulations. The analysis of testicular sperm, taken directly from the gonads, and sperm activated by the introduction to sea water through the swim-up technique was determined. Using the CASA motility module, the behaviour of sperm was studied using *Choromytilus meridionalis* that was thermally induced to spawn and the activity of sperm in the presence of egg-water was determined using *Parechinus angulosus* as a model. A helical swimming pattern was a distinctive feature found in all species studied. However, species-specificity was found in the diameters and kinematic differences of the helical pattern, with *Parechinus angulosus* sperm creating the largest diameter tracks and *Crassostrea gigas* sperm the smallest diameter tracks, including a characteristic serrated helix. *Parechinus angulosus* and

*Haliotis midae* maintained the progressive helical pattern, post-activation of 60 min, while mostly straight-line forward progressive sperm was evident in *Choromytilus meridionalis* and *Crassostrea gigas* sperm. The CASA quantitative sperm track features had a negative association with sperm morphology (head width, tail and total length), but has a strong positive association with sperm speed. It was evident from the *Parechinus angulosus* model that a decrease (thinner) in sperm head-width and shorter tail resulted in faster swimming sperm, creating a swimming track with a larger circumference and diameter which covered a bigger surface area. In conclusion, this finding shows that head shape played a significant role in sperm hydrodynamics and how it could relate within the fertilization environment. The different patterns of sperm motility could be related to a search strategy in quickly locating eggs and behaviour to reproductive strategies such as broadcast spawning and spermcasting. It is with great interest that this study elucidates the immense contribution CASA has made to being able to correlate so many of these sperm features and that these associations make biological sense.



**Keywords:** sperm motility, sperm morphometry, computer-aided sperm analysis, broadcast spawners, helical swimming patterns

# LIST OF ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| **$2^D$** | Two-Dimensional |
| **$3^D$** | Three-Dimensional |
| **Acrm** | Acrosome |
| **ALH** | Amplitude of Lateral Displacement |
| **ANOVA** | One-Way Analysis of Variance |
| **AR** | Acrosome Reaction |
| **ATP** | Adenosine Triphosphate |
| **ATPase** | Adenosine Triphosphatase |
| **BCF** | Beat-Cross Frequency |
| **Ca** | Calcium |
| **CASA** | Computer-Aided Sperm Analysis System |
| **Cg** | *Crassostrea gigas* |
| **CITES** | Convention on International Trade in Endangered Species of Wild Fauna and Flora |
| **Cm** | *Choromytilus meridionalis* |
| **DAFF** | Department of Agriculture, Forestry and Fisheries |
| **DNA** | Deoxyribonucleic Acid |
| **DNC** | Dance |
| **Ds** | *Donax serra* |
| **EC** | Egg Cell |
| **e.g.** | For Example |
| **Ellip** | Ellipticity |
| **Elong** | Elongation |
| **Fig** | Figure |
| **fps** | Frames Per Second |
| **FSP** | Sulphated Fucose Homopolymers |

| | |
|---|---|
| **HIK** | Hermanus Abalone Farm |
| **Hm** | *Haliotis midae* |
| **hrs** | Hours |
| **Hz** | Hertz |
| **ICUN** | International Union for Conservation of Nature |
| **KCl** | Potassium Chloride |
| **km** | Kilometre |
| **L** | Length |
| **LIN** | Linearity |
| **m** | Meter |
| **M** | Medium |
| **min** | Minutes |
| **mm** | Millimeter |
| **mM** | Millimolar |
| **MMP** | Mitochondrial Membrane Potential |
| **Mp** | Midpiece |
| **Na** | Sodium |
| **NDIC** | Nomarski Differential Interference Contrast Microscopy |
| **°C** | Degrees Celsius |
| **°E** | Degrees East |
| **°S** | Degrees South |
| **Pa** | *Parechinus angulosus* |
| **Pp** | Principal Piece |
| **R** | Rapid |
| **r** | Correlation Coefficient |
| **S** | Slow |
| **SAP** | Sperm-Activating Peptides |
| **SCA** | Sperm Class Analyzer |
| **sec** | Seconds |

| | |
|---|---|
| **TEM** | Transmission Electron Microscopy |
| **USB** | Universal Serial Bus |
| **VAL** | Average Path Velocity |
| **VCL** | Curvilinear Velocity |
| **VSL** | Straight-Line Velocity |
| **W** | Width |
| **WHO** | World Health Organization |
| **WOB** | Wobble |
| **YE** | Egg Jelly |

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# CHAPTER 1

# INTRODUCTION TO THE STUDY

## 1.1 General Introduction

Theoretical and practical comprehension of reproduction is the foundation in understanding gamete biology and the evolution of any species. It is critical in exploring and anticipating the effects of over-exploitation, climate change, such as temperature changes and ocean acidification, as well as organic, inorganic and light pollution (Hodgson, 2010). The measure of an animal's biological fitness is its ability to produce offspring, which can constitute a great part of any animal's lifespan and sometimes body composition, as in some marine invertebrates.



Broadcast spawners were generally considered to be resilient to over-exploitation and infertility. This can be ascribed to their ancestral mode of fertilization (involving the most simplistic sperm form and function), that has been providing high fecundities with great larval dispersal and development of new populations (Jamieson, 1993; Roberts and Hawkins, 1999). However, accumulating evidence indicated a steady decline and extinction of some broadcast-spawning marine invertebrates, through overfishing, polluting of natural ecosystems and effects of climate change. An example of such decline is the white abalone, *Haliotis sorenseni* located along the United States western coastline that has been placed on the International Union for Conservation of Nature (IUCN) Red list in 2001.

*Haliotis sorenseni* was the first marine invertebrate to be listed (Davis, 1996; Hobday *et al*., 2001). Similarly, in 2007 abalone endemic to South Africa, *Haliotis midae* was placed on the list of species under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), to prevent illegal trade and harvesting.

In the present study computer-aided sperm analysis system (CASA) has been used to better understand the biology of the sperm cell, sperm-swimming behaviour and sperm functionality in marine invertebrates. This automated system was initially used to enhance the research of sperm through measuring sperm concentration, sperm motility and later the focus moved to sperm morphology. It was successfully used to evaluate sperm in domestic animal production laboratories, such as bull stud farming, to establish procedural standards useful for production in a field where subjective evaluations were relied on (Amann and Hammerstedt, 1980; Yániz *et al*., 2108). It was later used in the field of clinical andrology, to assess male fertility based on the objective evaluation of sperm concentration, morphology and motility (Amann and Waberski, 2014). However, human seman analysis on the earlier CASA systems had some difficulties, with sperm clumping, background debri and limitations in the image analysis (Mortimer, 1994). The development of technology has improved the objective analysis of CASA systems as a clinical tool (Mortimer *et al*., 2015), it is used in varicocelectomy (Ariagno *et al.,* 2017) showing decreased sperm motility (van der Horst and du Plessis, 2017) and Mortimer *et al*., (2015) had suggested CASA systems should be used for sperm functional studies. A study evaluating patients exposed to cigarette smoking and heavy metals, showed normal semen parameters in routine

semen analysis. However, CASA analysis of the semen showed a significant reduction in the average swimming speed as compared with patients not exposed to cigarette smoking and heavy metals (Mukhopadhyay *et al.,* 2010), indicating a probable disruption in sperm functionality.

CASA systems measure sperm motility by examining the two-dimensional pattern of the sperm head in motion, along with capturing changes in the shape of the wave form of the beating flagellum (Amann and Waberski, 2014; Lu *et al*., 2014). The images that are captured provide visual assessments and computer calculated measurements of the sperm motility. The motility of sperm infree-spawning invertebrates has been achieved when sperm is exposed to sea water. Sperm motility has been used as a reliable predictor for sperm quality and a possible indicator of fertility (Au *et al.,* 2002). This was demonstrated in *Cyprinus carpio,* fish sperm that had been exposed to hypoxic conditions which caused a decline in sperm motility and fertilization rate (Wu *et al.,* 2003). CASA sperm morphology is used to aid inthe assessmentof sperm quality; however, the selection of an appropriate fixative and staining technique for CASA morphologyis important in retaining the original morphology. The correct technique should allow for the differential staining of spermatozoa, providing clear borders indicating the head, acrosome, midpiece and tail, in order to assess the normality of each sperm component (Maree *et al*., 2010). Previous studies have shown that sperm morphology plays a crucial role, in determining sperm quality which often has been linked to sperm competition and reproductive fitness (Snook, 2005; Møller *et al*., 2009; Maree *et al*., 2010; Van der Horst *et al*., 2018).



Fertilization success in marine broadcast spawners have been influenced by sperm

motility and is often regarded as another important factor in quantifying sperm quality (Cosson *et al*., 2008). Various other sperm traits have been reported to also influence fertilization rate and sperm competition, such as sperm size, accessory structures, longevity, swimming velocity and chemoattractants (Rothchild and Swann, 1951; Vogel *et al*., 1982; Levitan, 1993; Levitan and Pertersen, 1995; Snook, 2005; Serrão and Havenhand, 2009).

For example, in humans and rodents an increase in sperm length has been associated with faster swimming sperm, providing an advantage in sperm competition and possible fertilization success (Gomendio and Roldan (1991)). However, this positive association between sperm length and sperm swimming speed appears to be species-specific as several studies have reported positive, negative or no associations between sperm morphology and motility (Malo *et al*., 2006; Pitcher *et al*., 2007; Skinner and Watt, 2007; Fitzpatrick *et al*., 2012). A study by Humphries *et al.,* (2008) found a more consistent pattern, than using sperm lengh and speed, when using sperm component ratios (head:flagellum) and sperm swimming speeds. Other studies on internal and external fertilizing species also found relationships between sperm length and speed by assessing multiply morphological traits (Simpson *et al*., 2013).



Sperm motility allows sperm to search for the egg, an essential marker for determining sperm quality for fertilization success. The helical swimming patterns created by sperm of many marine invertebrate species increase the spatial potential of sperm to ‘track’ or ‘knock-into’ the egg through the chemoattractant concentrations created by the jelly coats of the eggs (Riffell *et al*., 2004; Fitzpatrick *et al*., 2012). This mechanism of klinotaxis allows sperm to either continuously or intermittently and gradually adjust

their swimming path as a result of $Ca^{2+}$ bursts that changes the beating pattern of the flagellum (Alveraz, 2012, Jikeli, 2015). This allows the swimming trajectory of the sperm to be altered by creating tight loops and wide circular arcs, depending on the distance from the chemoattractant source (Miller and Brokaw, 1970; Miller, 1977, 1985; Kaupp *et al*., 2008). Sperm movement is further highlighted as an essential characteristic in human semen evaluation and is a vital tool in commercial animal productions (Mocé and Graham 2008; Gallego and Asturiano, 2017). CASA technology has been used for many years in *in-vitro* fertilization clinics, for animal breeding production, especially the bull artificial insemination industry, and research laboratories (Mortimer, 2002; Yeste, 2017). Compared to the substantial volume of CASA information on various vertebrate species, a limited number of broadcasting invertebrates have been investigated. The focus has been mainly on commercially exploited species such as mussels, oysters and abalone (Suquet *et al.,* 2003, Fitzpatrick *et al.,* 2008, Fabbrocini *et al.,* 2016). This has created a gap in understanding the uniqueness of the aquatic sperm biology and performance.



The application of CASA is useful as it evaluates sperm quality of various species and motility of individual sperm. CASA easily and readily provides huge datasets that allow various statistical analyses to be performed and determines sperm subpopulations through default settings in the program, that divide the sperm into subpopulations based on swimming speed. The in-depth analysis of the helical swimming patterns of the study species and sperm motility could contribute to a better understanding of the variation in interspecific and intraspecific sperm movement and the implications it may have on the rate of sperm-egg collisions. The datasets can also be utilized to explore

the kinematic parameters of commonality within and between species to offer biological meaning for fertilization success and sperm vitality (Fauvel, 2010; Martinez-Pastor, 2011). CASA technology is therefore being used extensively in the current study.

CASA results could assist to examine how physical, chemical and biological factors affect sperm concentration, swimming behaviour and possible fertilization success; the practical application of CASA for aquaculture research is important. This study is designed to add to the knowledge base, by evaluating and quantifying the various sperm traits, how these sperm traits can be used, what does it mean and what is the value for aquaculture? This nvestigationwill assist to define sperm traits and sperm quality and use it as a baseline for the future. CASA sperm velocities may form a valuable basic evaluation, relating to healthy sperm functionality with a fertilization potential. Accordingly, are the differences in aquatic habitats reflected in the sperm traits of these five broadcasters and does it relate to their swimming behaviour?



## 1.2 Aims and Objectives

This is a baseline study to try to define sperm traits of broadcast spawners sperm. It is proposed that cutting-edge and sophisticated technology be used to establish which sperm traits including sperm functionality, better assist to define sperm quality andfertilization potential. Therefore, these fundamental studies of sperm of broadcast spawners are important to assess the value of baseline values on which to build applications for possible aquaculture and future research studies.

The objectives of this investigation were to:

- Optimize and standardize the CASA experimental procedures utilized to evaluate sperm of marine broadcast spawners,
- Analyse the baseline sperm motility parameters of the selected marine invertebrate species to determine species-specific differences in these parameters,
- Assess and compare the nature of the helical swimming tracks among species and determine the degree of variation,
- Determine the sperm motility behaviour in the presence of egg-water and during induced natural spawning and assess its relation to hyperactivation,
- Assess the sperm morphological structure and how it relates to function by using both light microscopy techniques and ultrastructure.



## 1.3 Overview of Thesis Chapters

This thesis is divided into five main chapters, including a literature review and three research chapters. A general conclusion and a complete reference list are included at the end of the thesis. A final Chapter 6 has been added to show the potential of new developments in CASA.

### 1.3.1 Chapter 1: Introduction to the Study

This chapter provides a general introduction to the study, the aims and objectives as well as an outline of the thesis chapters.

### 1.3.2 Chapter 2: An Introduction to CASA and the Study Species

This chapter contains a review of the literature relating to computer-aided sperm analysis studies and highlighting its development and importance in spermatology in both mammalian and invertebrate animals. The sperm kinematic parameters are introduced as well as the marine invertebrate species studied.

### 1.3.3 Chapter 3: Material and Methods

In this chapter, the collection of marine samples, housing of laboratory experimental animals and dissections are described. The extraction of sperm samples and techniques used for the selection of motile sperm are explained, along with the induction of spawning methods using both chemicals and temperature. Additionally, the effect of egg-water and whole eggs to establish potential capacitation changes in sperm of marine invertebrates were tested. Thermal spawning is described for one invertebrate species to compare sperm motility parameters of sperm extracted from the testes versus sperm after induced spawning. The sperm morphology assessment protocol and the various methods of staining and microscopy are described.



### 1.3.4 Chapter 4: Results

The sperm concentration, percentage motility, sperm morphology and sperm motility swimming patterns and nine sperm kinematic parameters are assessed individually and compared among the invertebrate species. The quantification of differences in the sperm morphology structures is reported. Various statistical analyses are performed in order to identify differences and/or similarities among species and reveal possible relationships between sperm parameters.

### 1.3.5 Chapter 5: Discussion and Conclusion

In this chapter, all the data from the previous chapters are used to explain the importance of the systematic and detailed data generated by the CASA technology for invertebrate application. The unique helical swimming pattern will be discussed to highlight selected kinematic parameters and to determine a possible relationship between marine sperm structure, function, including a general conclusion with a view to future studies.

### 1.3.6 Chapter 6: Futuristic Perspectives on Sperm Motility Analysis

Current CASA of sperm motility is based on head centroid movement and accordingly sperm track reconstruction is based on x and y coordinates over time which is two-dimensional and represent the movement of the sperm head in time. This future preliminary work quantifies sperm movement in three-dimensions by means of the z-axis to construct a more realistic three-dimensional sperm swimming track.



## 1.4 Research Output

The following research article and conference presentations were generated during this study.

### 1.4.1 Research Article

- van der Horst G, Bennett M, Bishop JDD, (2018) CASA in invertebrates. *Reproduction, Fertility and Development*, 30: 907-918.

### 1.4.2 Conference Presentations

- van der Horst G, Bennett M, van der Horst M, (September 2009), Poster presentation at the 37th Congress of the Physiology Society of South Africa,

titled “Sperm motility patterns: Species and relationship to fertilization environment”.

- van der Horst G, Bennett M, van der Horst M (September 2011). Poster presentation at the 11th Biology of Sperm Conference, Sheffield, England, titled “Sperm motility quantified in broadcast spawners? Activation, motility patterns and sperm competition”.

- van der Horst G, M Bennet M, de Kock M (September 2012). Oral paper presented at Aqua 2012 International conference, Prague, titled “Computer-aided sperm analysis in broadcast spawners: baseline studies in assessing sperm quality quantitatively”.

- van der Horst G, Bennett M, de Kock M (May 2015). Oral presentation, Invited speaker, at the to Aqua 2015 International conference; Jeju Island, South Korea, “Sperm functional tests in broadcast spawners”.

# CHAPTER 2

# AN INTRODUCTION TO CASA AND STUDY SPECIES

## 2.1 Introduction

The South African coastline of over 3000 km has a vast variety of marine invertebrates with approximately 9000 species (Gibbons *et al*., 1999; Acuña and Griffiths, 2004; Hodgson, 2010). This relatively short coastline boasts four ecological distinctive regions: the tropical Maputaland and the northernmost part of the Kwazulu-Natal coastline, the subtropical East Coast, the warm, temperate South Coast and the cold, temperate West Coast, which stretches from Cape Agulhas to the Orange River. The ecosystem along the West Coast is greatly influenced by the cold Benguela system which is normally subjected to periodic strong winds, causing upwelling of cold nutrient-rich water that helps to increase phytoplankton growth (van Erkom Schurink and Griffiths, 1991; Olivier *et al*., 2013).



This rocky shore ecosystem also features extensive beds of *Ecklonia maxima* and *Laminaria pallida* kelp, providing a safe habitat and a food source for most shoreline invertebrates and as a food source. The cooler current produces species with a higher biomass value, such as limpets, black mussels, white mussels, abalone, rock lobsters and a number of fish species. The majority of reproductivestudies on species along the West Coast falls between the Kommetjie and Bloubergstrand regions (Hodgson, 2010) and along the coastal town of Saldanha.

The bay area of Saldanha has become the prime site for mussel and oyster mariculture, as pulses of nutrient-rich Benguela sea water move into this region (Monteiro *et al*., 1998; Olivier *et al*., 2013).

Mariculture is defined as the cultivation of marine organisms within an enclosed section of the open ocean through the construction of specialised cages and suspended rope culture in the case of mussels (Olivier *et al*., 2013). Over the past few years, many coastal and surface ocean studies have shown the negative impact of pollution, ocean acidification, elevated sea-surface temperature and reduced salinity on the overall fitness of the marine ecosystems which have the potential of changing marine invertebrate physiology (Morita *et al*., 2009; Somero, 2009; Byrne *et al*., 2010). However, a study by Sunday and her team (2014) disputed this idea and suggested that marine species possibly have the capacity to adapt through evolutionary change. The uncertainty of climate change within the scientific realm provides a gap for alternative solutions and procedures. The mariculture industry has made great strides in being an alternative source to harvesting wild stocks that may reach maximum sustainability limits. The over-harvesting of wild stocks has shown significant impact on both commercial and coastal fishing communities that rely on the wild stocks as a source of income and sustenance (Olivier, 2013).



The global trend in population growth and healthy living has seen an increase in consumption of seafood, comprising mainly of finfish, but the demand for marine molluscs has grown, especially bivalves, with a developing market for echinoderms (De Silva, 2001; Britz *et al*., 2009). Growth potential within mariculture research relies on the establishment of reliable and appropriate technology for commercial

production, focusing on breeding, brood-stock conditioning and spawning, gonad enhancement, larval-rearing, inducing morphological and molecular diagnosis treatment and impacts of pathogens on farmed and wild-caught animals. In South Africa, the government has come to view mariculture as a necessity to address the rising unemployment rate in rural fishing communities, thus being important economically as well as preserving the biodiversity and ecology of its rich coastline (Joemat-Pettersson, 2010; Olivier, 2013).

To have a greater understanding of fertilization kinetics and fertilization success in free-spawning invertebrates, we need to have more quantitative information on sperm movement and motility. Free-spawning marine invertebrates are ideal to study male reproduction; by evaluating its sperm biology and motility patterns; determining the relationship of spermatozoa at an intraspecific level, as well as its interaction with eggs, within a laboratory setting. Computer-aided/assisted sperm analysis (CASA) has been used in some marine invertebrate sperm studies with the focus on environmental pollutants and environmental factors, such as osmolality, pH and temperature (Lewis and Ford, 2012).



## 2.2 CASA Development

The sperm cell has always intrigued scientists, especially the need to understand its functional components and how it is related to its distinctive movement pattern. Moreover, this is evident from the volume of publications produced between 1940 and 1970 discussing the quantification of sperm motion (Boyers *et al*., 1989), which established the early stages of CASA development.

Automatic CASA systems were initially designed to visualise and digitize images of sperm to provide meaningful information about sperm concentration, individual sperm cell kinematics, and perform statistical analysis of the sperm population, by looking at the mean values (Lu *et al*., 2013; Amann *et al*., 2004). However, advances in technology have allowed CASA systems to surpass its initial expectations.

The first commercial CASA system was developed in the late 1980s with the arrival of the personal computer and emerging digitization and quantification of computer video imaging (Mortimer, 1990). CellSoft™ and Hamilton-Thorn HTM-2000® (Hamilton-Thorn Research) were the initial CASA systems available on the market, designed specifically for the evolution of sperm motion (Amann and Katz, 2014), and aimed predominantly as a research tool. Publications soon spiked, describing the application of these CASA systems on human, rat, mouse, bull and stallion sperm (Budworth *et al*., 1988; Mack *et al*., 1988; Mathur *et al*., 1986; Amann and Katz, 2004).



These brands of CASA provided meaningful laboratory output data (Gill *et al*., 1988), sparking the interest of clinical laboratories processing sperm for artificial insemination in humans and commercially viable domestic animals. However, the earlier-generation systems did not provide accurate results and the resolution of the digitized images were limited for clinical human semen analyses (Mortimer and Mortimer, 1988; Mortimer *et al*., 2015).

The progression of CASA into the andrology laboratories directed the improvement of CASA technology through the hardware, the software algorithms (Amann and Katz, 2004) and increased computational power. This development of the modern CASA

system, and the concomitant increase in publications over the past thirty years relating to human and animal sperm analysis (van der Horst *et al*., 2018) has advanced the confidence in CASA as an objective, dependable research and diagnostic tool in the medical, veterinary, laboratory and wildlife fields of study (Holt *et al*., 2007). The basic CASA technology has had applications that have remained relevant with the new generation systems, such as sperm concentration, motility and morphology analysis. The latest systems are fully integrated with modules to quantify sperm DNA fragmentation, vitality, hyperactivation, acrosome status and hypo-osmotic swelling. This improvement in the operational parameters allows the user to adjust the analytical properties and settings for species-specific sperm motility assessments. The implementation of the current CASA techniques has rapidly progressed into the scientific studies over the years, to include various primates (Maree, 2011; van der Horst *et al*., 2018), domesticated animals (e.g. ram, goat), fish (Cosson *et al*., 1985; Wilson-Leedy and Ingernmann, 2007), birds (e.g. penguin, zebra finch), invertebrates (e.g. sea urchin, black mussel) (Au *et al*., 2001, Van der Horst *et al*., 2018) and wildlife (e.g. lion, elephant).



Mammalian species, however, have monopolised CASA investigations over the years, as a result of the impact it has on human andrology clinical studies and the profitable commercial domesticated animal production units which rely chiefly on artificial insemination. A small percentage of CASA studies have been dedicated to invertebrate species, with a large percentage of CASA work being executed mainly on species classified under Mollusca and Echinodermata (van der Horst *et al*., 2018).

### 2.2.1 General Operation Principles of CASA

Before meaningful and accurate information can be gathered from a semen sample, the individual spermatozoon has to be recognised by the system. Microscopically capturing the sperm swimming pattern is the key operational principle of most brands of CASA available on the market. Clear images need to be captured by a camera, identified as spermatozoa, and followed or tracked while swimming before data measurements is determined by proprietary specific computer software (Boyers *et al*., 1989). The CASA instrumentation has to track spermatozoa from within the microscopic field of view, which was sent from the camera and this is best achieved through either dark-field or negative phase-contrast images. These images enable the translucent cells to be visualised by displaying the sharp-edged white sperm head against a black background. The moving sperm head centroid generates the coordinates, needed to construct the sperm tracks created (Mortimer, 1990; 2000). Using the centroids as reference points, the images of each sperm head are digitized and enables the computer to 'see' the sperm and allows the software to search for the successive image of the particular sperm head (Mortimer, 1994).



This is achieved through an algorithm which constructs, within a zone of probability a predicated radius that circles the sperm head with each movement made, based on the maximum distance the spermatozoa would have travelled within a given time period (Mortimer, 2000; Lu *et al*., 2014). These motility tracks are reconstructed through the software, integrating the successive positions created by the moving centroids. The frequency of acquiring images, referred to as the frame rate, determines the number of consecutive images a sperm trajectory will have within a

set time period, and which assists to shape the reconstructed track. This reconstructed sequence assists to analyse the specific sperm motility characteristics, known as the sperm kinematics.

### 2.2.2 Sperm Kinematics

Sperm motility parameters are comprised of three main velocity values based on the movement of the sperm centroid along the trajectory of the sperm path (Mortimer, 1990). These include the curvilinear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP) (Figure 2.1). To further describe the sperm patterns, three velocity ratios were developed, namely: linearity (LIN) is calculated as VSL/VCL x 100; straightness (STR) is calculated as VSL/VAP x 100; and wobble (WOB) is calculated as VAP/VCL x 100 (Mortimer, 1997). Measurements reflecting sperm wobble characteristics from the kinematics include: amplitude of lateral head displacement (ALH); dance (DNC), calculated as the product of VCL and maximum ALH; and beat-cross frequency (BCF) which shows the number of times the curvilinear path crosses the average path (Amann and Waberski, 2014). Sperm kinematic parameters can be used to explain the swimming speed of sperm in various media, the changes made in the sperm path in the precence of a chemoattractant and if its swimming pattern is more or less straight. Information useful when evaluating the dynamics of sperm when searching for eggs within the spawning environment and fertilization success.

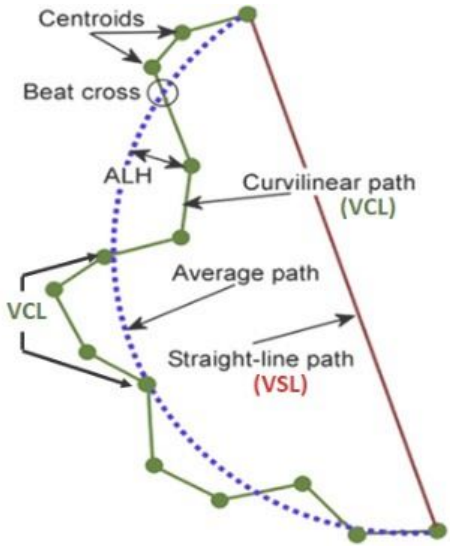



**Figure 2.1:** Illustration showing the kinematic parameters used in CASA. By linking the centroid positions created by the sperm head, the curvilinear path is created, shown in green; the time-average along this path is termed the curvilinear velocity (VCL). The path created by the first and last centroid positions, shown in red, reflects the straight-line velocity (VSL) with the average path velocity (VAP) shown in blue. The points where the centroid deviates from the average path is termed amplitude of lateral head displacement (ALH) and beat cross points indicate where the centroid crosses the two paths. (modified after Krí zková *et al*., 2017)



CASA determines the values of the kinematic parameters for each motile spermatozoon captured; using this data the velocity of progression and pattern of movement for individual spermatozoa can be analysed. Combinations of these values can be used to classify a specific population or subpopulation's movement pattern, such as sperm cells showing hyperactive movement (Robertson, 1988; Mortimer 1990; Mortimer, 2000). Several studies have shown the benefits of sperm parameters, for example VCL and ALH have been linked to active sperm motility in eel sperm longevity studies (Gallego *et al*., 2014). In the sea urchin *A. crassispina,* VAP and VCL were significantly correlated with fertilization rates (Fabbrocini *et al.,* 2010) and proved

to be better predictors of fertilization than percentage motiliy (Au *et al*., 2002). Considering the free movement of sperm in an aqueous medium these parameters, along with VSL, are reliable predictors of the chances of finding eggs (Levitan, 2000, Cabrita *et al*., 2014, Gallego *et al*., 2014). Many studies have evaluated selected sperm kinematic parameters, as shown above, to determine if one or group of kinematic parameters could be used to compare good quality from poor quality sperm which could be related to fertilization potential.

### 2.2.3 Frame Rate

The motility rate and kinematic parameters calculated depends on the video image acquisition rates, referred to as frame rates (expressed as frames per second; Hz), and relies on the quality of the camera and software capabilities (Bompart *et al*., 2018). Earlier CASA instruments and some standard digital video cameras could capture images at frame rates of 16, 25 or 30 fps. Some of the modern CASA systems have an advantage of using any frame rate within the capability of cameras with firewire or gigabit ethernet technology (Irvine, 1992; Holt *et al*., 2007; Contri *et al*., 2010) or digital USB3 cameras such as the Basler series (van der Horst *et al*.,2018). These cameras provide frame rates from 50, 60, 75, 100 fps or higher (Mortimer *et al*., 2015).



Visually, a sperm cell track created at 30 fps could appear relatively simple when compared to the detailed sperm cell track at 50–60 fps (Figure 2.2). To enhance the quality of a sperm trajectory, some CASA systems have intelligent filter systems that further optimize sperm recognition with advanced analysis in black and white mode to give sharper image outlines in debris filled samples, as well as a filter to decrease Brownian movement, sperm drifting and sperm tail recognition (Mortimer *et al*., 2015).

Higher frame rates are recommended for species with faster swimming sperm such as fish, merino ram, rabbit and hyperactivated human spermatozoa (Mortimer, 2015). However, some studies have shown conflicting results when using various frame rates and are particularly observed in the VCL measurement, depicting motility speed, and the amplitude of lateral head displacement but have no significant effect on the total sperm motility (Mortimer *et al*., 1995; Morris, 1996; Castellini *et al*., 2011)

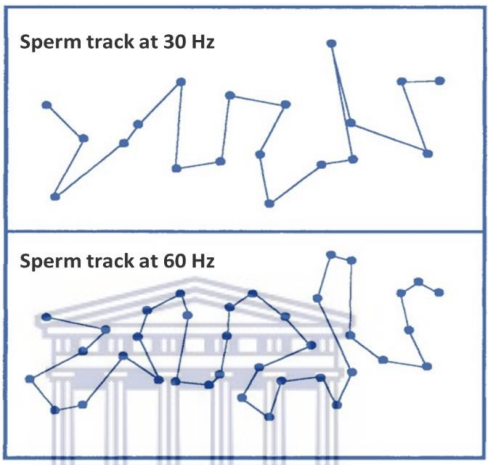



**Figure 2.2:** The reconstruction of the same sperm track provides a more detailed description of the track at 60 Hz when compared to the 30 Hz track. (modified after Mortimer, 2000)

Nevertheless, most mammalian sperm studies published used commercial CASA systems with a frame rate of 30-60 Hz, while spermatozoa evaluated at both 50 and 100 fps is cited in the methodology (Mortimer *et al*., 1999; Iguer-ouada, 2001; Castelli *et al*., 2011; Maree *et al*., 2013; Lu *et al*., 2014; Cojkie, 2017; Neumann, 2017). Reliable CASA motility data can be obtained when using high-definition cameras, with a wide range of frame rates from the lowest 50 fps, for non-hyperactivated human sperm and most mammals, and 100 fps and higher for extremely fast, nonlinear

swimming spermatozoa (Martinez-Pastor *et al*., 2011; Boryshpolets *et al*., 2013; Neuman *et al*., 2017).

### 2.2.4 Sample Chamber

Conventional slides are not effective for CASA technology and special fixed-depth sample chambers are required for these analyses as chamber depth can influence sperm dynamics (Le Lannou *et al*., 1992; Massányi *et al*., 2008; Lu *et al*., 2013). The haemocytometer has a chamber depth of 100 micrometers (µm) and is regarded as the gold standard for measuring sperm concentrations by the World Health Organization (WHO 1999) and andrology labs. This method can be time consuming and requires the use of dilution techniques which could lead to possible errors (Bailey *et al*., 2007; Bompart *et al*., 2018).



CASA systems have provided a more convenient method for measuring sperm concentration by using specific counting chambers. There are two chamber loading methods, namely: by capillary action in most disposable chambers and by drop-displacement in reusable chambers (Coetzee and Menkveld, 2001; Del Gallego *et al*., 2017; Bompart *et al*., 2018). A specific loading method and type of counting chamber are recommended for each commercial CASA system. Bailey and co-workers (2007) performed a study comparing two CASA counting chambers, Leja and Makler, to the gold standard, haemocytometer.

The study by Bailey *et al*., (2007), suggested that the Leja slide sperm concentration could be considered interchangeable with the haemocytometer counts, provided that the Leja sample is modified through dilution of the sample. Leja slides also had a

closer degree of repeatability or reliability with the gold standard and analysis of limits of agreements was also favourable with the haemocytometer (Bailey *et al*., 2007). Whereas for the Makler slides, the drop-displacement method showed the widest inconsistency, both when samples were undiluted and diluted (Johnson *et al*., 1996; Bailey *et al*., 2007). Nevertheless, there is insufficient research to determine whether one chamber is more suitable than the other (Kuster, 2005; Bompart *et al*., 2018).

## 2.3 Spawning

During sexual reproduction, for external fertilizers, the exchange of genetic material can be complex for slow-moving, sessile or sedentary adult marine invertebrate species. The precise timing of spawning events is critical in terms of population dynamics (Levitan and Petersen, 1995), especially for broadcast spawners that have to release gametes within high-energy environments where spawning conspecifics' densities vary spatially.



The possibility of sperm-egg collisions within turbulent flow environments leading to efficient fertilization was considered deleterious by Denny and Shibata (1988, 1989), as turbulence rapidly diluted the concentration of gametes. Yet, broadcast spawning species, in natural field and laboratory experiments, have shown successful fertilization under these conditions, through a range of physical, chemical and biological factors (Desrosiers *et al*., 1996; Serrão *et al*., 1996; Kregting *et al*., 2013; Crimaldi *et al*., 2014). The importance of adult aggregation and synchronization of gamete release increases the likelihood of egg and sperm meeting. As a result of passive transport by the turbulent flow or by active sperm swimming (Crimaldi *et al*., 2014).

The mathematical fertilization model designed by Denny and Shibata (1989) highlighted the turbulent dilution effect on broadcast spawned gametes. This model estimated the lower fertilization rates obtained as a result of the dilution of egg and sperm concentrations, as was seen in studies by Pennington (1985) and Levitan *et al*. (1992). Other experiments have shown that some water movement could enhance fertilization success through mixing of eggs and sperm (Pennington, 1985; Denny, 1988; Mead and Denny, 1995). This is especially the case in small scale embayments along the surf zone and rocky shore that allows for dilution of gametes in limited volumes and as a result increases egg fertilization (Denny *et al*., 1992). Other species spawn at low-tides when water volumes are reduced to increase gamete concentration and fertilization success (McDowell, 1969).

### 2.3.1 Gamete Traits

Variations in gamete traits have been linked to gamete fitness, the ability of an egg to be fertilized by sperm, and have been shown to enhance fertilization fitness in marine broadcast spawners, without being influenced by environmental conditions (Levitan, 1996, 1998). The evolution of gamete traits has increased the probability of fertilization through gamete viscosity, morphology, both egg-size and sperm structure, receptiveness of the egg surface to sperm, sperm chemoattractants, sperm velocity and sperm longevity (Rothschild and Swan, 1951; Levitan, 1998; Lotterhos and Levitan, 2010).

The first field experiments correlating results of laboratory tested species-specific gamete performance and gamete traits were documented by Levitan (1998). These experiments showed how gametes from three closely related sea urchins species

responded differently for optimal fertilization, based on the uniqueness of their gamete traits. Field studies demonstrated that when sperm was dispersed before eggs, species with larger eggs and slower, but longer-lived sperm had more positive fertilization fitness than those species with smaller eggs and faster but short-lived sperm (Levitan, 1998). These male-female gamete interactions further support the importance and the variations of gamete recognition proteins, facilitating the selection of genetically compatible mates.

### 2.3.1.1 Egg Traits

Egg traits vary across taxa, with differences in cell size, and the presence of jelly coats, or other structures that can capture sperm, influencing the number of sperm-egg collisions that result in fertilization (Levitan, 1995, 1998; Podolsky and Strathmann, 1996). Several coral species have been shown to release buoyant gamete bundles that initially float to the surface and then rupture, dispersing eggs and sperm (Harrison *et al*., 1984).



This adaptation allows for maximum sperm-egg collisions and ultimately increases chances of fertilization before gametes are adverted away from the release site. Marine invertebrate egg jelly coats play a role in protection against environmental stressors, preventing polyspermy and increasing the diameter of the egg for better sperm-egg collision (Thomas and Bolton, 1999; Farley and Levitan, 2001; Inamdar *et al*., 2007; Marshall and Bolton, 2007).

### 2.3.1.2 Sperm Traits

Differences in sperm traits, such as sperm behaviour and buoyancy, longevity and

velocity (Levitan, 1995), have influenced sperm functionality and fertilization success. The dispersal and dilution of gametes in the water column can be reduced by the viscosity of the spawned material. Some species release sperm in a very viscous fluid, allowing the slow 'wisping' away of sperm from the release site (Marshall, 2002; Marshall *et al*., 2004). When the viscosity of spawned material is increased by adults during high turbulence and water flow, it reduces the rapid dilution of gametes (Thomas, 1994). Some sea urchin gametes remain 'trapped' at the aboral surface, between the spines, before they are transported away by water movement (Lotterhos and Levitan, 2010). Laboratory results of gamete traits have shown subtle influences on fertilization, revealing the potential for sexual selection based on possible gamete traits (Levitan, 1998). The relationship between gamete traits, especially sperm traits, such as swimming behaviour, speed, sperm shape and chemokinesis is central to regulating sperm-egg collisions and fertilization success.



Broadcast sperm longevity, like sperm velocity, varies between species and velocityis known to vary within a single ejaculate (Crean *et al*., 2012). In sea water, sperm of some species can remain actively motile for a few minutes to several days, while maintaining their fertilization capability (Pennington, 1985; Williams and Bentley, 2002; Campbell *et al*., 2017). It was suggested that species with greater sperm swimming velocity could possibly increase fertilization fitness (Gary, 1955; Levitan, 1993), with sperm longevity attributing to fertilization success, especially when sperm is limited, spawning synchrony is low and female conspecifics are located further downstream (Levitan, 1995). Motility is essential for all flagellated sperm cells that require propulsion to actively swim to the egg for fusion (Kamp *et al*., 1996). A study

completed by Au *et al*., (2001) had shown that the exposure of adult sea urchins to cadmium decreased the percentage of motility, velocity and normal swimming patterns. This was accompanied by a reduction in the fertilization rate of sperm. Kupriyana and Havenhand (2002) observed that polychaete, *Galeolaria caespitose*, sperm swimming behaviour was not significantly different when activated with egg-water solution relative to filtered sea water. Although sperm velocity varied significantly in the presence of male conspecifics, this produced a positive correlation with fertilization success (Kupriyana and Havenhand, 2002). The change reported in sperm velocity highlighted the possible post-ejaculatory selection interactions between females and males. Chemokinesis had no effect on these sperm's swimming behaviour, which was unique, as chemical communication has a major influence on most species' sperm-egg fusion.



CASA research has enhanced the significance of swimming speed and motility patterns of sperm compared to previously described models of fertilization kinetics, that assumed the motion of eggs and sperm to be random (Farley, 2002). Helical swimming patterns, often seen in marine invertebrate sperm, have been associated with egg-collisions. This swimming pattern has been previously described by many investigators (Miller and Brokaw, 1970; Miller, 1973; Levitan,1995, 1998; Au *et al*., 2001; Riffell and Zimmer, 2007; Fitzpatrick *et al*., 2010; Fabbrocini *et al*., 2016) or analysed by CASA or another objective sperm motility system (Basti *et al*., 2013; Lymbery *et al*., 2016).

There have been few detailed descriptions of the helical nature of the swimming patterns and sperm motility of the species investigated in the present study. By using

the CASA system, sperm traits such as sperm concentration, morphology, sperm motility, and longevity and sperm behaviour, characterized by the helical swimming patterns, are analysed in detail for the first time in the five broadcast spawners.

## 2.4 Marine Invertebrate Sperm Morphology

Sperm cells have adapted to their ever-changing environments. To understand the cellular events that are necessary for the development of sperm motility and sperm behaviour, one therefore has to consider the basic sperm form. To favour its' motility and speed, the sperm head of each species has a unique shape; with a highly compacted nucleus. Spermatozoa also retain very little cytoplasm to provide space for substrates that would provide energy for movement (Anderson and Personne, 1970). Invertebrate spermatozoa have been classified as either primitive or modified and is established by the mode of fertilization.



## 2.5 Primitive Sperm Morphology

Primitive sperm are typically produced by external fertilizers, of which most are invertebrates, while internal fertilizers and all mammals have been classified with modified sperm (Figure 2.3) (Franzen, 1956; Fawcett, 1970; Hodgson, 1986). Rouse and Jamieson (1987) however, renamed sperm types to aquasperm and introsperm, based on their polycheate studies as the morphology was close to the idealized 'primitive' type. They concluded that there were two types of aquasperm- ect-aquasperm and ent-aquasperm, produced by species with true external fertilization for example broadcast spawners and spermcasters, respectively (Jamieson and Rouse, 1989). This sperm classification correlated with the mode of fertilization demonstrated by Mollusca, in which most bivalves have primitive sperm (Hodgson,

1986), whereas marine invertebrates that are internal fertilizers, such as the opisthobranchs, pulmonates, and cephalopods all have modified sperm (Hodgson, 1986; Rosati, 1995).

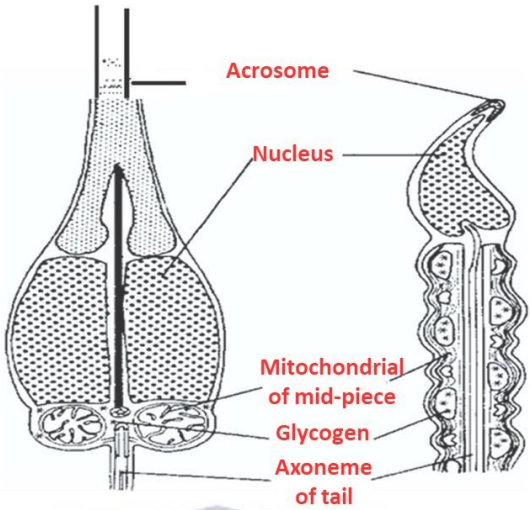



**Figure 2.3:** An illustration demonstrating the structure of the primitive *Mytilus* (mussel) sperm on the left and on the right a modified *Achatina* (land snail) sperm. (modified after Hodgson, 1986)



The small sperm are composed of three regions: a short, rounded-to-conical shaped head; a short midpiece containing a few spherical mitochondria, normally four or six, which surrounds the proximal and distal centrioles, and a flagellum with an endpiece (Hodgson, 1986; Rosati, 1995; Levitan, 1998).

The sperm head typically has two main features, namely, a highly condensed nucleus with the acrosome forming the anterior cap. Although primitive sperm are similar across invertebrate taxa, there are variations that are displayed within the sperm head size and organization of the acrosome (Figure 2.4) (Fawcett, 1970; Rosati, 1995). Eckelbarger *et al*. (1989) stated that the variations in primitive sperm head size

showed a positive correlation with egg size in echinoderms. In some echinoderm species, the acrosome takes the form of a sphere inside a cavity in the nucleus. In the mollusc, *M. galloprovincialis*, it forms an elongated conical shape in the head above the nucleus; and in the horseshoe crab, *Limulus*, it appears as an inverted bowl on top of a circular nucleus (Rosati, 1995).

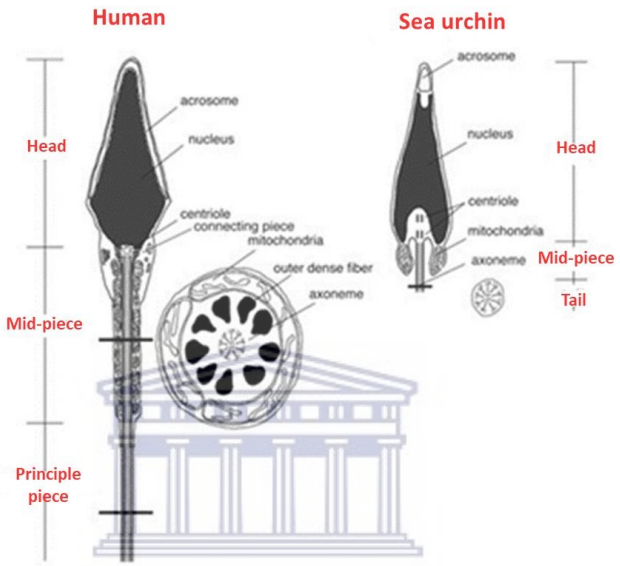





**Figure 2.4:** Comparison of structures between modified human spermatozoa and the primitive spermatozoa of the sea urchin, trout and tunicate, showing variations in primitive head size and acrosome. (modified after Inaba 2011)

Compared to the acrosome, the mitochondria of primitive spermatozoa are conservative in structure, with developed cristae similar to that of somatic cells (Hodgson, 1986). The flagellum consists of an axonemal complex with the basic nine double microtubule arrangement around a central pair of single microtubules (9+2 arrangement) (Fawcett, 1970; Hodgson, 1986). Motile sperm rely on a single flagellum for propulsion, which varies between species and is adapted to its environment. The

microtubules of the flagellum are supplied with energy via the mitochondrial ATP, and maintained by the dynein motor adenosine triphosphatase (ATPase), in order to create movement of the flagella (Shinyoji *et al*., 1998).

## 2.6 Chemotaxis and Sperm Motility

Various marine invertebrate sperm react differently to the introduction to sea water. While the majority of species' spermatozoa are immediately activated, some are completely quiescent in sea water or a small population is motile, with full activation only completed in response to several signals (Morisawa, 1994; Yoshida *et al*., 2002; Liu *et al*., 2011). Marine invertebrate spermatozoa typically rotate about their long axis, creating a helix as they swim (Gray, 1955; Jekeli *et al*., 2015). The helical pattern allows spermatozoa to sample the chemoattractant concentration either continuously or intermittently, and permits gradual adjustments to the swimming path (Jekeli *et al*., 2015; Guerrero *et al*., 2011).



Helical patterns have been reported in many animals, including sea urchins, corals, starfish and some fish (Liu *et al*., 2011; Kaupp *et al*., 2008). The helical nature of their sperm swimming patterns may indicate an efficient fertilization strategy, through allowing sperm to collide with eggs within its immediate vicinity (Farley, 2002). Chemotaxis is a species-specific mechanism that is created through chemical factors released from jelly coats of eggs that aims to guide the sperm towards the egg (Figure 2.5) (Miller, 1985; Márton and Dresselhaus, 2008).

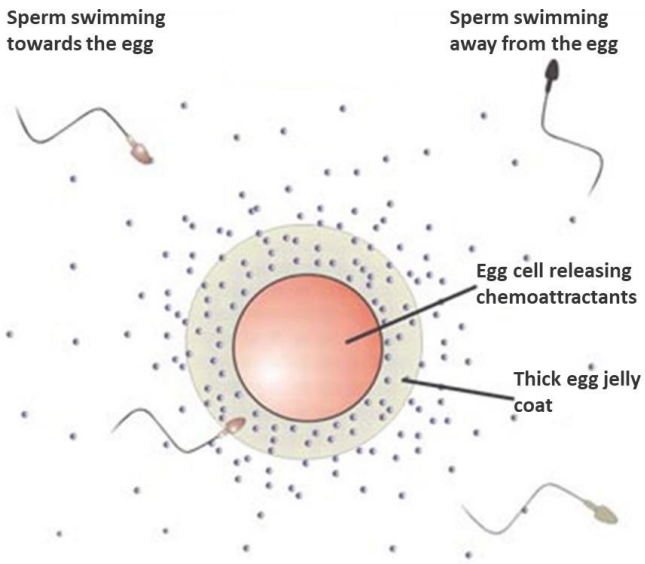



**Figure 2.5:** Diagram showing species-specific sea urchin sperm cells (left) moving towards the chemoattractants (dots) released from the sea urchin egg cell's egg jelly. Non-species-specific sperm (on the right) swims in the opposite direction. (modified after Márton and Dresselhaus, 2008)



Invertebrate sperm adjust their swimming path in relation to the chemoattractant gradients created by the eggs (Kuapp *et al*., 2006). These motility responses of spermatozoa rely on the flagellum as it gathers sensory information, the flagellum acts as a motor to propel and as a rudder to steer the cell towards its target, the egg. Receptors and gated channels along the flagellar surface convert the sensory information into cellular signals that modulate the whip-like motion of the beating flagellum and steers the swimming spermatozoa (Kuapp *et al*., 2006, Alverez *et al*., 2014).

Previous research has shown that chemoattractant signals which initiated sperm chemotaxis are synthesised and secreted by the egg or somatic cells associated with the egg (Márton and Dresselhaus, 2008). Chemotaxis is believed to enhance sperm-

egg fusion, but with the rapid dilution of the chemoattractants in sea water, spermatozoa must possess a high level of sensitivity in order to be guided to the correct egg. Current knowledge of sperm chemotaxis originates mostly from the sea urchin and starfish as model organisms (Kaupp *et al*., 2006; Hirohashi *et al*., 2008; Márton and Dresselhaus, 2008).

Approximately 80 peptides that affect sperm motility have been identified from Cnidaria and Echinodermata. These species-specific peptides are referred to as sperm-activating peptides (SAPs) (Miller, 1985; Suzuki, 1995). The chemotactic properties of only two SAPs, namely, resact and asterosap, have been clearly demonstrated (Ward *et al*., 1985; Vacquier, 1998; Kaupp *et al*., 2003; Böhmer *et al*., 2005). Although, the functioning of all SAPs has not been established, it has been tacitly assumed to be involved in chemotaxis (Kaupp *et al*., 2006; Márton and Dresselhaus, 2008). It has been suggested that chemoattractants and SAPs, like resact, activate multiple cellular reactions through common signal transduction pathways to stimulate and align sperm motility.



For example, these chemical factors evoke changes in intracellular pH, concentrations of cyclic nucleotides, $Na^+$ and $Ca^{2+}$ ions, membrane potential and phosphorylation pattern of several proteins (Cook *et al*., 1994; Darszon *et al*., 2001, 2005; Wood *et al*., 2007). Through these signal pathways, ion fluxes across the sperm membrane are created that affect flagellar motion and, by altering the waveform created by their flagellum, enable the spermatozoa to steer (Ramarao and Garbers, 1985; Bently *et al*., 1986; Neill and Vacquier, 2004).

Due to swimming in a chemoattractant gradient, spermatozoa are periodically stimulated, triggering $Ca^{2+}$ bursts that modify the waveform of the flagellar beat (Alverez *et al*., 2014). Spermatozoa respond by synchronizing the $Ca^{2+}$ concentration fluctuations with that of the constantly changing chemoattractant concentration, as they swim towards the egg (Böhmer *et al*., 2005; Guerrero *et al*., 2010).

This swimming behaviour has the advantage of sampling a larger area to collide into an egg, but the disadvantage is that sperm chemotaxis is distance dependent (Darszon *et al*., 2008). However, marine invertebrates release a high concentration of gametes, thus enhancing the probability of sperm-egg collision (Guerrero *et al*., 2011).

## 2.7 Marine Invertebrates: Mollusca and Echinodermata

Invertebrates form one of the largest groups of organisms, ranging from multicellular animals with no backbone to unicellular protistans. This diverse group incorporates over 30 phyla and is found in terrestrial and aquatic habitats. Some of the most common marine invertebrates are the Porifera, cnidarians, marine worms, molluscs, arthropods, echinoderms and crustaceans. Five shallow-water marine invertebrate species was selected for this study, including four species from the Mollusca phylum, of which there are 3 154 known species along the southern African coastline, and one Echinodermata of 419 known species.

### 2.7.1 Aquatic Reproduction

External fertilization is the most common and ancestral method of mating for many marine invertebrate species (Lotterhos and Levitan, 2010; Gallego-Parez). Aquatic organisms have two basic mechanisms of reproducing, either asexually or sexually;

although there are some species that require a combination of the two mechanisms (Hodgson, 2011). The greater majority of invertebrate taxa rely on sexual reproduction, which is regarded as being energetically more expensive. Two main routes of invertebrate sexual reproduction have been recognised; organisms could be either dioecious or gonochoristic, having separate sexes or hermaphroditic; where individual species produce both male and female gametes within its lifespan.

There are various aspects of hermaphroditisim some species have a single ovotestis or separate femaile and male gonad, while other species have the ability to adapt their sexuality based on the necessities of the population, producing male and female gametes simultaneously or sequencially (Hodgson, 2011). Oysters often change their sex, by beginning life as a male before becoming a functional female (Orton, 1921, Park *et al*., 2012). In addition to this diverse range of reproductive modes, often depended on the fertilization strategy, organisms could be either external or internal fertilizers. Dioecious internal fertilizing invertebrates for example some gastropods and most hard substratum taxa such as crustaceans, reproduce through copulatory or pseudocopulatory modes. These strategies include using their feeding structures to transfer sperm from the male directly into the maternal individual, avoiding contact with the sea water. Fusion of the male and female gametes occurs within the maternal organism, and is often followed by egg-brooding and the release of larvae or the direct release of fertilized eggs into the environment (Swart, 2014).



Almost all marine invertebrates are dioecious with the majority of species releasing eggs and sperm into the aquatic environment, which is frequently referred to as broadcast spawning. Broadcast spawning is defined as both the release of male and

female gametes and fertilization in the external environment. Some authors have proposed the term free-spawning to be used for males that released sperm into the environment independent from the presence of eggs or when eggs are retained by the female (Levitan, 1998; Yund, 2000).

Levitan (1998) suggested that broadcast spawning is always accompanied by free-spawning, but not vice versa, as some taxa have males that release sperm into the water column with fertilization occurring internally or on some external surface of the female. Although many authors have used these terms interchangeably (Giese *et al*., 1987; Pemberton, 2003; Bishop *et al*., 2006), this study will primarily use the term broadcast spawning.

A third aquatic fertilization method that was relatively recently acknowledged by Pemberton *et al*. (2003) is referred to as spermcast mating. Through this form of spawning male gametes are dispersed to female conspecifics and the fertilized eggs are retained by the originator/conceiver. Johnson *et al*. (2004) and Phillipi *et al*. (2004) did not fully agree with the spermcast mating term and referred to species showing this pattern as being egg-brooding free-spawners. Nevertheless, this diversity and variety of reproductive patterns in aquatic invertebrates, which are often seen as primitive, offers additional information that indicates the intensity of sexual selection within these species, which has been previously considered absent (Darwin, 1876; Palumbi, 1999; Levitan, 2004; Lotterhos and Levitan, 2010).

Broadcast spawning and spermcast mating have been recognised in sessile and sedentary organisms, often from filter-feeding species, that are living in large

populations (Serrão *et al*., 2009). Bishop and Pemberton (2006) envisaged a possible continuum between the two modes of spawning. This concept was reemphasised by other authors, noting that some species had the ability to fertilize the retained, unfertilized eggs in a sea water-filled compartment, such as the mantle of the flat oyster, (Ostreinae); through which water is pumped rather than in a true body cavity (Havenhand *et al*., 2010).

### 2.7.2 Echinoderms

The term echinoderm refers to 'spiny-skinned' marine animals with a radial symmetry, and has been classified as the invertebrates closest to the vertebrates, belonging to the same deuterostome evolutionary lineage (Zou, 2010). Five echinoderm classes have been identified, namely sea urchins, sea lilies, sea stars, brittle stars and sea cucumbers (Su *et al*., 2000). Sea urchins have a high economic value as a luxury food and this has intensified harvesting of wild populations along with the culturing of edible species.



Several populations have already decreased in Asian and South, Central and North American countries. Ecologically they assist to maintain kelp overgrowth and serve as protection for many smaller organisms (Binet *et al*., 2014). The Cape sea urchin, *Parechinus angulosus* (Leske, 1778), is a gonochoristic species with a variation in test colours among individuals, ranging from pink-purple to green and red. This dominant herbivore is located along the wave–exposed rocks and rocky-pools through-out the Western and Eastern Cape, forming dense populations of up to 90 animals per $m^2$ (Fricke, 1979). These iteroparous animals (species that reproduce several times in their lifespan) are broadcast spawners that display a bimodal spawning cycle,

however, there have been animals within this species with gravid gametes throughout the year (Fricke, 1980; Wynberg *et al*., 1989).

*Parechinus angulosus* plays an important ecological role in coastal ecosystems as many studies have demonstrated, covering topics such as population dynamics, respiration, ecological energetics (Greenwood, 1974; Stuart and Field, 1980), its grazing impact on kelp beds (Fricke, 1979), feeding habits, defecation, absorption rates (Buxton and Field, 1982), and the effect of organic pollutants on fertilization success (Wynberg *et al*., 1989).

The Cape sea urchin also plays an important role in the rocky-pool ecology as studies have shown that the removal of this species leads to an increase in siltation of the area and decreased the quantity of abalone recruits, who seek protection under their spiny bodies (Tarr *et al*., 1996; Day and Branch, 2002). More recently, phylogeographic population structures were investigated and showed high levels of population differentiation in *P. angulosus* from eighteen different sites along the southern African coastline (West-South-East coast) (Muller, 2012). A wealth of literature documents the sea urchin as a model species in developmental biology and current knowledge on generalized spermatozoan physiology, form and functioning has its origins in echinoderm morphology (Rothschild *et al*., 1951; Bernstein, 1961; Epel, 1975; Tilney, *et al*., 1978; Lewis *et al*., 2012; Fabbrocini *et al*., 2017). Sea urchins have been widely used in several CASA studies as they are easy to obtain and maintain within the laboratory.

CASA has been applied on sea urchin gametes by Fabbrocini *et al*. (2016) to optimize

protocols aimed at the development of gamete management of the Mediterranean population of *Paracentrotus lividus*. The group evaluated sperm quality as a function of the gonad stage, through assessing changes in sperm motility parameters. Another CASA study was conducted to offer rapid information about sperm quality of marine invertebrates for possible use in the culturing of sea urchins as well as ecotoxicology and climate change research (Au *et al*., 2002).

Binet *et al*. (2014) reported on using a carbocyanine fluorescent probe, popular for assessing the mitochondrial membrane potential (MMP) of mammalian sperm, for assessing changes in MMP of sea urchin sperm. The stain's uptake was validated through demonstrating its effect on the sperm motility and speed of the sea urchin sperm. Toxicology investigations have been one of the focus areas for the application of CASA in studies using invertebrate species. Sea urchin gametes and embryos are sensitive to environmental stressors making these species popular in toxicological studies which date back to the 1920s (Hoadley, 1923; Fitzpatrick, 2008; Lewis *et al*., 2012).



Recently, more toxicity bioassay tests have implemented CASA in its studies by using the sensitivity of sperm motility and its various velocity parameters as an assessment tool (Au *et al*., 2000; Fabbrocini *et al*., 2010). A toxicity study correlated the sensitivity of sea urchin sperm to certain compounds that altered the morphology of the spermatozoa and reduce sperm speed as well as the overall percentage of motile sperm (Au *et al*., 2002). These studies suggest the change in swimming ability could lead to an increase in sperm limitation and decrease in fertilization success (Au *et al*., 2002; Ghirardini *et al*., 2005).

Despite the growing number of CASA investigations, there is minimal data establishing baseline values to determine the effects of contaminants on sperm motility in a natural population of sea urchins (Lewis *et al*., 2012) and no specific CASA studies on *P. angulosus* (van der Horst *et al*., 2018).

### 2.7.3 Molluscs

Molluscan species belong to the second largest phylum with over 90 000 recognized species. Molluscan diversity can easily be compared to insects and vertebrate species and ranges from terrestrial to freshwater and marine species. They are generally soft-bodied animals enclosed, partly or wholly, in a calcium carbonate shell. The shell is usually secreted by its soft mantle covering the body. Three major classes are found: Bivalvia such as oysters (*Crasstrostrea gigas*), mussels (*Choromytilus meridionalis*) and clams (*Donax serra*); Cephalopods including squids and octopi; and the most diverse class, the Gastropoda (*Haliotis midae*) (Davis-Coleman, 2006; Benkendorff, 2010).



#### 2.7.3.1 Bivalvia

Bivalves are enclosed by two protective shell-valves, connected by a strong elastic ligament acting as a hinge. This group forms significant colonies in the intertidal and subtidal communities. Bivalves can also be found inhabiting exposed sandy beaches where they burrow into the soft-sediment. While there are few bivalves that are mobile, most adult live a sedentary life attached to the rocky substratum by strong filaments (Bally, 1983; Laudien *et al*., 2003; Blackburn *et al*., 2014). Various mussel species have been identified in aquaculture farming and many studies have utilized mussels in biological, biochemical and genetic research worldwide (Gosling, 1992)

### 2.7.3.2 *Choromytilus meridionalis* (Krauss, 1848)

*Choromytilus meridionalis* are endemic to South Africa and colonize the coastlines along with four other mussel species, namely the warm-water brown mussel, *Perna perna*, another indigenous ribbed mussel, *Aulacomya atra* and two invasive species—the Mediterranean mussel, *Mytilus galloprovincialis* and the recently discovered bisexual *Semimytilus algosus* (van Erkom Schurink and Griffiths,1991; Bownes *et al*., 2008).

*Choromytilus meridionalis* have a patchy distribution along gentle sloped, often sand covered slow draining rocky surfaces that have moderate to large wave exposure (Marshall and McQuaid, 1993; Steffani and Branch, 2003). Previous research on *C. meridionalis* includes studies on their reproductive cycles, assessment of gamete production, population genetics, as well as physiological, ecological and toxicology studies (Griffiths, 1981; Griffiths and Hockey, 1987; Grant *et al*., 1984; van Erkom Schurink and Griffiths, 1991; Dahms, 2014). Research projects focussing on *C. meridionalis* spermatozoa and egg morphology are limited, and include a study by Hodgson and Bernard (1986), who reported on the ultrastructure of spermatozoa of three mussel species, including *C. meridionalis*.

Only few wild species have been studied and more attention has been placed on commercial species, such as *M. galloprovincialis* (Grant *et al*., 1984; Robinson and Griffith, 2002; Branch and Steffani, 2004). Not many investigations have focused on sperm morphology, spermatology and sperm motility patterns. As noted by Hodgson (2010), the lack of empirical work has left a gap in the controls of breeding patterns and experimental work is needed to examine the factors that affect reproduction,

fertilization fitness and larval development.

### 2.7.3.3 *Donax serra* (Röding, 1778)

The Donacidae family inhabits the macrozoobenthos of the intertidal exposed sandy beaches worldwide. *Donax serra*, is the largest wedge-shaped surf clam inhabiting sandy intertidal and upper subtidal beaches (Laudien *et al*., 2003). These sand-burrowing bivalves are most abundant on exposed beaches where the sand is not too coarse and contains a high phytoplankton production (Donn, 1987). Many species of *Donax* have been shown to migrate with the tides along the surf zone. Once sexual maturity and adult size was attained *D. serra* migrate to the subtidal zone (Bally *et al*., 1983; De Villiers, 1975).

Spawning season differences have been observed between Eastern Cape and Western Cape white mussels, with Eastern Cape white mussels spawning in summer (Hanekom, 1975) and winter (McLachlan and Hanekom, 1979; van der Horst, 1986), whereas Western Cape *D. serra* have not shown a distinct spawning period and had mature gametes year-round (Griffiths, 1977; De Villiers, 1975). This contrast between these populations resulted in speculation of an interspecific and intraspecific variation (Laudien *et al*., 2003), which may indicate that these populations belong to a different subspecies (Wade, 1967; Donn, 1990). Differences between morphological and behavioural characteristics of species are believed to be determined by changes in the physical and or biological environments (Levitan, 1988, Grant, 1991 Laudien *et al*., 2003). Although their shell morphology and soft tissue colour differences have been significant there were no differences detected in sperm morphology and growth rate (van der Horst, 1988; Schoeman, 1997).

Many ecological studies have been carried out on *D. serra* communities and this species is one of the only South African marine invertebrate species previously evaluated using CASA (van der Horst, 1988). Sperm motility in the latter study was measured using classic videographic methods (Katz and Overstreet, 1981), that included a developing frame-lapsed method, in conjunction with computer analysis to provide accurate and reliable data of sperm swimming speeds. Evaluating sperm motility using videotaped recordings in the 1980s was unique in comparison with the development of computerized laboratory equipment of today.

### 2.7.3.4 *Crassostrea gigas* (Thunberg, 1793)

*Crassostrea gigas* has been referred to by several common names, including Pacific oyster, Japanese oyster and Pacific cupped oyster. However, the World Register of Marine Species (WoRMS) has registered and is in the process of accepting a new genus *Magallana*, with *Magallana gigas* being designated as the new accepted species name (Salvi and Mariottini, 2017). Nevertheless, for the purpose of this study the well-established common names will be used, and is well documented in many publications. *C. gigas* has been described as filter-feeding, sedentary invertebrates normally attached to hard substrates and are protandrous hermaphrodites (Pieterse 2012). Most oyster species can undergo a sex change during their life, normally by spawning as male first and then change to female after the first spawning season. These changes are reversible and depend on the availability of resources, such as food and space, while the sex ratio is skewed toward females during favourable environmental conditions and males predominate when resources are scarce (Orton, 1921, FAO, 2012).

Following global trends, the South African oyster industry has been based on *C. gigas*, (Robinson *et al*., 2005) due to its adaptability and the low cost of growth. A small volume of CASA studies has been conducted on this commercially important species, especially its gametes and larvae have been widely used during cryopreservation studies (Suquet *et al*., 2012). CASA has shown disruption in oyster sperm motility when this species is exposed to aquatic pollutants. The number of motile sperm when exposed to alkylphenol, an organic industrial compound, was significantly reduced when compared to non-exposed oysters (Nice, 2005).

The effects of ocean acidification and seasonal variation on the sperm quality of these oysters were also measured, as fertilization fitness may be affected in the future. Ocean acidification studies using CASA by Havenhand *et al*. (2009) have shown no significant changes in sperm swimming behaviour when comparing the percentage motility and sperm swimming speed measurements of sperm exposed to filtered sea water and acidified filtered sea water. These results are in contrast to a study by Havenhand *et al*. (2008) that had found a species of sea urchin that had reduced fertilization success as a result of the impact of acidic sea water on sperm swimming behaviour.



### 2.7.3.5 *Haliotis midae* (Linnaeus, 1758)

*Haliotis midae* is commonly referred to as abalone or perlemoen in South Africa. It is the largest of five endemic abalone species, the others are *Haliotis parva*, *H. spadicea*, *H. queketti* and *H. speciosa* (Evans *et al*., 2004), and it is the only species commercially cultivated (Raemakers *et al*., 2009). Abalone are dioecious broadcasters, (Boolootian *et al*., 1962), with spawning events occurring between April

and December.

East Asian countries have the greatest demand for abalone and assisted in establishing the most lucrative mariculture industry in South Africa (Tarr, 1989). Conversely, this great need caused overfishing, severe poaching and reducing the commercial quotas of this species (Tarr *et al*., 1996; Day and Branch, 2002). This forced the government to add *H. midae* to the list of Convention of International Trade in Endangered Species (CITES) in 2007 that prevents the export of abalone without a permit. The governmental restrictions on harvesting natural stocks motivated the farming of abalone in the 1990s, which produced viable spat and juveniles from wild-caught abalone (Genade *et al*., 1988). South African studies have previously been focused on reproductive biology (Newman, 1967), induced spawning (Genade *et al*., 1988) and histology of the gonads (Wood and Buxton, 1996).



More recently Roux *et al*. (2013, 2014) focused on histological evaluations of gonadal tissue of cultured wild abalone along with developing a protocol for laboratory fertilization procedures for cultivated *H. midae*. Other ecological aspects, such as nutrition, disease, growth rate and genetics have also been investigated (Day and Branch, 2002; Roodt-Wilding, 2007). There are only limited studies on marine invertebrates' gamete fertilization, characterization of sperm motility behaviour and sperm quality, including a publication that carried out preliminary cytotoxicity studies on cryoprotectants on *H. midae* embryos (Roux *et al*., 2008).

It is envisaged that the use of CASA sperm kinematic parameters could examine how physical, chemical and biological factors affect sperm swimming behaviour. By using

their sperm traits, kinematic subpopulations information would provide information that relates to sperm functionality that could assist with fertilization efficiency. This baseline study will be needed to build up the knowledge base of the sperm biology and swimming behaviour of these study species, to gain useful information that would be beneficial for commercial, aquaculture and laboratory research purposes.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 General Aspects

Experimental procedures were standardized among the study species to ensure accurate comparison of sperm parameters measured. Where necessary, some procedures were modified to simplify the sperm sample collection presented and are indicated in the appropriate sections. Wild-caught samples were stored and maintained using standard procedures and all holding containers were stored in a temperature-controlled environment of 17-18˚C. This environment complemented the surface sea temperature at the collection sites, which fluctuated between 12-19˚C. Sea water in holding containers was changed every 12 hours (hrs). The animals were kept under constant aeration using a Tetra silent aquarium air pump attached to air-stones, to assist with the oxygen exchange at the surface. All experimental work was completed within 48 hrs after animal collection.

This study adhered to the ethical guidelines of the University of the Western Cape. A yearly permit for the hand-collection of certain animal species was obtained through the Department of Biodiversity and Conservation at the University of the Western Cape from the governmental Department of Agriculture, Forestry and Fisheries (DAFF), Cape Town, South Africa (Appendix I). Cultivated animals were obtained from commercial aquaculture farms along the Western Cape coast lines.

## 3.2 Animal Collection and Housing

### 3.2.1 *Choromytilus meridionalis*

*Choromytilus meridionalis* (black mussels) were collected between January and April along the west coast of South Africa, at Blouberg strand (33.790 ˚S, 18.4588 ˚E) and at the inner bay of Saldanha (33.0197 ˚S, 18.1903 ˚E). Samples were hand-collected at low-tide along the sandy rocky intertidal zone in Blouberg strand (33.790 ˚S, 18.4588 ˚E). Black mussels, ranging from a shell size of 60-80 mm were collected by cutting the byssal thread strong hold and transported to the laboratory in a 20-l holding tank filled with sea water found at the site. In the laboratory, half of the sea water was removed from the holding container and refreshed with sea water collected from the site. Aeration was maintained with an air-stone attached with tubing to a Tetra aquarium air pump. Sex determination was done before experimental work to obtain ten male black mussels for the study.



Additionally, black mussels were obtained from an aquaculture farm that directly supplied to retailers within the Cape Town region. Mussels were packaged in plastic mesh bags and transported dry (without sea water) in a refrigerated truck to the distribution company in Maitland, Cape Town. Commercially grown samples were covered with sea-water soaked absorbent paper; dry-stored in the laboratory walk-in-fridge at 4˚C and was used within 48 hrs of harvesting. Mussels were dry-stored in the fridge once they had been exposed to air for a length of time as reimmersion in a volume of sea water can induce spawning (Vakily, 1989).

### 3.2.2 *Donax serra*

*Donax serra* (white mussels) were located along the sandy intertidal zone at Big Bay

beach, in Blouberg strand (33.7901 ˚S, 18.4588 ˚E). Ten male individuals of this species were used for the study collected during monthly low tides and spring tides, in late spring (October-November), summer (December-February) and early autumn (March-April); between 2009 and 2018. Samples were hand-collected as recommended by DAFF bywading out to the mean tide level during low tides to feel for white mussel in the soft sand. The mechanism of collecting involved moving the heel of the foot, side to side down into the wet sand, until sensing a white mussel. Once a mussel was located, the heel was held firmly against the white mussel and the mussel was quickly grabbed, before it burrowed further into the sand (Jerardina *et al*., 2014). The mussels were placed in a 20-l plastic holding container filled up three quarters full with sand and topped up with sea water collected from the site. The high sand level in the container allowed the mussels to burrow down into the sand column ensuring stress-free transportation to the laboratory, where they were maintained in the sand-filled container. To maintain aeration, a Tetra aquarium air pump was attached to an air-stone and placed in the water column above the sand.



### 3.2.3 *Crassostrea gigas*

Cultured *C. gigas* (oysters) were obtained from a marine farm in Saldanha bay located off the west coast of South Africa (33.0197 ˚S, 18.1903 ˚E). The oysters were grown using the long-line culturing technique. This technique involves suspending long rope lines into the ocean that are attached to floating buoys or barrels which support plastic mesh baskets (DAFF, 2012).

Oysters were harvested daily, in the morning, and cleaned before being packaged in plastic mesh bags. Mesh bags filled with 30 oysters were transported in a refrigerated

truck to the distribution company in Maitland, Cape Town. Oysters were collected from the distribution factory in Maitland and placed on top of a plastic sheet barrier covering ice which prevented direct contact between fresh water and the oysters. Within the laboratory, the oysters were immediately dissected for sex determination and gamete collection of ten males were used for experiments.

### 3.2.4 *Parechinus angulosus*

Adult *P. angulosus* (sea urchins) with a test diameter ranging from 40-55 mm were hand-collected at monthly low-tides of 0.25-0.15 m, along the intertidal rock pools at Blouberg strand, Cape Town (33.8025 ˚S, 18.4601 ˚E). The surface sea water temperature within the rock pools were measured on the day using a digital pocket thermometer (Flinn Scientific, Canada) and ranged between 17-19˚C.

Cluster communities of urchins were located along crevices of the rocks; where animals were quickly picked off the rocks in one smooth movement. This method prevented the anchoring of the tiny tube feet to the substratum and reduced trauma to the sea urchin and injury to the collector. Five sea urchins were collected and stored in 20-l holding containers filled with sea water collected from the sampling site and transported immediately to the laboratory. Additional sea water was collected at the site for the laboratory maintenance of animals and experimental use. Laboratory room temperature was maintained at 17-18˚C and aeration in holding containers were maintained with an air-stone attached to a Tetra aquarium air pump. Ten male sea urchins were sampled for the experiments and egg-water was prepared on the day from one to three female sea urchins before each experiment requiring egg-water.

### 3.2.5 *Haliotis midae*

Owing to the embargo on the collection of wild *H. midae* (abalone), CASA analysis was performed on-site, using sperm from wild-harvested abalone broodstocks grown at the HIK abalone farm in Hermanus on the south-west coast of South Africa (34.4349 ˚S, 19.2203 ˚E). The analysis of additional abalone experiments could not be achieved as no dry-sperm samples were acquired. Furthermore, collecting samples from this species is highly regulated and no broodstock males were sacrificed. Commercially cultured abalone were grown in oyster net baskets suspended in 50-l tanks; which were filled supplied with aerated and filtered (1 µl) sea water, provided through a mechanical flow-through sea water system (Roux, 2011). Five broodstock males were kept at a room temperature of 18˚C in a rectangular 40-l plastic holding tank, with the photoperiod set on 12 hrs light and 12 hrs dark. Abalone can be clearly distinguished by the colour of the gonad; the female has a green gonad and when ripe the male gonad is cream-coloured.



## 3.3 Gamete Collection

### 3.3.1 Dissection and Sperm Extraction

#### 3.3.1.1 *C. meridionalis*

The *C. meridionalis* were cleaned by removing the excess byssal threads. All living material adhering to the mussel shells were scrapped off and the mussels were thoroughly rinsed with room temperature (18˚C) filtered sea water and then blotted dry. Using a scalpel, the posterior adductor muscle and foot retractor muscle were cut by inserting the scalpel between the shell-valves near the byssal threads, on the ventral side. The shell-valves were fully pried open to expose the internal contents.

Ripe engorged gonads were clearly visible within the inner shell-valve and sex distinction was possible by the colour of the gonads presented in Figure 3.1.

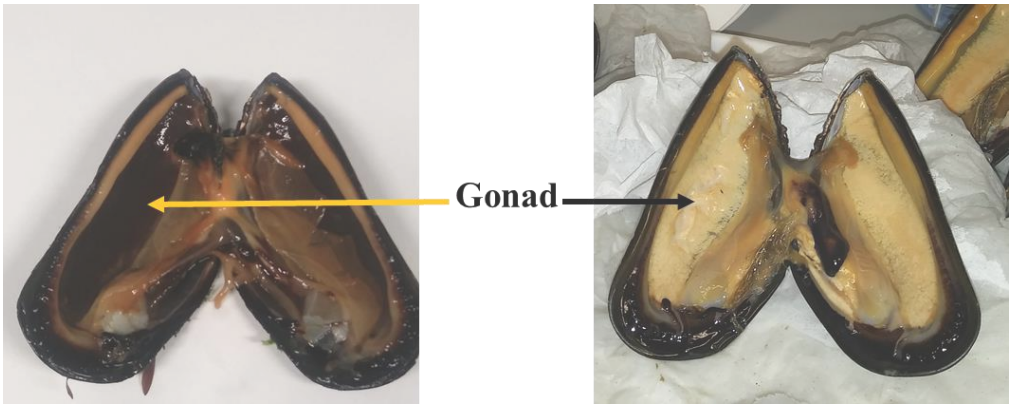



**Figure 3.1:** The sex of *C. meridionalis* is distinguished by the colour of the gonads, showing brown gonads (yellow arrow) in female black mussels and male gonads (black arrow) filled with creamy white sperm.



Female gonads were brown in colour, while cream, white-yellowish gonads belonged to males. Sex determination was further verified microscopically by placing a drop (2 µl) of gonadal fluid on a slide with a cover slip and viewed with a Nikon Eclipse 50i microscope (IMP, Cape Town, South Africa) using negative phase contrast microscopy. Testes were dabbed dry with towelling paper to remove excess sea water to prevent the activation of sperm motility before CASA evaluation, as sperm age rapidly and become less viable when diluted in sea water (Levitan, 1993). A downside of obtaining testicular sperm is the possible presence of immature sperm. Nevertheless, CASA kinematic parameters were set at specific cut-off values for sperm swimming speed to counter this possibility. There are other methods for obtaining sperm, through chemical-induced spawning and thermal shock (see section 3.5.5.2 on thermal-regulated spawning). These methods were not appropriate for all

CASA experiments and some were more time consuming. Too extract sperm a small incision was made in the testes with a sterile disposable blade (#24) attached to a scalpel handle, which allowed sperm to ooze out of mature gonads. Thick, white sperm was extracted from the testicular tissue, using a displacement pipette with a 10-µl tip. Concentrated sperm was stored in a clean Eppendorf tube, free from sea water and was referred to as dry-sperm in a previous study (Vacquier *et al*., 2004). In contrast, sperm taken out directly from the testicular tissue in this study is referred to as testicular-sperm. Testicular-sperm was stored at 4˚C for 24 – 48 hrs when not immediately used for experiments.

### 3.3.1.2 *D. serra*

*Donax serra* were removed from the sand-filled holding container, cleaned with filtered sea water and held firmly down on the dissecting board. The scalpel was placed between the valves on the ventral surface and the posterior adductor muscle was cut, which released the muscle attachment to the shell. The valves were cracked open at the hinge area. *Donax serra* gonads do not have the distinct colour differentiation between male and female gonads observed in the black mussels (Figure 3.2).



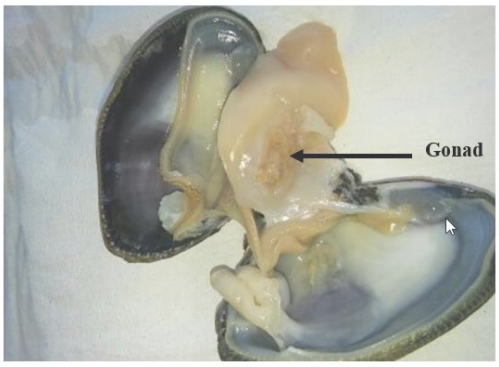



**Figure 3.2:** Exposed internal structure of *D. serra*, arrow showing the gonad from which testicular-sperm was extracted.

The gonad is located between the digestive gland and the muscular foot, where a small incision was made to remove the gonadal fluid to determine the sex. A slide was simply prepared, as explained in section 3.2.1 for the black mussel, and the sperm viewed using a Nikon Eclipse 50i microscope (IMP, Cape Town, South Africa) using negative phase contrast microscopy. Highly viscous sperm was extracted from males, using a displacement pipette with a 10-µl tip and testicular-sperm was stored in an Eppendorf tube at 4˚C.

### 3.3.1.3 *C. gigas*

Before gamete extraction, *C. gigas* were removed from the container with ice, cleaned with filtered sea water and allowed to acclimatise to the laboratory temperature of 18˚C for one hour. The curved lower shell of the *C. giga* was securely held down, while a straight-edged paring knife was firstly inserted between the two shells near the hinge area and then twisted until the hinge 'popped'. This method provided sufficient space to allow the scalpel to slide between the shells cutting the adductor muscles which completely released the flat top shell.



The liquid inside was drained and the visceral mass dried by dabbing with absorbent paper. *Crassostrea giga* gonads have minimal colour distinction between sexes, but were always white in colour. The gonads formed part of the visceral mass, alongside the circulatory and digestive organs. Sex determination was achieved microscopically; by preparing a slide using a small amount (2 µl) of extracted gonadal fluid and placing a cover slip on top to evenly distribute the fluid for viewing. The 10X negative phase objective of a Nikon Eclipse 50i microscope (IMP, Cape Town, South Africa) was used to verify the gametes. The highly viscous sperm of male oysters were pipetted from

the testicular tissue, using a displacement pipette, through an incision made in the gonad and testicular-sperm were stored at 4˚C in an Eppendorf tube for later use.

### 3.3.2 Chemical-Induced Spawning for Gamete Extraction—*P. angulosus*

Sea urchin species have been described as favourable experimental animals to use in various research fields, including understanding cell division, gene regulation, fertilization and sperm motility to acrosome reaction (Vacquier, 2011; Fabbrocini *et al*., 2016). Accordingly, *P. angulosus* were used as a good experimental model for CASA motility experiments and served as a baseline for intraspecific comparisons. In contrast to the other species, eggs were also collected in this species to test the effect of eggs and egg-water on various sperm characteristics (see below).

Sexually mature *P. angulosus* gametes were extracted through chemical induction with 0.55 mM potassium chloride (KCl) (Palmer, 1937; Tyler, 1949). Sea urchins from the holding containers were cleaned by rinsing off any debris found on the aboral surface with filtered sea water. A volume of 1-2 ml 0.55 mM KCl was injected using a 1-ml syringe into the soft tissue surrounding the masticating apparatus on the oral surface. The whole urchin was gently agitated by tilting it from side to side, allowing the distribution of the KCl within the body cavity. The presence of KCl stimulated the contractile tissue within the mesenteries, which induced spawning of the gravid gonads within 30 sec to 5 min after being administered. The distinctive colour difference of the gametes provided easy identification, with either orange eggs or white sperm being ejected through the five gonopores on the aboral surface.

Urchin eggs were collected by inverting the spawning female over a 100-ml glass

beaker, filled to the brim with filtered sea water, allowing the negative buoyant eggs to settle at the bottom as shown in Figure 3.3. Sperm was pipetted using a 20-µl Gilson pipette and stored as dry-sperm in a 2-ml Eppendorf tube, until needed. Dry-sperm from spawned sea urchins will be referred to as dry-spawned sperm for this study purpose and is used for all sea urchin experiments and when not immediately used, was stored at 4˚C for a period of 12-48 hrs.

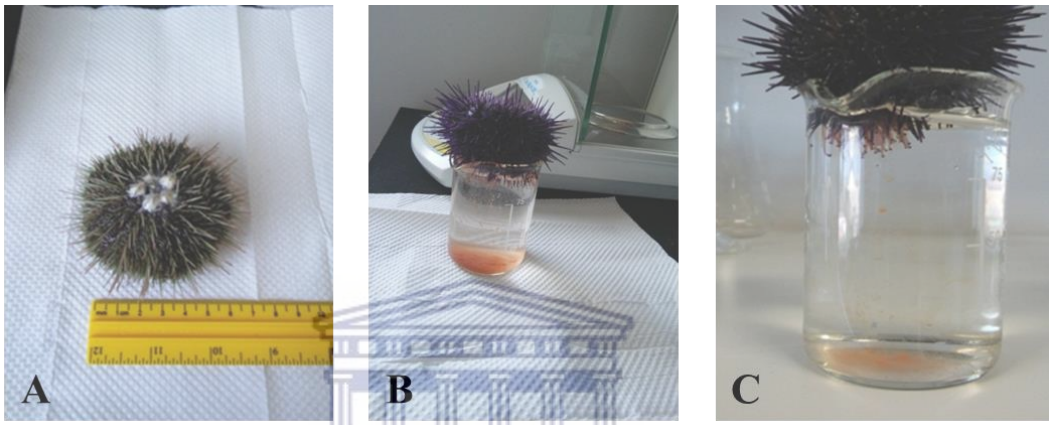



**Figure 3.3:** Photographs displaying male and female *P. angulosus* spawning induction within a laboratory set-up. Male sea urchin (A) with whitish sperm appearing at the five gonopore openings. The female sea urchin (B and C) was inverted and allowed to spawn its orange eggs into filtered sea water.



## 3.4 Sperm Biology and Morphometric Assessments

### 3.4.1 Sperm Smear Preparations

The morphology of the spermatozoa was evaluated using sperm smears and light microscopy for the following experiments: testicular- and dry-spawned sperm motile sperm from swim-up populations (see section 3.5.3 swim-up technique), sea urchin dry-spawned sperm exposed to egg-water alone, and sperm in the presence of eggs. To prepare the sperm smears, a small aliquot, approximately 7 µl, of sperm in sea

water suspension was placed on a slide. A second clean slide was used to make a thin smear, by holding the second slide at a 45˚ angle and placing it in front of the sample. This slide was slowly moved backwards of the sample, allowing the sample to spread across the edge of the slide. Subsequently, with one quick motion the second slide was pushed forward to create a smear of the sample across the slide towards the frosted side. The smeared slides were allowed to air dry completely before staining the preparation.

### 3.4.2 Staining Procedure

SpermBlue® fixative and staining solutions utilised were obtained from Micropitic, S.L. (Barcelona, Spain), for light microscopy work. Both solutions were modified for marine invertebrate studies, by using filtered sea water as a diluting medium, as described by van der Horst and Maree (2009). Air-dried sperm smears were gently placed in vertical polypropylene Coplin jars containing the fixative solution for 10 min at a room temperature of 20˚C. Slides were carefully removed and held at a vertical angle, to allow the excess fixative to slowly drip off onto absorbent paper. Wet fixed slides were placed in the SpermBlue® staining Coplin jar for 10-12 min.



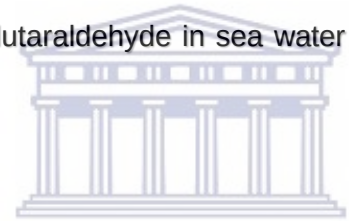
Once stained, slides were gently removed from the jar; excess stain was allowed to roll off the slide before washing. The washing step involved dipping the slide once for 3 sec into filtered sea water (filtered using a 0.2-µm Millipore filter) and a final 2 sec dip into distilled water. While holding the slide vertically, it was carefully dabbed on absorbent paper to remove excess liquid.

Stained slides were left overnight for drying; afterwards, coverslips were mounted with

DPX mounting medium and allowed to completely dry before viewing. Sperm blue stained slides was used to verify different sperm components with light microscopy.

### 3.4.3 Fixation of Samples

Samples for light microscopy and transmission electron microscopy were prepared using testicular sperm, dry–spawned sperm, sperm from swim-up populations, sea urchin sperm exposed to egg-water and sea urchin eggs. A volume of 100-200 µl of the prepared samples were placed into a microcentrifuge tube and centrifuged for 5 min at 500 rpm. The supernatant was removed and the sperm pellet re-suspended in 500 µl of 2.5% glutaraldehyde, diluted with filtered sea water (0.2-µm Millipore filtered). Filtered sea water was diluted with distilled water to 850 mOsm and the final osmotic concentration of 2.5% glutaraldehyde in sea water fixative was approximately 1050 mOsm.




### 3.4.4 Morphometry Assessment: Light Microscopy and CASA

#### 3.4.4.1 Brightfield Microscopy

Sperm structure of SpermBlue® stained spermatozoa was captured using the Morphology module of the Sperm Class Analyser (SCA®) system version 6.2.0.16 (Microptic S.L., Barcelona, Spain). Images were captured using a Basler A312fc digital camera (Microptic, S.L., Barcelona, Spain) mounted on an upright Nikon Eclipse 50i microscope (IMP, Cape Town, South Africa). Brightfield imaging was performed on stained spermatozoa using a 40X objective and a 100X oil immersion objective. The microscopic illumination was adjusted for each objective used.

### 3.4.4.2 Phase Contrast Microscopy

Five microlitres of the fixed unstained sperm preparations, as described in section 3.3.1 was pipetted onto a cleaned slide, covered with a coverslip and allowed to settle for 10 min before viewing. Sperm morphometry was measured with the Cell counter module of the SCA® system version 6.2.0.16 (Microptic S.L., Barcelona, Spain). Images were captured using a Basler Ace ACA 1300-200UC digital camera, C-mounted on a Nikon Eclipse 50i microscope (IMP, Cape Town, South Africa), equipped with a phase contrast condenser. Phase-contrast imaging of the fixed spermatozoa was observed, using a green filter and a 40X Ph2 objective or a 100X Ph3 oil immersion objective.

Spermatozoa were displayed on the monitor with brightness adjusted for each individual sample preparation. All cells were accepted for analysis, however, cells that overlapped or that were obstructed by debris were excluded. Analyses were carried out on black mussel, sea urchin, white mussel and oyster sperm. Morphometric parameters of a total of 50 non-overlapping spermatozoa were measured per sample per animal, a number found by Maree *et al*. (2010) to be a good representation of the morphometry of the whole sperm population.



### 3.4.4.3 Nomarski Differential Interference Contrast (NDIC) Microscopy

Microscopic examination of unstained fixed slides was additionally performed using Nomarski differential interference contrast (NDIC) microscopy to illustrate and observe the surface structure of the various sperm components in more detail. Micrographs were taken using an Olympus Astra 20 high resolution camera (Wirsam, Cape Town, South Africa) fitted onto a Zeiss Photomicroscope III (Zeiss, Cape Town, South

Africa). Slides were prepared similarly to the phase contrast slides as above. Images were captured with a 100X objective positioned at DIC III and evaluated with the semi-automated analysis® FIVE soft imaging system (Wirsam, Cape Town, South Africa).

### 3.4.4.4 Sperm Morphological Parameters Measured

Length and width measurements of the sperm head, acrosome, midpiece and tail were performed. The 40X objective was used to measure the total sperm length, while the 100X oil immersion objective was used to measure the length and width of the head, acrosome, midpiece and tail. Sperm head length (HL) was measured along the major axis of the head and sperm head width (HW) was taken at the shortest axis. The total sperm length was determined by adding the sperm head length and the total tail length measurements. For the morphometric analysis a total of 50 non-overlapping spermatozoa were measured per sample per animal (Maree *et al*., 2010).

### 3.4.5 Sperm Ultrastructure: Transmission Electron Microscopy (TEM)

Preparations of the fixed motile spermatozoa samples as described were used for transmission electron microscopy. A total of five animals per species were used. The spermatozoa were fixed in 2.5% glutaraldehyde solution prepared in filtered sea water. After fixation the samples were postfixed in 1% osmium tetroxide in the same buffer. The fixed preparations were processed for TEM by enhancing contrast with lead citrate and uranyl acetate.

A Reichert ultramicrotome (SMM Instruments, Johannesburg, South Africa) with either a glass or diamond knife was used to cut silver to gold sections (Agar Scientific, Randburg, South Africa). Thin sections were mounted on copper grids and examined

using a Jeol JEM 1011 transmission electron microscope at 80 kV (Advance Laboratory Solutions, Johannesburg, South Africa). Samples were viewed at magnifications ranging from 5000X to 80 000X and detailed micrographs of longitudinal sections of whole spermatozoa and cross-sections of various sperm components were produced. A Megaview III digital camera was fitted to the microscope and all images were captured using the ITEM software package (Advance Laboratory Solutions, Johannesburg, South Africa) and saved as either 'jpeg' or 'tiff' files. It should be noted that the sperm pellets did not section well and were prone to cutting artefacts.

## 3.5 Sperm Concentration and Percentage Motility

Sperm analysis included measurements of sperm concentration, total motility and progressive motility in motile populations. Motility of sperm was determined following two techniques, namely flush and swim-up. Testicular-sperm and dry-spawned sperm from the marine invertebrate specimens studied, except abalone, were used during the swim-up technique and the flush technique (Figure 3.4).

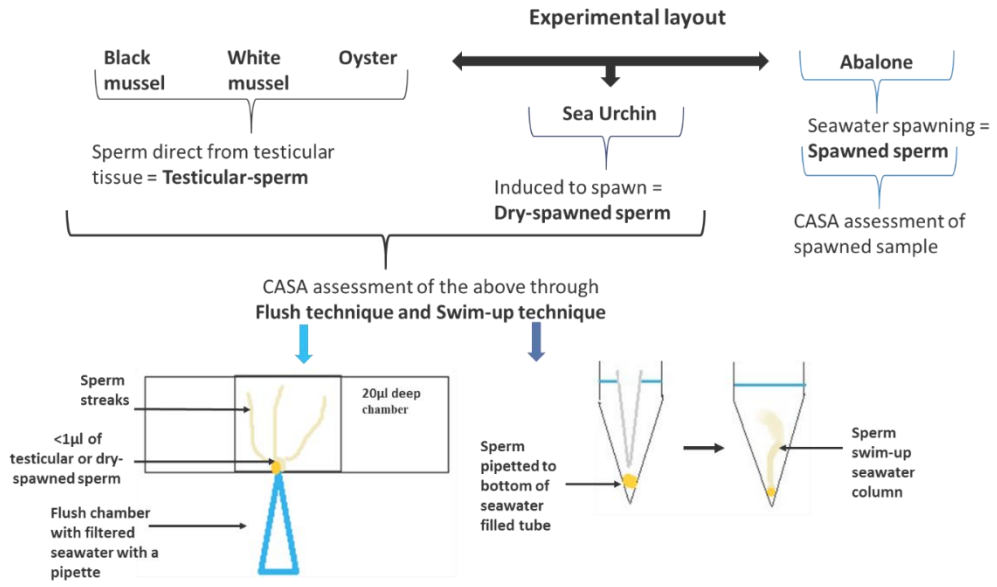



**Figure 3.4:** Flow chart illustrating the study animals and the various sperm samples obtained, including a visual description of the two study techniques used, namely flush and swim-up.



### 3.5.1 Determination of Sperm Concentration and Motility

The sperm concentration and motility were assessed by pipetting ≤ 1 µl sperm in 5 µl sea water into a 20-µm deep chamber of a Leja slide (Leja Products B.V., Nieuw Vennep, The Netherlands), for automatic SCA CASA analysis. Motility parameters for flush were assessed within 2-7 min of sea water introduction for *C. meridionalis*, *D. serra* and *C. gigas*. However, *P. angulosus* dry-spawned sperm was immediately activated in sea water. The swim-up samples were evaluated after a 10 min incubation at time intervals of 5, 15, 30 and 60 min. This technique was repeated approximately 24 hrs later, after concentrated testicular and dry-spawned sperm samples of *C. meridionalis*, *D. serra*, *C. gigas* and *P. angulosus* were stored at 4˚C. Since *H. midae* was induced to spawn, no flush measurements were obtained.

Laboratory equipment and Leja slides were kept at 18˚C for all experimental evaluations, to mimic the natural cooler sea water temperature, since sperm motility and longevity is affected by temperature. Two to five fields were captured randomly to eliminate bias toward best motility areas; however, fields that included sperm clumps or debris were avoided to decrease faulty analysis. Tracks recorded from captured sperm were visually observed and incomplete tracks, caused as a result of sperm tracks overlapping, were deleted. Accurate analysis of each track was verified by performing the replay function of SCA.

### 3.5.2 Flush Technique

The flush technique evaluated sperm motility at the interface of sperm exposed to sea water. Testicular-sperm from black mussel, white mussel, oyster and dry-spawned sperm from sea urchin was used for this technique. Contact between the concentrated sperm and sea water allowed the sperm to swim-out. A small volume (approximately <1 µl) of undiluted sperm was transferred to a 20-µm deep two chamber Leja slide (Leja Products B.V., Nieuw-Vennep, The Netherlands). Through capillary action a slight sperm dome was created at the mouth of the chamber. In addition, 5 µl of filtered sea water was pipetted or flushed, into the chamber producing columns of sperm inside the chamber. This technique demonstrated the difference in activation time of sperm between the invertebrate species as motile sperm swam-out of the sperm column and could possibly best mimic what happens during actual spawning (micro-clumps of sperm dispersing as motile sperm are activated in *C. meridionalis*, *C. gigas* and *D. serra*).

### 3.5.3 Swim-Up Technique

This technique was conducted on testicular-sperm from black mussel, white mussel, oyster and dry-spawned sperm from sea urchin. All experimental work was executed at 17-18˚C. Equipment, solutions and invertebrates were allowed to acclimatise for approximately 1 hr at this temperature. Fifty microlitre of filtered sea water was pipetted into a 5-ml Eppendorf tube, using a Gilson pipette, to which 10 µl of dry-sperm was added with a displacement pipette. Sperm was slowly released into the Eppendorf tube to create a sperm pellet at the bottom of the sea water column and to prevent disturbing the pellet. The 10-min activation time allowed a motile population of spermatozoa to swim-up into the water, creating a sperm cloud just above the pellet of sperm. A small volume of motile sperm was taken from above the sperm cloud to measure sperm motility and the kinematic parameters over time at 5, 15, 30 and 60 min. The swim-up technique was repeated approximately 24 hrs later, after concentrated testicular-sperm and dry-spawned sperm samples were stored at 4˚C. Another method of mixing the sperm with sea water to produce an even distribution of sperm was used to compare sperm motility reaction. A pellet of 10 µl sperm was pipetted into an Eppendorf tube, to which 50 µl of filtered sea water was sprayed on top of the pellet. A small volume of this mixed-sperm was pipetted for CASA analysis.



### 3.5.4 Spawned-Sperm

No dry-sperm was obtained from abalone as none of the broodstock males were sacrificed. Sperm was acquired through chemical-induced spawning. Abalone sperm motility was assessed from samples taken directly from the spawning tank and was termed spawned sperm for this study. Broodstocks underwent a two-step chemical

induction procedure adapted from Morse *et al*. (1977), whereby sodium hydroxide solution was initially added, followed by hydrogen peroxide (25 mg/kg) after 15 min (Visser-Roux, 2011).

The tanks were emptied an hour later, cleaned and refilled with aerated filtered sea water followed by males starting to spawn. This procedure generally occurred 4 hrs after induction and is the routine procedure employed in the abalone industry to induce spawning (Roux *et al*., 2014). Sperm was ejected when the adductor muscle contracted pressing against the gonadal tissue close to the foot. This resulted in gametes being released through the respiratory pores by means of the nephridiopore duct (Purchon, 1977; Hahn, 1989). At the start of spawning, the aeration and flow-through sea water system were subsequently switched off to prevent dilution of gametes. Sperm samples were collected, using a Pasteur pipette, from the middle of the holding tank, close to the spawning abalone, as sperm was released from the respiratory pores directly into sea water.



### 3.5.5 Variables Affecting Motility Patterns

In these investigations, *C. meridionalis* was easily accessible and served as a model for induced spawning through thermal regulation and partly aided as a model for *D. serra* and *C. gigas*. Spawning via thermal regulation was applied to simulate the natural environment and prevent any possible effect of chemical induction on sperm motility. A comparative study between spawning induced sperm and testicular sperm of *C. meridionalis* was considered to test the validity of using testicular sperm for the various CASA experiments. In contrast to the other species, the sea urchin model was readily acquired and gametes easily obtainable. *Parechinus angulosus* sperm was

therefore used to assess the sperm motility between the chamber depths of 20-µm and 100-µm slides; as they shared a similar helical swimming pattern to the other species investigated. Sea urchin eggs were used to determine the potential differences of various environments on sperm motility patterns.

### 3.5.5.1 Chamber Depth and Motility Characteristics

The sea urchin helical swimming patterns were assessed when the sample chamber depth was increased from 20-µm to 100-µm. Two chamber Leja slides (Leja Products B.V., Nieuw-Vennep, The Netherlands) with a chamber depth of either 20-µm or 100-µm depth were compared. Swim-up and flush techniques were used to compare and measure the diameters of the helical pattern, the curvilinear velocity (VCL) and sperm motility patterns. The swim-up technique as described in section 3.5.3 was used, with minor adjustments. A 10-µl sperm pellet was pipetted in an Eppendorf tube containing 100 µl of filtered sea water and analysis occurred after the 10 min activation period. A swim–up sperm sample of 5 µl was pipetted into the 20-µl deep chamber and 30 µl swim-up sample filled the 100-µl deep chamber.



### 3.5.5.2 Thermal-Regulated Spawning

Analyses were based upon commercially cultivated samples of *C. meridionalis* obtained from Saldanha Bay between May 2016 and May 2017. To stimulate spawning a temperature difference of approximately 16˚C was created which would provide a thermal shock to the bivalves (Honkoop *et al*., 1999; Helm *et al*., 2004). The success of thermal shock spawning required mature adult bivalves with viable gametes.

Large volumes of sea water were filtered through grade 1 medium flow filter paper (Whatman®, Merck, Germany), half of the filtered sea water was poured into 2-l glass beakers covered with sealing film (Parafilm® M, Sigma-Aldrich, Germany) and stored at 4˚C. The rest of the sea water was covered and left at laboratory temperature for experimental use.

Large holding tanks, consisting of clear plastic containers with 370 x 300 x 100 mm dimensions, were used to hold the cold or warm water into which smaller spawning chambers (140 x 140 x 60 mm) were submerged as shown in Figure 3.5. The holding tanks were placed on top of large black plastic sheeting; which provide a dark bottom for easier identification of gametes being liberated (Helm *et al*., 2004). The warm water holding tank was filled with distilled water and heated using an aquarium 50 W glass heater (Via Aqua, Commodity Axis Inc.®), to approximately 20˚C. The cold-water holding tank temperature, was maintained at 4–6˚C with crushed ice.



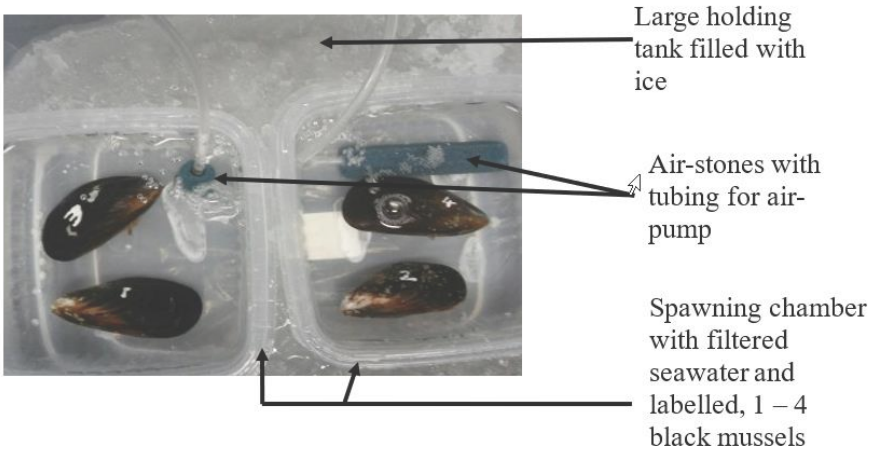



**Figure 3.5:** Larger ice-water holding tank, containing the cold spawning chamber with aerated filtered sea water and black mussels labelled 1-4, to assist with spawning identification.

The smaller spawning chambers, filled with approximately 500 ml of filtered sea water, were placed into individual temperature-controlled tanks until the set temperatures (either 20˚C or 4–6˚C) were reached. To complete the preparation, air-stones attached with tubing to a Tetra aquarium air-pump were placed into each of the spawning chambers. Using a digital pocket thermometer (Flinn Scientific, Canada) the temperature of individual spawning chambers was maintained over the entire experimental period.

The area within the spawning chamber was large enough to support 2 to 3 black mussels and still allowed adequate space to clearly observe the start of the mussels spawning. Prepared black mussels were cycled, initially between the cold chamber (4–6˚C) for 40 min and the 20˚C warm chamber for 40 min until spawning was observed. The amount of thermal cycling events depended on individual black mussel maturity. When an individual male started spawning, it was left in the chamber and non-spawning black mussels removed, along with the aerator. This was done to prevent the dilution of sperm. On the occasion when a female spawned first the non-spawning mussels and spawning sea water was discarded and the chamber washed 3X with distilled water. This was done to prevent contamination with the presence of eggs, which naturally stimulates group spawning in males (Helm *et al*., 2004).



Once gametes were ejected from the exhalent siphons located near the posterior dorsal region of the mussel, shown in Figure 3.6, 2 ml of sperm with filtered sea water was extracted using a Pasteur pipette. This extraction was placed in a plastic 10-ml blue-capped measuring cylinder for immediate analysis. Sperm with sea water was referred to as spawned-sperm for experimental purposes. Spawned-sperm samples

were collected every 10 min over a period of one and a half hours for analysis.


**Figure 3.6:** Spawning male black mussel, ejecting clouds of white sperm.

### 3.5.5.3 Whole Eggs and Egg Water

*Parechinus angulosus* were randomly selected, induced to spawn and the females were allowed to shed eggs into a beaker filled with sea water. Spawned-sperm stocks were prepared, as described in section 3.3.2. Eggs in the spawning beaker were gently agitated by aeration, using the Tetra aquarium pump to gently bubble air into the beaker, mixing the eggs and sea water. Egg-water was freshly acquired by allowing 5 ml of eggs, to settle to the bottom of a 100-ml sea water-filled beaker. Approximately 10 ml of sea water was pipetted from above the layer of eggs and placed in a 15-ml plastic blue-capped vial. The suspension was centrifuged at 500 rpm for 5 min to remove any other debris, and 500 µl of the top layer of the supernatant was used. The egg-water was used as the medium for the swim-up technique (see section 3.5.3). Five microlitre of sperm was added to the egg-water volume and motile sperm was sampled for analysis from the population within the cloud above the sperm

pellet.

After spawned eggs were washed with filtered sea water, 100 ml of fresh filtered sea water was added and eggs were allowed to settle in the beaker. A 1:10 dilution was made; 1 ml of the egg stock was added to 9 ml of filtered sea water and mixed to form the egg suspension. One millilitre of the egg suspension (± 200 eggs/ml) was added to a 5-ml filtered sea water vial, to which 5 µl of dry-spawned sperm was pipetted and gently swirled to mix gametes. Sperm motility and sperm morphology assessments were performed for both egg-water and whole egg solutions.

## 3.6 CASA Equipment, Acquisition Properties and Sperm Functional Parameters

All microscopic analysis measuring the sperm motility populations was performed with the Sperm Class Analyser® (SCA) version 5.4.0.0, CASA system (Microptic S.L. Barcelona, Spain), using the Motility module. The slides were viewed with a Basler A312fc digital camera (Microptic S.L. Barcelona, Spain), mounted (C-mount) on either a Nikon E50i microscope or an Olympus CH2 microscope (Wirsam, Cape Town, South Africa), both equipped with negative phase contrast objectives. To divide sperm into sub-populations based on swimming speed, default SCA® settings for Fish/Invertebrate and curvilinear velocity (VCL) were used (manufacturer settings). The SCA® acquisition parameters were set as follows: number of images captured = 50; images per sec = 50; optics = Ph-(negative phase contrast) and chamber = Leja 20; automatic analysis. The percent rapid spermatozoa were determined using the default settings for VCL cut off values of 38<50>120 µm/s to establish slow (non-progressive), medium and rapid swimming spermatozoa.

Therefore, sperm swimming less than 38 µm/s was considered to be immotile since such slow motion was ascribed to potential flow, Brownian movement and collisions of motile sperm with immotile sperm. The acquisition settings for SCA® motility analysis remained unchanged for all samples. Two to five fields were randomly captured to remove any bias towards high motility and to standardize measurements for all invertebrate specimens.

Sperm motility parameters were determined based on the automatic detection of the sperm heads by the SCA® system; by using negative phase contrast optics (Maree and van der Horst, 2012). The following kinematics parameters were assessed: VCL, straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), wobble (WOB), amplitude of lateral head displacement (ALH), beat cross frequency (BCF) and an additional derived motility parameter DANCE (DNC = VCL x ALH). Table 3.1 summarises the various sperm motility parameters and their derivatives (see Figure 2.1, a diagrammatic presentation of these parameters).

**Table 3.1:** Definitions of the standardized sperm kinematic parameters measured by the SCA® system

| Parameter | Description | Measurement |
|---|---|---|
| VCL (μm/s) | Curvilinear velocity | Time-average velocity of the sperm head along its actual trajectory |
| VSL (μm/s) | Straight-line velocity | Time-average velocity of the sperm head along a straight line from its first detected position to its last |
| VAP (μm/s) | Average path velocity | The time-average velocity of the sperm head along its average trajectory. |
| LIN (%) | Linearity | Linearity of the curvilinear trajectory (VSL/VCL) |
| STR (%) | Straightness | Linearity of the average path (VSL/VAP) |
| WOB (%) | Wobble | Measure of oscillation of the actual path about the average path (VAP/VCL) |
| ALH (μm) | Amplitude of lateral head displacement | Magnitude of lateral head displacement of a sperm head about its average path |
| BCF (Hz) | Beat-cross frequency | Average rate at which the curvilinear path crosses the average path |
| DNC (μm²/s) | DANCE | Two dimensional space occupied by a motile sperm during one second (VCLxALH) |

All the kinematic measurements were presented in a SCA® system's motility report and data of individual spermatozoa can be created, along with reports of all images captured. The diameters of individual sperm tracks were measured using the SCA® system version 6.2.16 (Microptic S.L. Barcelona, Spain) with the SCA® Motility module using the Toolbox function. Capturing of data involved the Basler A312fc digital camera (Microptic S.L. Barcelona, Spain), mounted (C-mount) on a Nikon E50i microscope (IMP, Cape Town, South Africa), equipped with phase contrast objectives. Sperm motility parameters are evaluated by capturing sperm tracks at 50 frames/second. Random fields were captured to eliminate any bias and captured helical tracks were visually verified to remove any over-lapping tracks. Assessments were made on the helical sperm tracks of 100 individual sperm of each animal including black mussel, white mussel, sea urchin and abalone. The measuring tool of SCA 6.3 was used to determine the diameter of each individual sperm captured and

used to calculate the perimeter including the area of and used to calculate the helical track occupied by each sperm. Reports of individual tracks provided the VCL and ALH of each sperm measure, to calculate the DANCE parameter. ALH max (2 x ALH) x VCL was used to calculate DANCE.

## 3.7 Statistical Analyses

The Medcalc® software version 17.2 (Mariakerke, Belgium) was used for standard statistical analyses. Descriptive statistics was used for calculation of averages and standard deviations. Normality of distribution tests were performed and most data sets expressed normal distribution. As a result, one-way analysis of variance (ANOVA) was used to compare more than two sets of data to determine statistical significance. The Kruskal-Wallis test was performed in data sets with non-parametric data distributions, with appropriate post hoc tests such as Student Newman-Keuls and Tukey. Data are represented as the mean ± standard deviation (SD) in the tables and $P<0.05$ was considered as significant. Spearman rank correlation coefficient and Pearson Product-Moment correlation coefficient and multivariate analyses (Andrews Plots, Star-sunray plots, Chernoff faces) were performed to identify differences and/or similarities when only very small differences were evident among parameters for different treatments or species differences using Statgraphics Centurion XVII, version 17.2.07 (64-bit), Statpoint Inc.).

# CHAPTER 4

# RESULTS

## 4.1 Sperm Structure: Light Microscopy

The sperm of *C. gigas*, *C. meridionalis*, *D. serra* and *P. angulosus* were viewed by phase contrast and NDIC microscopy. The sperm of these marine invertebrate species are of the ect-aquasperm type with a small head containing a nucleus with an acrosome, a simple midpiece and a tail. *C. gigas* testicular sperm have a typical short-spherical head when viewed with phase contrast and NDIC microscopy as shown in Figure 4.1 A and C. Anteriorly, the head is capped by a circular-shaped acrosome that forms a slight apical bump on the head as shown in Figure 4.1 A. Posterior to the sperm head is the narrow and small midpiece, to which a long and thin flagellum is centrally attached.



*Donax serra* spermatozoa of testicular origin displayed similarities to the *C. gigas* sperm. *Donax serra* sperm have a short-barrel shaped head that is apically capped with a bulging, circular-shaped acrosome as shown in Figure 4.1 B and D. Light microscopy revealed a short narrow midpiece and posteriorly it is attached to thin, long principal piece ending with a thin endpiece (Figure 4.1 B). *Parachinues angulosus* sperm have a conical-shaped head. In contrast to the other species, the acrosome of the sea urchin is not clearly distinguished under phase contrast and NDIC microscopy.

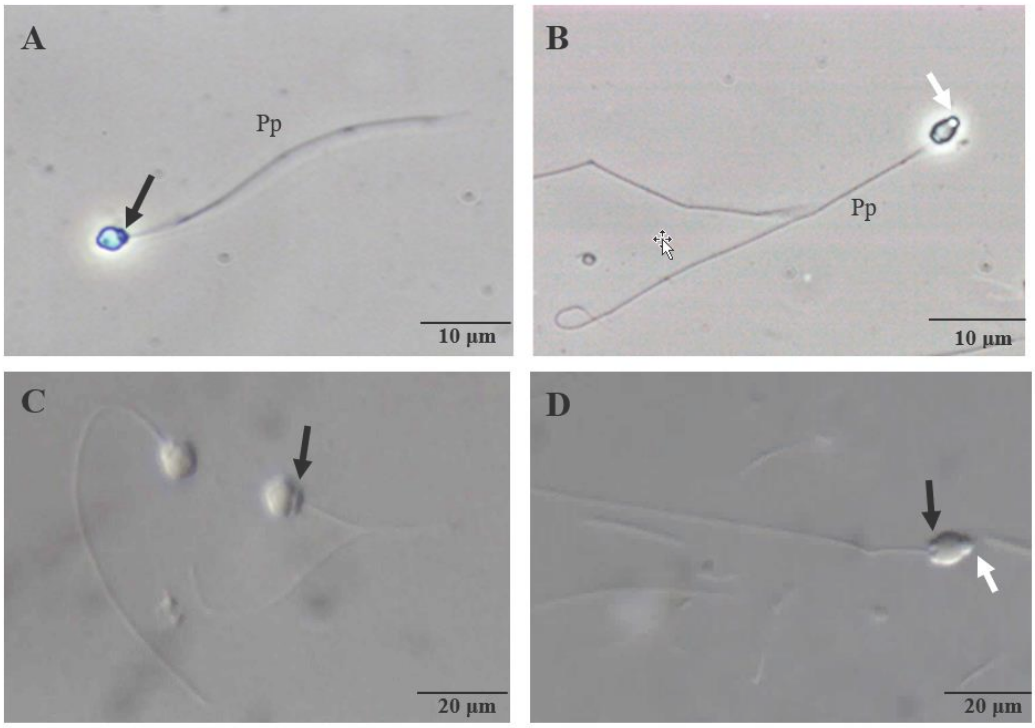



**Figure 4.1:** Phase contrast microscopy of testicular sperm of *C. gigas* (A) and *D. serra* (B) and NDIC microscopy of *C. gigas* (C) and *D. serra* (D), showing the overall sperm structure. These micrographs highlight the prominent acrosome (white arrow) and narrow bulging posterior midpiece (black arrows) with their thin long principal piece (Pp).



*P. angulosus* sperm, like *C. gigas* and *D. serra* sperm, has a clearly bulging and tube-shaped midpiece at the end of the conical head as shown in Figure 4.2 A and B. Typical of primitive sperm, it has a principal piece that emerges from the centre of the midpiece and ends with a thinning endpiece (Figure 4.2 A and B).

Black mussels, *C. meridionalis* and *M. galloprovincialis*, and *P. angulosus* sperm heads showed the most well-defined structural features of the four species (Figure 4.2 and 4.3). *C. meridionalis* sperm showed the most substantial morphological difference between swim-up and testicular sperm among the species. *Choromytilus meridionalis*

testicular sperm have a pear-shaped head with a relatively long anteriorly postioned conical-shaped acrosome attached to the apical surface of the head (Figure 4.3 A and C).

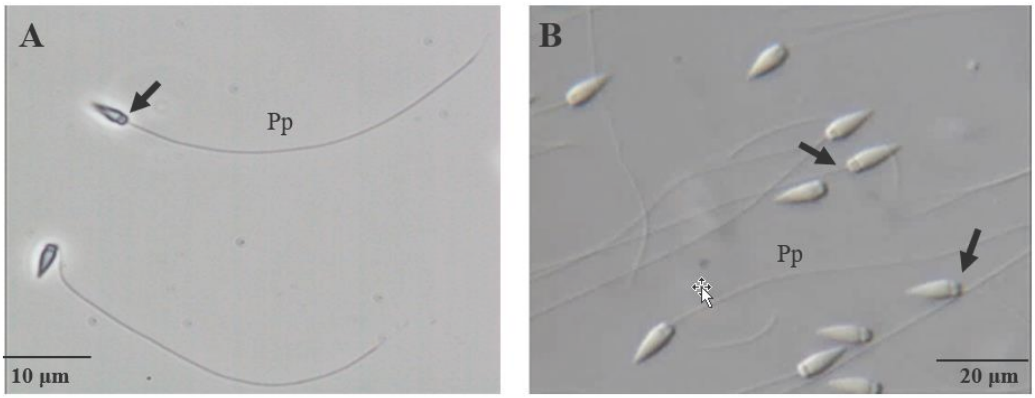



**Figure 4.2:** *P. angulosus* dry-spawn sperm viewed using phase contrast (A) and NDIC (B) microscopy showing the long principal piece (Pp) and prominent midpiece (black arrows).



In comparison, both phase contrast and NDIC microscopy of the swim-up *C. meridionalis* sperm appear to possibly have a marginally shorter conical shaped acrosome as presented in Figure 4.3 B and D. The midpiece of *C. meridionalis* is not discernible with light microscopy and the long, thin principal piece is located at the round, posterior region of the head (Figure 4.3 B).

In comparison, NDIC microscopy shows *M. galloprovincialis* testicular sperm appear to have a round head, the nucleus is round, attached apically with a distinctive narrow long, tubular-shaped acrosome as presented in Figure 4.3 E. The narrow bulging midpiece of *M. galloprovincialis* can be distinguished from the head and the long thin principal piece is centrally attached to the midpiece.

Sperm structure in sperm motility is evident at the light microscopy level, showing differences in size and shape of the head and acrosome. Whereas there was variation in sperm structure among species, there was minimal to no variation between testicular or dry-spawn sperm and swim-up sperm using light microscopy techniques for most of the study species (Table 4.1).

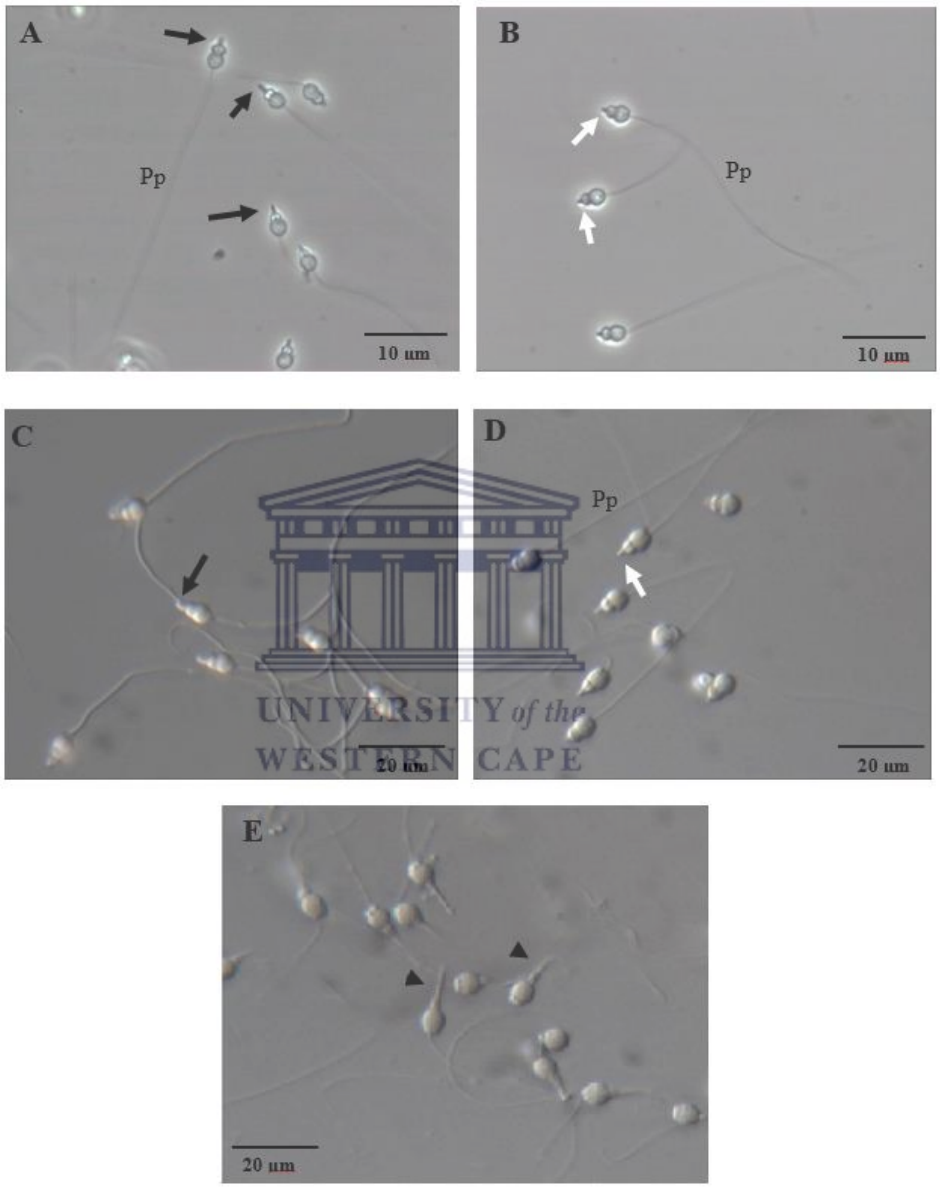



**Figure 4.3:** Phase contrast images of *C. meridionalis* testicular (A) and swim-up sperm (B) and NDIC microscopy of *C. meridionalis* testicular (C) and swim-up sperm (D), including *M. galloprovincialis* testicular sperm (E). *C. meridionalis* has a conical-shaped acrosome (black arrows) in the testicular sperm and a slightly shorter and thinner acrosome (white arrows) in the swim-

up samples and a long principal piece (Pp). *M. galloprovincialis* (E) has an exceptionally longer acrosome (black arrow-heads) in contrast to *C. meridionalis*.

**Table 4.1**: Light microscopic sperm morphometry features found between the testicular or dry-spawned sperm and swim-up sperm of four marine invertebrates

| | | Head (L) | Head (W) | Ellip | Elong | Acrm (L) | Acrm (W) | Mp (L) | Mp (W) | Tail | Total Length |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ***P. angulosus*-Dry spawned sperm** | **Mean** | 4.14 [a] | 1.28 | 3.25 [a] | 0.53 | - | - | 0.69 [a] | 1.00 | 42.66 | 47.50 |
| | **± SD** | 0.11 | 0.05 | 0.07 | 0.01 | - | - | 0.02 | 0.04 | 1.9 | 1.43 |
| ***P. angulosus*-Swim-up sperm** | **Mean** | 4.41 [b] | 1.39 | 3.41 | 0.53 | - | - | 0.74 [b] | 1.02 | 43.64 | 48.25 |
| | **± SD** | 0.10 | 0.17 | 0.05 | 0.04 | - | - | 0.04 | 0.05 | 1.05 | 0.99 |
| ***C. meridionalis*-Testicular sperm** | **Mean** | 4.39 [a] | 2.05 | 2.15 [b] | 0.36 | 2.49 [a] | 1.43 | - | - | 44.74 [a] | 49.39 |
| | **± SD** | 0.05 | 0.04 | 0.04 | 0.01 | 0.83 | 0.06 | - | - | 0.87 | 0.97 |
| ***C. meridionalis*-Swim-up sperm** | **Mean** | 3.98 [b] | 2.07 | 1.93 | 0.32 | 1.79 [b] | 1.47 | - | - | 45.81 [b] | 50.05 |
| | **± SD** | 0.10 | 0.04 | 0.02 | 0.00 | 0.07 | 0.03 | - | - | 0.69 | 0.65 |
| ***D. serra*-Testicular sperm** | **Mean** | 3.22 | 2.08 | 1.56 [c] | 0.21 | - | - | 0.60 | 1.34 [a] | 49.75 | 53.32 |
| | **± SD** | 0.18 | 0.10 | 0.08 | 0.03 | - | - | 0.03 | 0.13 | 1.04 | 1.11 |
| ***D. serra*-Swim-up sperm** | **Mean** | 3.27 | 1.98 | 1.65 | 0.24 | - | - | 0.58 | 1.26 [b] | 48.82 | 52.35 |
| | **± SD** | 0.04 | 0.11 | 0.08 | 0.02 | - | - | 0.04 | 0.09 | 1.02 | 1.06 |
| ***C. gigas*-Testicular sperm** | **Mean** | 2.18 | 1.88 | 1.16 [d] | 0.07 | - | - | - | - | 28.98 [a] | 31.47 [a] |
| | **± SD** | 0.05 | 0.04 | 0.03 | 0.01 | - | - | - | - | 1.48 | 1.46 |
| ***C. gigas*-Swim-up sperm** | **Mean** | 2.19 | 1.90 | 1.16 | 0.07 | - | - | - | - | 31.22 [b] | 33.70 [b] |
| | **± SD** | 0.04 | 0.06 | 0.05 | 0,02 | - | - | - | - | 0.94 | 0.98 |

Measurements in µm, n = 50 sperm per species, mean ± standard deviation (SD), Head (L) = total sperm head length, Head (W) = head width, Ellip = ellipticity, Elong = elongation, Acrm (L) = acrosome length, Acrm (W) = acrosome width, Mp (L) = midpiece length, Mp (W) = midpiece width, Total length = Total sperm length. a, b, c, d = values labelled with different superscript letters were significantly different ($P < 0.05$)

## 4.2 Sperm Morphometric Analysis

The morphometric parameters of testicular, dry-spawned and swim-up sperm components were measured using the SCA® morphology module. Morphometric features of four representative species, *P. angulosus*, *C. meridionalis*, *D. serra* and *C. gigas* are presented in Table 4.1.

For all species, the components measured included length and width (measured across the broadest part of the head) of the acrosome, head and midpiece, as well as ellipticity and elongation of the head. At a highest magnification (1000X), individual midpiece and acrosome were measured for selected species. Tail length and total sperm length was assessed for all species.

The morphometric assessments between individual species' testicular or dry-spawned and swim-up sperm showed significant changes in certain sperm features, such as the combined head length (head and midpiece), acrosome length, midpiece and tail. Ellipticity is an indication of whether the sperm head is thin or tapered; the higher the value obtained, the thinner the head. Significant differences were found between all four species as presented in Table 4.1. Ellipticity and elongation of the *C. meridionalis* swim–up sperm head decreased in comparison to the testicular sperm. *Crassostrea gigas* sperm showed no changes in ellipticity and elongation after becoming motile in comparison to the testicular sample. Head width showed no to minimal changes ($P > 0.05$) for all the species as well as total sperm length between the testicular or dry-spawned sperm of *P. angulosus*, *C. meridionalis* and *D. serra* when compared to the swim-up sperm samples. A significant increase ($P < 0.05$, $H = 4.81$) was found in the swim-up *C. gigas* total sperm length of 33.70 µm when

compared to the testicular total sperm length of 31.47 µm. The anatomical features that differed most among the species for testicular versus swim-up sperm were within the *C. meridionalis* species. *Chromytilus meridionalis* testicular sperm had a head length of 4.39 µm, with a prominent acrosome length of 2.49 µm and a tail measuring 44.74 µm. *Chromytilus meridionalis* swim-up sperm displayed a total head length of 3.98 µm and an acrosome length of 1.79 µm, while the tail increased to 45.81 µm.

*Parechinus angulosus* dry-spawned sperm head length was significantly shorter ($P < 0.05$, $H= 6.818$), 4.14 µm, in contrast to the 4.41 µm head length found after sperm were motile in the swim-up sample. The midpiece length of 0.69 µm of dry-spawned sperm displayed a significant decrease ($P < 0.05$, $F= 8.782$), compared to the 0.79 µm of *P. angulosus* swim-up sperm. *Donax serra* testicular and swim-up sperm showed minimal change; a significant decrease ($P < 0.05$) was found in the midpiece width of the swim-up samples.



Species-specific sperm morphometric assessments were evident as significant differences ($P < 0.05$) among species were established. Overall, the smallest mean testicular sperm head length analysed were those from the *C. gigas* and *D. serra* sperm, 2.18 µm and 3.22 µm respectively. *Crassostrea gigas* and *D. serra* had tails, 13 and 15 times longer than the head, respectively (Table 4.1). *Choromytilus meridionalis* and *P. angulosus* sperm had the largest total mean head length (4.39 µm and 4.14 µm, respectively) with a tail 10 times longer than the head length.

## 4.3 *Parechinus angulosus* Sperm Morphometric Analysis After Exposure to Various Media

Table 4.2 summarizes the sperm components measured for *P. angulosus* sperm

exposed to sea water (swim-up and mixed techniques), in egg-water and eggs with sperm.

**Table 4.2**: Sperm morphometric assessment of *P. angulosus* sperm components after exposure to sea water (dry-spawned, swim-up, mix), egg-water and eggs.

| *P. angulosus* | | Head (L) | Head (W) | Ellip | Elong | Mp (L) | Mp (W) | Tail | Total Length |
|---|---|---|---|---|---|---|---|---|---|
| **Dry spawned** | **Mean** | 4.14 | 1.28 [b] | 3.25 [b] | 0.53 | 0.69 [a] | 1.00 [b] | 42.66 | 47.50 |
| | **± SD** | 0.11 | 0.05 | 0.07 | 0.01 | 0.02 | 0.04 | 1.09 | 1.43 |
| **Swim-up** | **Mean** | 4.41 | 1.39 [b] | 3.41 [a] | 0.53 | 0.74 [b] | 1.02 [b] | 43.64 | 48.25 |
| | **± SD** | 0.10 | 0.17 | 0.05 | 0.04 | 0.04 | 0.05 | 1.05 | 0.99 |
| **Egg water** | **Mean** | 4.26 | 1.25 [a] | 3.41 [a] | 0.44 | 0.67 [a] | 0.91 [a] | 42.91 | 47.56 |
| | **± SD** | 0.13 | 0.02 | 0.14 | 0.23 | 0.01 | 0.05 | 0.52 | 0.50 |
| **Egg** | **Mean** | 4.25 | 1.33 [b] | 3.26 [b] | 0.53 | 0.74 [b] | 0.91 | 41.90 | 46.43 |
| | **± SD** | 0.11 | 0.06 | 0.11 | 0.01 | 0.03 | 0.08 | 0.63 | 0.77 |
| **Mix** | **Mean** | 4.30 | 1.37 [b] | 3.15 | 0.52 | 0.76 [b] | 1.00 [b] | 43.76 | 48.39 |
| | **± SD** | 0.19 | 0.03 | 0.17 | 0.02 | 0.03 | 0.05 | 2.45 | 2.42 |

Measurements in µm, n = 50 sperm per species, mean ± standard deviation (SD), Head (L) = total sperm head length, Head (W) = head width, Ellip = ellipticity, Elong = elongation, Mp (L) = midpiece length, Mp (W) = midpiece width, Total length = Total sperm length. Superscript letters indicate a significant difference ($P < 0.05$); a, b = values in columns labelled with different superscript letters were significantly different ($P < 0.05$)

Sperm exposed to egg-water showed the most significant changes within the sperm components measured. The mean ellipticity of the sperm head for the swim-up and egg-water sperm showed a similar increase, 3.41 µm, in contrast to the dry-spawned value of 3.25 µm, which indicates that the sperm head had gotten thinner after exposure to egg-water and after being motile in the swim-up samples. A significant decrease was found ($P < 0.05$, $H= 12.395$) in the mean sperm head width after egg-

water exposure, in comparison to the head width of swim-up, eggs only and mixed sperm samples. The exposure of sperm to sea water through swim-up, eggs only and mixed samples caused an increase ($P < 0.05$, $F= 8.782$) in the midpiece length to 0.74-0.76 µm. In contrast, egg-water sperm shared similar lower values (0.67 µm, $P > 0.05$) in the length of the midpiece to that of the typical dry-spawned sperm (0.69 µm). *Parechinus angulosus* sperm midpiece width was significantly lower ($P < 0.05$, $F= 4.303$) after egg-water and eggs only exposure, both having a value of 0.91 µm. The midpiece width of sea water exposed dry-spawned, swim-up and mixed sperm all showed a similar average measurement ($P > 0.05$) of approximately 1.00 µm. Sperm components with the least changes ($P > 0.05$) were total head length, tail and total sperm length for samples of all media.

## 4.4 Sperm Ultrastructure: Transmission Electron Microscopy

The various sperm components described under section 4.1 are described in more detail in this section dealing with the ultrastructure of the sperm of the species investigated. The typical features of ect-aquasperm are presented in Figure 4.4, Figure 4.5 and Figure 4.6 and include: the shape and electron-density of the nuclei; the shape and space occupied by the acrosome; the number and structure of mitochondria; and the arrangement of the inner mitochondrial membrane in the midpiece. Variations may have been found in the acrosome and head of the typical sperm of the five species and when sperm was exposed to other environments. Sperm tails and midpieces of all the investigated species share similar arrangement and will be described here.

Sperm tails of these external fertilizers (*C. meridionalis*, *D. serra*, *C. gigas* and *P.*

*angulosus*), consisted of the classical axonemal structural arrangement, composed of a ring of nine double microtubules around a central pair of single microtubules (9+2 pattern is shown in Figure 4.5 C). Similarly, the midpiece shared a simple structure, of a ring of mitochondria surrounding the proximal and distal centrioles near the posterior region of the nucleus. Transverse sections of *C. gigas*, *D. serra* and *C. meridionalis* sperm showed four to five spherical mitochondria displaying prominent tubular cristae (Figure 4.6 B, C), while six spherical mitochondria are found in *M. galloprovincialis* (Figure 4.6 F).

Figure 4.4 A and B shows the typical ultrastructure of the *D. serra* testicular sperm, with a barrel-shaped nucleus containing condensed electron-dense chromatin. The apical region of the nucleus was slightly curved, housing the acrosome. The acrosome had a slight bulge at its apical region and showed areas of different electron-density. The acrosome contained an outer dark region, with a posterior invagination forming a narrow tube that contained a lighter granular material in the sub-acrosomal space, as shown is Figure 4.4 A and B. In comparison, the swim-up *D. serra* sperm showed some variation in the acrosome.



The swim-up sperm acrosome had a prominent electron-dense outer ring and a posterior invagination that formed a narrow tube containing less-dense material internally. Protruding into the apical region was a wider curved less-dense acrosome bulge as shown in Figure 4.4 C. The nucleus of the *C. gigas* testicular sperm had typical features, including a short, spherical and electron-dense nucleus with a flat posterior region and an anterior region. The anterior region of the nucleus had a shallow U-shaped invagination that was capped with the acrosome (Figure 4.5 D and

E). The short, disc-shaped and electron-dense acrosome cap showed no differentiation. By contrast, in the nucleus of the swim-up sperm of the *C. gigas*, the anterior U-shaped invagination had a deep and wide sub-acrosomal space as shown in Figure 4.4 F. The apically capped acrosome was slightly longer and curved over the wide anterior invagination of the nucleus (Figure 4.4 F).

In Figure 4.5 A-C, the dry-spawned sperm of *P. angulosus* display features typically found in these species. The apical surface of the sperm head was capped with a small spherical acrosome that had a small posterior sub-acrosomal space created by the slight U-shaped anterior invagination of the nucleus. The conical-shaped nucleus contained condensed chromatin and the posterior region of the nucleus had a slight invagination. The posterior invagination of the nucleus housed the proximal centriole and formed the prominent flagellar canal as shown in Figure 4.5 A and D.



The midpiece of the typical dry-spawned *P. angulosus* sperm appeared to contain a single tube-shaped mitochondrial ring with tightly coiled tubular cristae (Figure 4.5 A and B). *Parechinus angulosus* sperm motile in egg-water appeared to exhibit the initial stages of the acrosome reaction but electron microscopy evidence was not sufficiently convincingas shown in Figure 4.5 E. The swim-up sperm and sperm placed in egg-water showed some variation in midpiece shape; these mitochondria appeared more elongated with slightly less-coiled tubular cristae as shown in Figure 4.5 D and E. The ultrastructural images of testicular *C. meridionalis* (Figure 4.6 A-C) and *M. galloprovincialis* (Figure 4.6 E-F) sperm displayed features found typically in these species. Typical for *C. meridionalis* was the electron-dense nucleus that was short and barrel-shaped. At the apical region of the nucleus, it was capped by a conical-

shaped acrosome and posteriorly the nucleus had a V-shaped invagination containing the proximal centriole as presented in Figure 4.6 A. *Mytilus galloprovincialis* (Figure 4.6 E-F), exceptionally long acrosome was a distinctive ultrastructure feature among the species of the *Mytilus*. The contents of the conical acrosome of the testicular *C. meridionalis* were electron-dense and had a slightly longer anterior extension. Posteriorly, the acrosome is invaginated and formed a narrow tube in the acrosome with less-dense material in the sub-acrosomal space. When comparing these sperm features to those of the swim-up *C. meridionalis* sperm, there appeared some variation in the acrosome. The swim-up sperm acrosome's anterior extension appeared shorter and the conical-shaped acrosome was less electron-dense and granular compared to testicular spermas shown in Figure 4.6 D.

Overall, particles of cell debris were observed in the swim-up sperm's ultrastructure images of oyster, black mussel and sea urchin, which was present after fixation and hampered the sectioning of the samples as shown in Figures 4.4 F, 4.5 D, and 4.6 D. In contrast, testicular or dry-spawned sperm, of the mentioned species had no obvious particles of cell debris present in the ultrastructure images (Figure 4.4 A, D; Figure 4.5 A; Figure 4.6 A). Micrographs of the sea-urchin sperm exposed to egg-water showed similar cell debris free images as those seen in testicular and dry-spawned samples, shown in Figure 4.5 E and F.

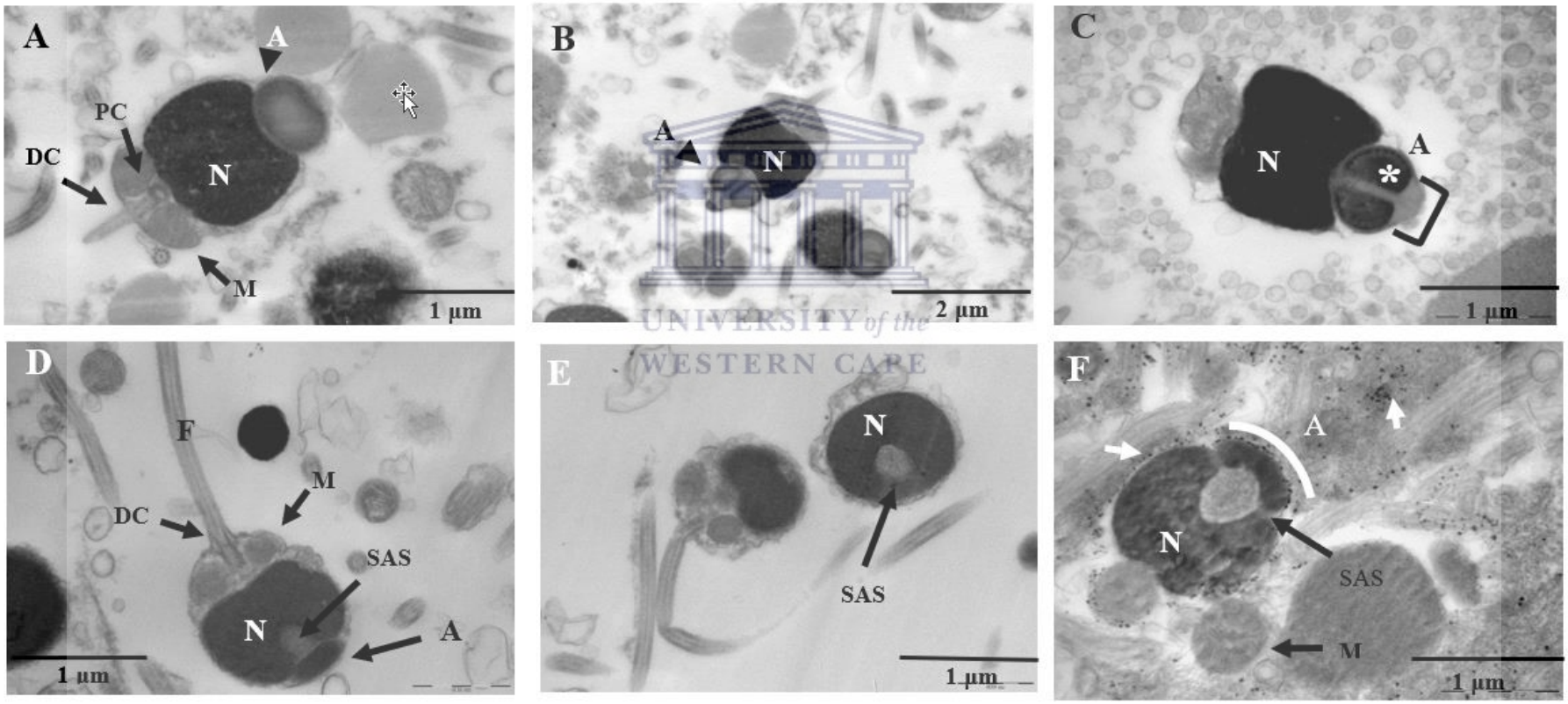





**Figure 4.4:** Longitudinal sections through the sperm components of *D. serra* (A–B = testicular sperm; C = swim-up sperm) and *C. gigas* (D-E = testicular sperm; F = swim-up sperm). (A) Developing sperm and (B) mature sperm found in the same animal showing a barrel-shaped and highly electron-dense nucleus (N) on which the acrosome (A) rest, containing electron-dense material on the borders (arrow head) and less dense contents internally. Swim-up sperm (C) showing acrosome with more electron-dense contents (white asterisk) and a wider curved apical bulge (black bracket). *C. gigas*, testicular sperm (D-E) and swim-up sperm (F) have the classical round electron-dense nucleus capped apically

with an electron dense acrosome. Swim-up sperm samples (F) contained cell debris after fixation, (white arrows). Acrosomes of testicular sperm (D-E) appear as a small spherical cap and swim-up sperm had a possible slightly curved flattened apical acrosome (white bracket). Longitudinal sections through acrosome (A), distal centriole (DC), mitochondria (M), nucleus (N), proximal centriole (PC), sub-acrosomal space (SAS).

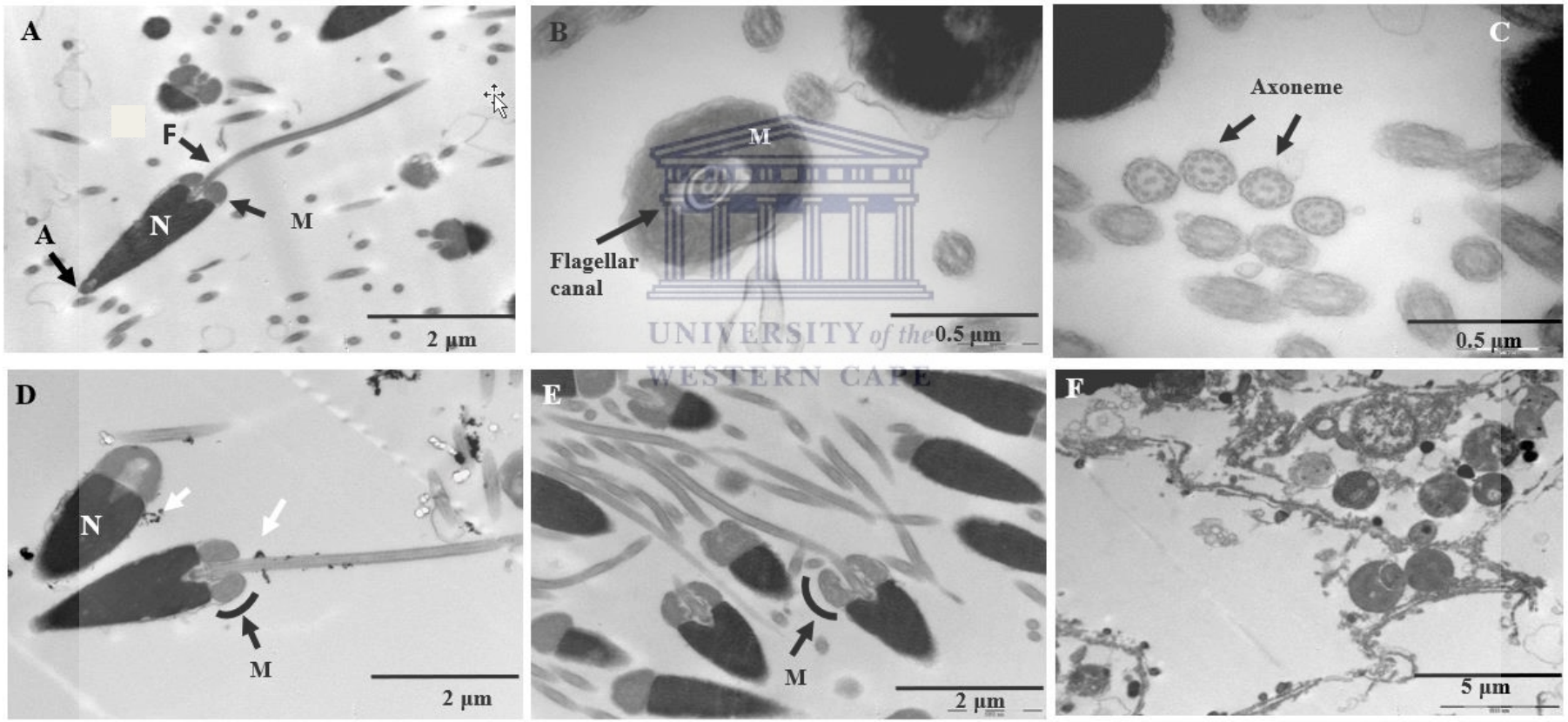





**Figure 4.5:** Longitudinal (A, D, E, F) and transverse (B-C) sections through *P. angulosus* dry-spawned (A-C), swim-up (D), egg-water (E) and eggs only (F) sperm. Typical sperm features of sea-urchin dry-spawned sperm (A): capped apically with a small acrosome on the conical-shaped nucleus; with spherical-shaped mitochondria. Cross section (B) shows the midpiece containing a single mitochondrion (M), a well-defined flagellar canal and tails with the basic 9+2 arrangement of microtubules (B-C). In contrast the swim-up (D) and egg-water sperm (E)

showed a midpiece with elongated mitochondria (black bracket) and contained particles of cell debris after fixation (white-arrows). Longitudinal/cross sections through acrosome (A), flagellum (F), mitochondria (M), nucleus (N).

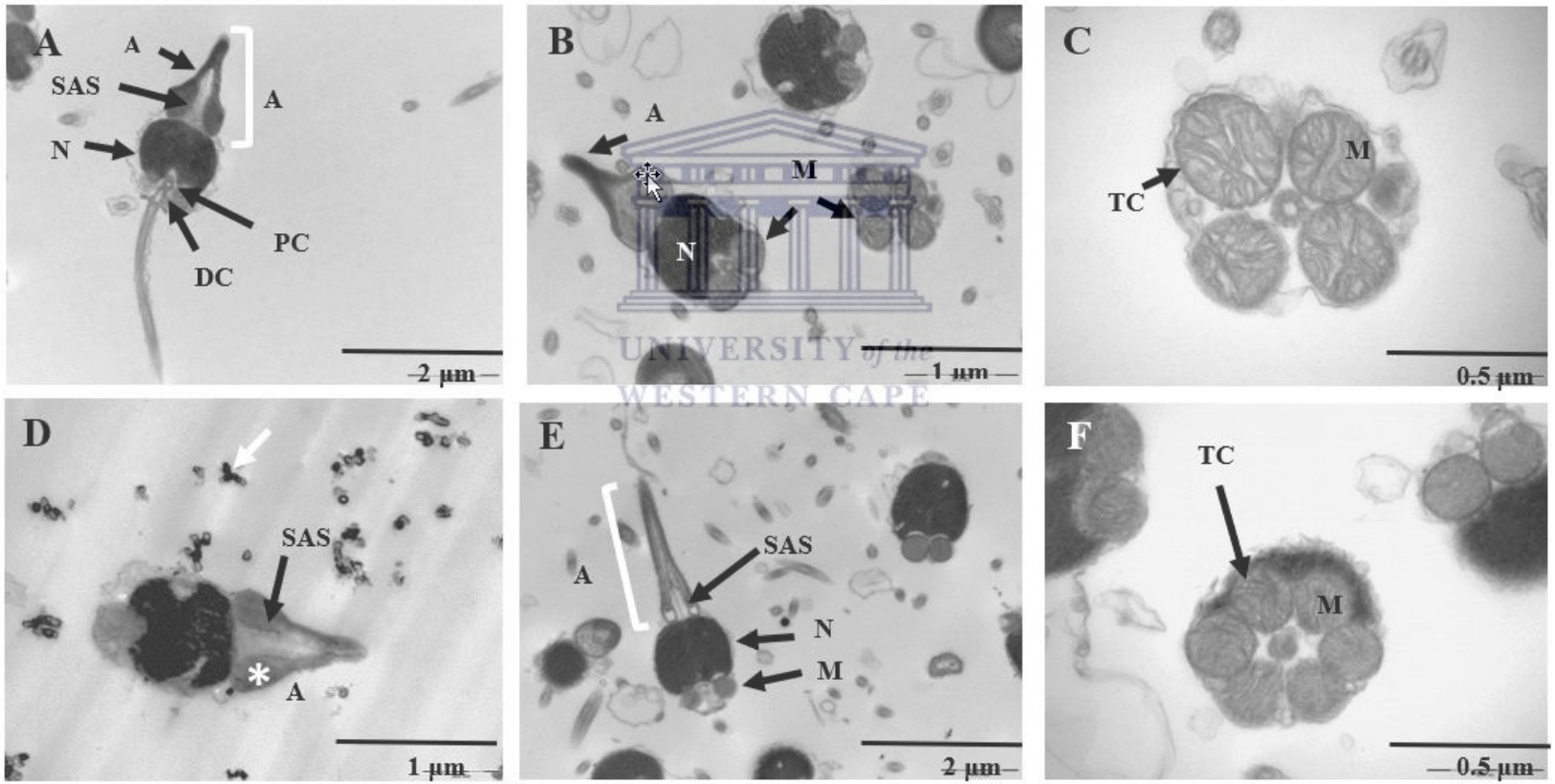





**Figure 4.6:** Longitudinal (A, B, D, E) and cross section (C, F) of the ultrastructure of *C. meridionalis* testicular (A-C) and swim-up (D) sperm and *M. galloprovincialis* testicular sperm (E-F). *C. meridionalis* testicular (A) sperm showing a round electron-dense nucleus, a prominent electron-dense conical acrosome (A), with an acrosome vesicle leading to the sub-acrosomal space, with a midpiece surrounding the proximal (PC) and distal centrioles (DC). Midpiece containing four spherical mitochondria (C). Swim-up micrograph (D) shows a less-dense conical

acrosome (white asterisk) and containing cell debris among the sperm (white arrows). Longitudinal sections through acrosome (A), acrosome vacuole (AV), distal centriole (DC), mitochondria (M), nucleus (N), proximal centriole (PC), sub-acrosomal space (SAS), tubular cristae (TC).

## 4.4 Morphology Summary

Figure 4.7 is a schematic diagram summarising the comparative aspects and the sperm similarities in terms of structure of the species studies. The diagram presents the representative species, namely *C. gigas*, *D. serra*, *C. meridionalis* and *P. angulosus*. Incorporated are *H. midae* and *M. galloprovincialis* to show some comparison. Unfortunately, not enough basic morphological information was acquired to compile an acceptable sampling number, but the diagram was drawn to scale. Quantitative sperm measurements were not established for the latter two species; however, we included some comparative information gathered from the literature (Hodgson and Bernard, 1986; Hodgson and Foster, 1992).

The diagram explores the range of variability found in these primitive broadcast spawners. The diversity is displayed in the size and shape of the acrosome and nucleus. The midpiece shows similarities containing between 4-6 spherical mitochondria for all species except the sea urchin. The *P. angulosus* has one tubular-shaped mitochondrion in the midpiece.



## 4.5 Comparison of CASA Parameters Between Testicular and Spawned Sperm.

The comparison of sperm from spawning *C. meridionalis* with testicular sperm (dry-sperm) were used as a guide to determine the validity of using testicular sperm for the different CASA assessments. As a baseline study all sperm traits were evaluated. Although, kinematic cut-off points of sperm velocities and the classification of rapid, medium and slow swimming subpopulations were used to further identify possible changes in sperm functioning between spawning and testicular sperm. It has been

demonstrated that grouping sperm in subpopulations on the basis of their velocity made it possible to identify distinctive motility changes occurring within the same semen sample (Gallego *et al*., 2014).

Table 4.3 indicates the percentage total motile sperm and the sperm kinematic parameters of *C. meridionalis* testicular sperm and thermally induced spawned sperm taken within the first 2-7 min of sea water activation. Testicular sperm from *C. meridionalis* sperm were assessed using the flush technique.

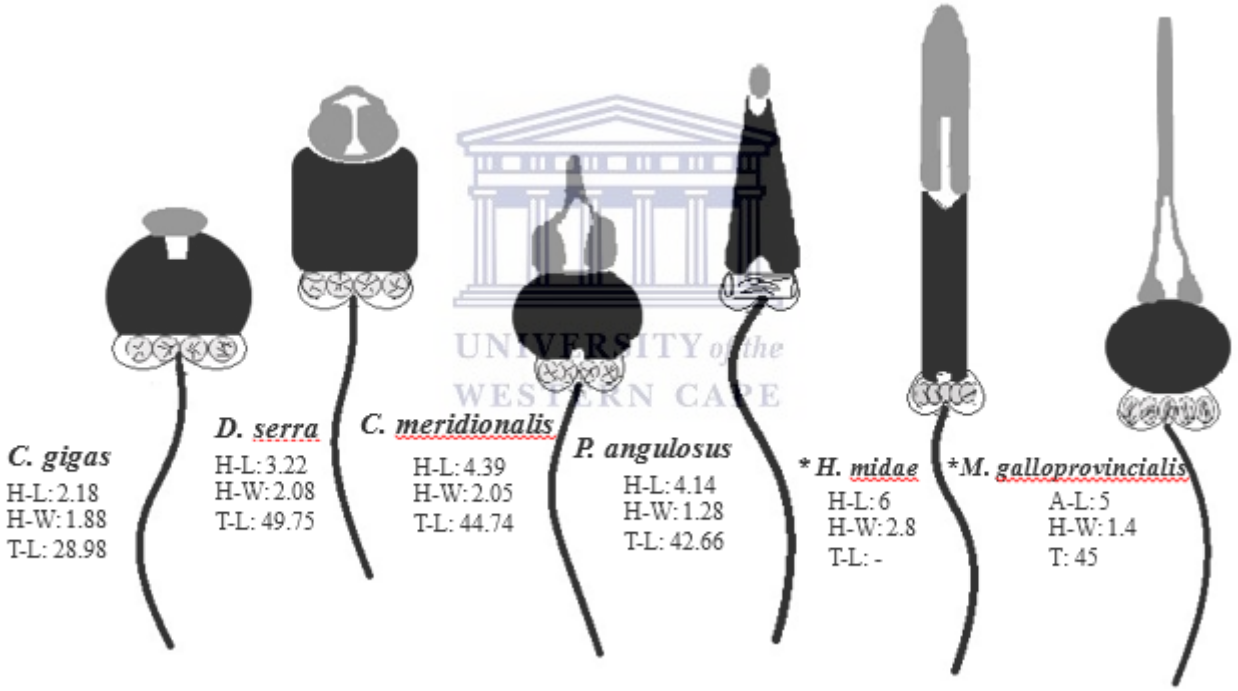



**Figure 4.7:** Diagrammatic representation of the various sperm components of the acrosome and head shape of the six broadcast spawners. *C. gigas*, *D. serra*, *C. meridionalis* and *P. angulosus* measurements obtained from this investigation. *Measurements for morphometry for *H. midae* = Hodgson and Foster, 1992 and *M. galloprovincialis* = Hodgson and Bernard, 1986. A-L= Acrosome-Length, H-L = Head-Length, H-W= Head-Width, T=tail, all measurements in µm.

**Table 4.3:** Sperm motility and kinematic parameter measurements comparing the activity of testicular sperm and spawned sperm from *C. meridionalis*

| Sperm Motility and Kinematics | | Testis (n=13) | Spawn A (n=10) |
|---|---|---|---|
| **Total motility (%)** | **Mean** | 53.2 | 57.2 |
| | **± SD** | 20.3 | 28.6 |
| **Rapid progressive motility (%)** | **Mean** | 26.4 | 27.3 |
| | **± SD** | 18.8 | 23.4 |
| **Medium progressive motility (%)** | **Mean** | 18.7 | 28.1 |
| | **± SD** | 13 | 17.4 |
| **Non-progressive motility (%)** | **Mean** | 8.2 [a] | 1.7 [b] |
| | **± SD** | 5.1 | 1.7 |
| **VCL (µm/s)** | **Mean** | 116.4 | 112.9 |
| | **± SD** | 41.3 | 14.1 |
| **VSL (µm/s)** | **Mean** | 27.4 | 27.1 |
| | **± SD** | 9.7 | 6.6 |
| **VAP (µm/s)** | **Mean** | 76.6 | 97 |
| | **± SD** | 28.2 | 18.1 |
| **LIN (%)** | **Mean** | 23.7 | 23.9 |
| | **± SD** | 3.3 | 5 |
| **STR (%)** | **Mean** | 36.8 [a] | 28.0 [b] |
| | **± SD** | 6.8 | 5.5 |
| **WOB (%)** | **Mean** | 65.3 [a] | 85.4 [b] |
| | **± SD** | 8.7 | 6.3 |
| **ALH (µm)** | **Mean** | 2.6 [a] | 1.5 [b] |
| | **± SD** | 1 | 0.8 |
| **BCF (Hz)** | **Mean** | 9.6 | 8.2 |
| | **± SD** | 4 | 4.1 |

VCL = curviliear 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. a,b = values labelled with superscript letters in the same row are significantly different ($P < 0.05$).

As laboratory spawning induction was not easy to achieve for all the study species, only *P. angulosus* and *C. meridionalis* spawning was induced. *Choromytilus meridionalis* thermal cycling resulted in approximately 80% of the *C. meridionalis* spawning in the warm bath within one hour of induction, after the initial cold bath incubation of 40 min. The males continued spawning intermittently for roughly 90min, squirting out streams of milky sperm.

No significant differences ($P > 0.05$) were found within the percentage total sperm motility, percentage rapid and medium progressive populations of testicular compared to spawned sperm of *C. meridionalis*. However, the percentage non-progressive population (slow) show a significant increase ($P < 0.05$) in testicular sperm compared to spawned *C. meridionalis* sperm that had a lower percentage (Table 4.3).

The sperm kinematic parameters indicating speed of sperm movement, namely; VCL, VSL, VAP and LIN, showed no significant differences ($P > 0.05$) when comparing the testicular and spawned sperm. In the sample of testicular sperm, a significant increase ($P < 0.05$) in the STR value was found when compared to the lower STR value of spawned sperm (Table 4.3). While spawned sperm showed an increase ($P < 0.05$) in WOB a significant decrease was found in WOB of testicular samples. The ALH increased ($P < 0.05$) for testicular sperm sample in comparison to the lower value found in spawned black mussel sperm.

Assessment of the motile pattern of the sperm collected from spawning *C. meridionalis* showed similar helical swimming patterns to those of testicular origin as well as to sperm from other species of mollusc and examples are presented in Figure

4.8. Spawned spermatozoa showed immediate motility upon sea water introduction, while testicular sperm of *C. meridionalis* showed a slower activation time when exposed to sea water and, in this case, sperm needed to be released from very concentrated clumps of testicular sperm as shown in Figure 4.8 (A). However, the testicular sperm maintained the helical swimming pattern as observed in sperm analysed from spawning *C. meridionalis* (Figure 4.8). After 60 min, the progressive helical swimming patterns of induced spawning *C. meridionalis* sperm remained the same.

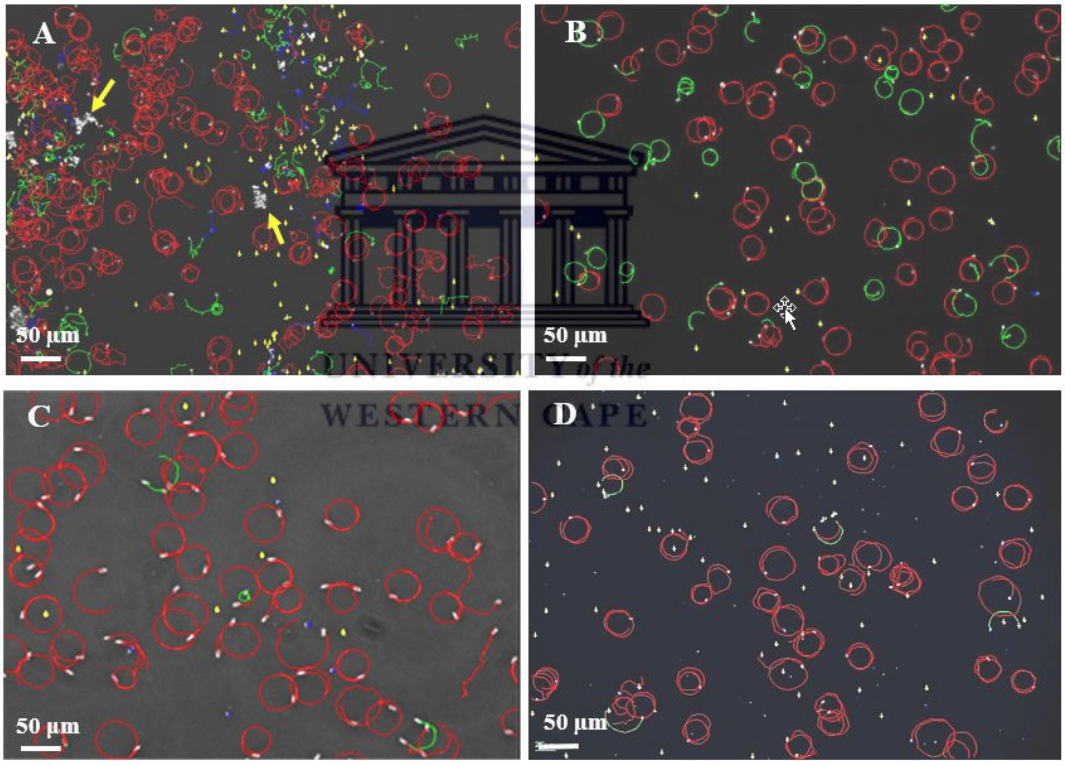



**Figure 4.8:** Typical sperm motility patterns from testicular origin (A) and induced spawning *C. meridionalis* (B) sperm, *H. midae* (C) and *D. serra* (D). Testicular and spawned black mussel sperm display a similar characteristic of helical swimming patterns found upon introduction to sea water as for most marine broadcast spawners (C and D). Yellow arrows indicate the clumped sperm from testicular origin. Scale bars apply to all figures. Red tracks = rapid

swimming sperm; green tracks = medium swimming sperm; blue tracks = non-progressive (slow) sperm; and yellow crosses = immotile sperm.

In contrast, sperm collected directly from the testis of *C. meridionalis* primarily showed forward progressive tracks after 60 min as stated in our paper (van der Horst *et al*., 2018) and are presented in Figure 4.14 (D).

## 4.6 Chamber Depth Effect on Sperm Motility and Kinematics

*Parechinus angulosus* was chosen as the model species from the five animals studied to assess motility charactertistics at different chamber depths, as it has the largest helical diameter (Figure 4.15). Furthermore, the swimming pattern of *P. angulosus* sperm has similarities to that of the other four species used in this investigation, showing sperm to swim in a compressed helix.



The sperm swimming patterns of four animals were visually assessed, to confirm the chamber depth slide recommended for SCA® and is presented in Figure 4.9. In both chamber depths the helical swimming pattern was maintained. In contrast, within the deeper chamber (Figure 4.9 B) a mixture of large or incomplete helical tracks and some ‘star-shaped’ sperm swimming patterns were found, when compared to the characteristic repetitive and completely helical tracks produced in the shallower chamber (Figure 4.9 A). However, it should be noted that the incomplete helical tracks relate to slower swimming speeds in the deeper chamber and not to real differences in the swimming patterns between the two chamber depths. The background of the 100-µm deep slide appeared ‘cloudy’ as shown in Figure 4.9 (B). Sperm were accordingly swimming in different focal planes in the deeper slide and for a particular

CASA capture and analysis, only sperm in a specific focal plane would be analysed, as sperm out of focus were ignored.

The 20-µm chamber allowed sperm to form an in-focus layer, providing a contrasting background for optimal capturing. The 20-µm Leja slide was subsequently used in all CASA analyses performed in this study. The 20-µm slide is also the recommended chamber depth for animal sperm studies using the SCA® systems.

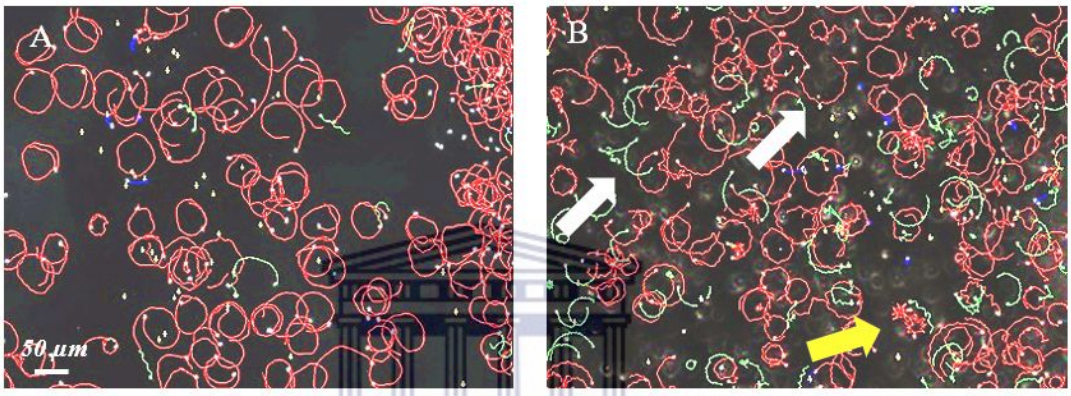



**Figure 4.9:** Sperm motility tracks of *P. angulosus* sperm in A) 20-µm Leja and B) 100-µm GoldCyto slides. Both slides, A and B, show the typical helical pattern, except slide B has some incomplete helical tracks (white arrows) and yellow arrow shows star-shaped pattern, similar to hyperactivation. In B, the background appears 'cloudy' as all spermatozoa appear not to be focused in the focal plane. Red tracks, rapid swimming sperm, green tracks, medium swimming sperm, blue tracks, slow swimming sperm and yellow cross, immotile sperm. Scale bars of 50µm apply to both figures.



## 4.7 Sperm Concentration and Total Motility of Sperm

Table 4.4 shows the testicular and dry-spawned sperm concentrations and the percentages of motility, progressive motility, and non-progressive motility captured at the initial introduction to sea water for the five marine invertebrate species.

*Choromytilus meridionalis*, *C. gigas* and *D. serra* sperm samples were taken directly from the testis and had an activation time that varied from 2-7 min. Accordingly, sperm were only captured after 7 min of dilution with sea water.

Dry-spawned *P. angulosus* sperm were collected from the external gonopore after induction to spawn, while *H. midae* samples were captured in sea water filled spawning tanks near the nephridiopore of the spawning animal. Both species sperm samples showed immediate motility in the presence of sea water.

The following results have been published and accordingly the data relates to the paper indicated in Appendix II. Sperm concentrations of testicular origin were extremely high ranging from >1554.3 x $10^6$/ml in *C. gigas* to ± 4642 x $10^6$/ml in *D. serra*, including dry-spawning 5057.2 x $10^6$/ml in *P. angulosus* (gonopore collection), compared to sperm spawned in sea water. *Haliotis midae* had the lowest sperm concentration but it represents sperm spawned in a large containment of sea water within a 40-l spawning tank.



However, *H. midae* sperm were collected very close to release at the nephridiopore. After flush and swim-up techniques, the sperm concentration in the other four species (excluding *H. midae*) varied from 15 to 30 million/ml. Accordingly, sperm motility was analyzed at the same range of sperm concentration for all species.

**Table 4.4:** Average sperm concentration and percentages total motility, progressive motility and non-progressive motility (mean ± SD). In all species, sperm concentration represents testicular samples except in *P. angulosus* (collected at external gonopores) and *H. midae* where sperm collection occurred (near nephridiopore) during induced spawning.

| **Species (n)** | **Sperm concentration ($x10^6$/ml)** | **Total motility (%)** | **Rapid progressive motility (%)** | **Medium progressive motility (%)** | **Non-progressive motility (%)** |
|---|---|---|---|---|---|
| ***P. angulosus* (14)** | 5057.2 ± 1353.3 | 66.4 ± 21.5 | 46.7 ± 23.4 [a] | 12.4 ± 18.3 | 7.3 ± 8.4 |
| ***C. gigas* (12)** | 1544.3 ± 1201.6 | 49.6 ± 19.6 | 23.6 ± 15.7 | 22.4 ± 8.7 | 3.5 ± 1.4 |
| ***C. meridionalis* (13)** | 3247.0 ± 2027.4 | 54.7 ± 23.3 | 26.8 ± 18.8 | 18.6 ± 12.9 | 8.2 ± 9.6 |
| ***D. serra* (13)** | 4642.0 ± 3702.1 | 50.3 ± 30.7 | 43.4 ± 31.1 | 3.03 ± 2.9 | 4.0 ± 4.1 |
| ***H. midae* (16)** | 22.1 ± 15.3 | 91.3 ± 7.9 | 50.6 ± 26 [a] | 29.3 ± 23.2 | 11.2 ± 8.1 |

a = values labelled with superscript letter show significantly different ($P < 0.05$)

Table 4.4 shows a large variation in the average percentage total sperm motility from 49.6 to 91% among the broadcast spawning species, while *H. midae* displayed a significantly higher percentage of total motile sperm ($P < 0.05$) than the other four species. *Parechinus angulosus* and *H. midae* sperm showed a significantly higher percentage of progressively moving sperm than the remaining three species ($P < 0.05$). *Donax serra* testicular sperm had ± 50% total motility of which 43.4% were rapidly progressive while having a medium to slow sperm motility subpopulation ranging from 3–4%, respectively, as presented in Table 4.4.

## 4.8 Sperm Motility and Sperm Kinematic Measurements of Testicular and Dry-Spawned Sperm Using the Flush Technique

Table 4.5 shows the percentage total motility, percentage motility subpopulations and kinematic parameter measurements of the testicular sperm of *C. meridionalis*, *D. serra* and *C. gigas*, as well as dry-spawned sperm of the *P. angulosus* using the flush technique. The results compare CASA measurements captured at the initial introduction of the sperm samples to sea water and after the concentrated sperm of all species were stored at 4°C for approximately 24 hrs. The test for longevity can be used for aquaculture insemination and when samples are couriered to a distant farm.



*D. serra* sperm were the only samples that showed no to very low motility after being stored at 4°C for ± 24 hrs and no CASA data could be captured. Overall, there was a decrease ($P > 0.05$) in percentage total motility of sperm after ± 24 hrs for *C. gigas* ($P > 0.05$, $F= 0.009$), *C. meridionalis* ($P > 0.05$, $F= 0.322$), *P. angulosus* ($P > 0.05$, $F= 1.335$) when compared to each species' initial total motility percentage (2 min), as shown in Table 4.5.

**Table 4.5:** Sperm kinematic parameters assessments (mean ± SD) of testicular and dry-spawned sperm samples of four marine invertebrates (n = 10 animals per species) to indicate the initial activation of sperm activity and then after being stored at 4°C for approximately 24 hrs.

| Parameter | *P. angulosus* | | | *C. meridionalis* | | | *C. gigas* | | *D. serra* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Time | Mean | ±SD | Time | Mean | ±SD | Mean | ±SD | Mean | ±SD |
| MOT (%) | 2 min | 69.9 | 12.1 | 2-7 min | 53.2 | 20.3 | 49.5 | 22.0 | 32.8 | 27.0 |
| | 24 hrs | 59.6 | 17.3 | 24 hrs | 51.3 | 24.0 | 36.5 | 23.3 | - | - |
| Prg Mot (%) | 2 min | 9.5 | 4.2 | 2-7 min | 6.5 | 5.6 | 7.8 | 4.7 | 4.7 | 4.9 |
| | 24 hrs | 7.2 | 4.1 | 24 hrs | 7.4 | 5.6 | 6.6 | 4.2 | - | - |
| Rpd Mot (%) | 2 min | 47.8 | 24.9 | 2-7 min | 26.4 | 18.8 | 23.5 | 15.8 | 23.7 | 21.8 |
| | 24 hrs | 40.0 | 26.7 | 24 hrs | 22.1 | 21.2 | 17.8 | 13.4 | - | - |
| Med Mot (%) | 2 min | 10.4 | 7.6 | 2-7 min | 18.6 | 13.0 | 22.4 | 8.7 | 4.0 | 2.9 |
| | 24 hrs | 7.3 | 5.2 | 24 hrs | 21.1 | 18.4 | 15.9 | 9.8 | - | - |
| Non-Prog (%) | 2 min | 11.8 | 11.5 | 2-7 min | 8.1 | 5.1 | 3.5 | 1.5 | 5.3 | 3.7 |
| | 24 hrs | 12.2 | 13.2 | 24 hrs | 8.0 | 3.9 | 2.7 | 1.2 | - | - |
| VCL (µm/s) | 2 min | 192.6 | 78.9 | 2-7 min | 116.4 | 41.3 | 110.4 | 15.7 | 191.5 | 49.8 |
| | 24 hrs | 177.7 | 74.4 | 24 hrs | 121.1 | 51.5 | 111.9 | 13.7 | - | - |
| VSL (µm/s) | 2 min | 66.5 | 26.9 | 2-7 min | 27.3 | 9.7 | 20.0 | 4.0 | 55.0 | 20.8 |
| | 24 hrs | 62.9 | 29.6 | 24 hrs | 32.6 | 12.9 | 22.0 | 4.0 | - | - |
| VAP (µm/s) | 2 min | 164.3 | 74.1 | 2-7 min | 76.6 | 28.2 | 48.6 | 4.2 | 133.3 | 35.8 |
| | 24 hrs | 149.9 | 68.6 | 24 hrs | 85.9 | 39.5 | 47.0 | 5.6 | - | - |
| LIN (%) | 2 min | 35.2 | 4.8 | 2-7 min | 23.7 | 3.3 | 18.2 | 3.7 | 28.3 | 6.5 |
| | 24 hrs | 34.3 | 5.7 | 24 hrs | 27.5 [a] | 4.7 | 20.0 | 4.6 | - | - |
| STR (%) | 2 min | 43.0 | 8.9 | 2-7 min | 36.8 | 6.8 | 40.9 | 6.4 | 38.5 | 6.9 |
| | 24 hrs | 43.1 | 6.7 | 24 hrs | 40.5 | 9.1 | 47.3 [a] | 9.3 | - | - |
| WOB (%) | 2 min | 74.7 | 24.4 | 2-7 min | 65.2 | 8.7 | 44.4 | 4.3 | 73.3 | 7.8 |
| | 24 hrs | 80.4 | 10.9 | 24 hrs | 70.4 | 10.3 | 42.1 | 2.3 | - | - |
| ALH (µm) | 2 min | 2.1 | 0.5 | 2-7 min | 2.5 | 1.0 | 3.5 | 0.6 | 2.2 | 0.8 |
| | 24 hrs | 2.3 | 0.7 | 24 hrs | 2.1 | 0.8 | 3.7 | 0.7 | - | - |
| BCF (Hz) | 2 min | 12.2 | 5.2 | 2-7 min | 9.5 | 4.0 | 9.5 | 1.9 | 18.5 | 5.9 |
| | 24 hrs | 12.2 | 7.3 | 24 hrs | 9.9 | 5.7 | 11.6 [a] | 1.4 | - | - |

MOT = total percentage motility, Prg Mot = progressive motility, Rpd Mot = rapid progressive motility, Med Mot = medium progressive motility, Non-Prog = non-progressive motility, VCL = curviliear 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. a = values labelled with superscript letter show significantly different ($P < 0.05$) for a species between time points.

This decrease in total motility after 24hrs coincided with the marginally lower values shown in the rapid, medium and slow sperm subpopulations of *P. angulosus*, *C. meridionalis* and *C. gigas*. Only the *P. angulosus* and *C. meridionalis* showed ± 50% active sperm samples after being stored at 4°C for approximately 24 hrs.

No significant changes (P > 0.05) were found in the speed of motility parameters (VCL, VSL, VAP) between the two time points of the individual species. However, some of the individual species showed an increase in all sperm kinematic parameters after being stored at 4°C for approximately 24 hrs. *Parechinus angulosus* sperm velocities, after being stored at 4°C for approximately 24 hrs, showed a decrease. Although, the speed of the motility parameters of *P. angulosus* remained higher than the other species at both time points.

A significant increase (P < 0.05) was found in the average LIN (P < 0.05, F= 1.693), slow (P < 0.05, F= 3.982) and rapid LIN (P < 0.05, F=0.175) sperm subpopulations, and the slow subpopulations of STR (P < 0.05, F= 3.182) and WOB (P < 0.05, F= 1.907) of testicular dry sperm of *C. meridionalis* sperm after 24 hrs. Testicular *C. gigas* sperm samples had a significant difference (P < 0.05) in the average STR (P < 0.05, F= 0.409) and BCF (P < 0.05, F= 0.983) values after 24 hrs.

## 4.9 Effect of Time on Swim-Up Sperm Motility and Sperm Kinematic Measurements

As stated in our article (Appendix II), significant differences among subpopulations of rapid, medium and slow sperm emphasise the importance of the subpopulation approach when assessing sperm kinematics. Figure 4.10 presents our published box and whisker plots of the sperm kinematic data of the swim-up sperm subpopulations

of three representative species, namely; *P. angulosus*, *C. gigas* and *D. serra*. There were significant differences (P < 0.05, F= 8.5) between rapid and medium swim-up sperm subpopulations in VCL and VAP for all representative species presented in Figure 4.10. Rapid swim-up sperm subpopulations for the *P. angulosus* had a mean VCL of 240 ± 51.9 µm/s and 207 ± 52.1 µm/s VAP. A similar trend was found in the *C. gigas* and *D. serra* swim-up sperm subpopulations, where there was a higher tendency of active sperm in the rapid subpopulation of VCL, VAP and WOB as presented in Figure 4.10.

Table 4.6 and Table 4.7 present the data for the percentage sperm motility groupings and sperm kinematics of four representative species, including *D. serra*, *C. gigas*, *C. meridionalis* and *H. midae*. In this section the motility and sperm kinematic measurements of swim-up sperm samples for all mollusc species were analysed over time ranging from 5, 15, 30, 60 min and after being stored at 4°C for approximately 24 hrs.



*Haliotis midae* sperm samples were collected within 2 min of ejection into the spawning tank and after 60 min of continuous spawning. Owing to *H. midae* being a protected species no testicular or dry-spawned sperm was obtained for the 24 hrs assessment. As mentioned above, *D. serra* sperm showed low to no motile sperm after being stored at 4°C for approximately 24 hrs and no data were captured.

No significant changes (P > 0.05) were found in the percentage total sperm motility and progressive motility measurements of the sperm activated during swim-up technique for *D. serra* (P > 0.05, F= 0.134), *C. gigas* (P > 0.05, F= 0.813), *C.*

*meridionalis* (P > 0.05, F= 0.823) and *H. midae* (P > 0.05, F= 1.662) as presented in Table 4.6. No significant difference was evident between the swim-up sperm samples of *D. serra* and *C. meridionalis* over all the time points. Nevertheless, their sperm showed a similar pattern, whereby the total sperm motility decreased (P > 0.05) after 15 and 60 min, while after 30 min the percentage motile sperm was high (P > 0.05) when compared to the initial value after 5 min for *D. serra* as shown in Table 4.6. Data captured for both *D. serra* (P > 0.05, F= 0.106) and *C. meridionalis* (P > 0.05, F= 0.45) showed a higher percentage of swim-up sperm within the rapid subpopulations, as well as the medium subpopulation of *C. meridionalis* (P > 0.05, F= 0.442) sperm over all time points measured. Spawning *H. midae* sperm had the highest percentage total motility; (95.16%) after spawning for 60 min, while *C. gigas* swim-up sperm had the lowest percentage total motility of sperm of all the molluscs presented in Table 4.6.

Overall, the average sperm velocities showed no significant changes among the time points of the individual species of molluscs as presented in Table 4.7. Donax serra swim-up sperm had the highest average VCL value of the molluscs, not significant however, it did decrease after 60 min activation and no swim-up sperm motility was achieved after being stored at 4°C for approximately 24 hrs. Nevertheless, swim-up sperm of *D. serra* and spawned *H. midae* sperm, shared a tendency of higher average VSL and VAP and in the average LIN, STR, WOB, ALH and BCF after being motile for 60 min (P > 0.05).

This similarity was not shared by activated *C. gigas* and *C. meridionalis* sperm; swim-up sperm samples from both displayed a steady lowering (P > 0.05) of the average VCL, VSL and VAP after 15 to 60 min activation. This lowering of swim-up sperm

activity continued for LIN, STR, WOB, ALH and BCF of *C. gigas* and *C. merdionalis*. After being stored at 4°C for approximately 24 hrs, *C. meridionalis* showed the most active swim-up sperm for all average sperm kinematic measurements captured as presented in Table 4.7.

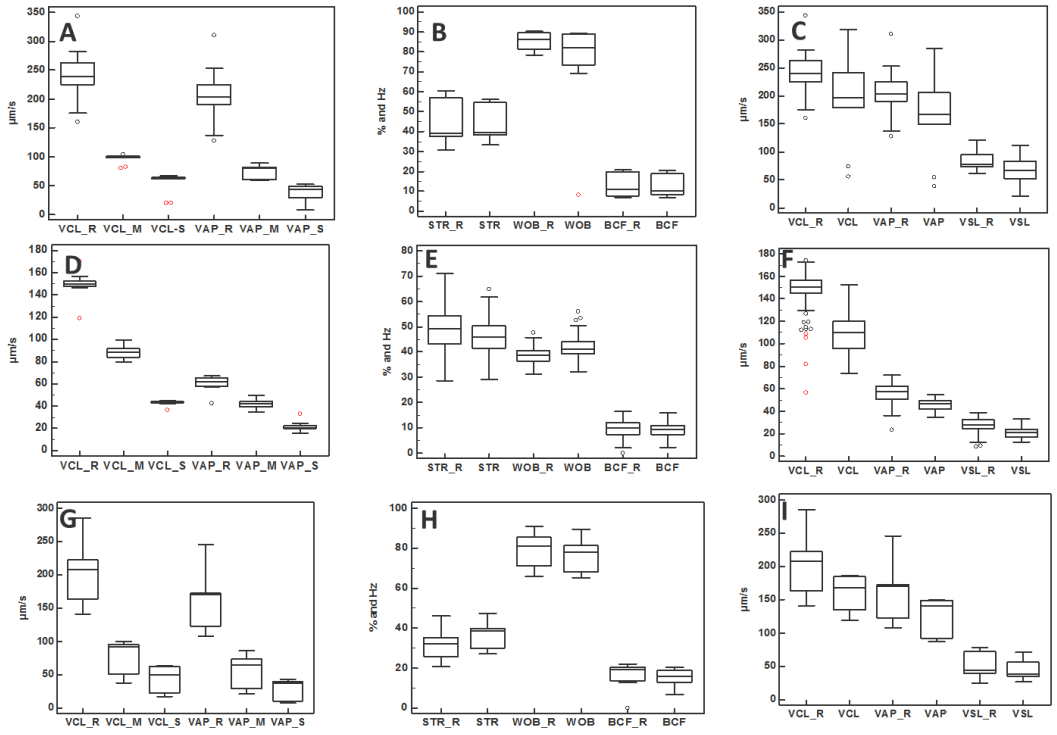



**Figure 4.10:** Box and whisker plots showing sub-population values of rapid, medium, slow sperm and average kinematic parameters for three representative species. A, B and C = *P. angulosus*; D, E and F = *C. gigas*; G, H and I = *D. serra*. VCL = curviliear velocity, VSL = straight line velocity, VAP = average path velocity, STR = straightness, WOB = wobble, VCL_R = Rapid; VCL_M = Medium; VCL_S = Slow; VCL = Average; VAP_R = Rapid; VAP_M = Medium; VAP_S = Slow; VSL_R = Rapid; VSL = Average; STR_R, WOB_R, BCF_R =Rapid; STR, WOB, BCF = Average



### 4.9.1 Comparison of Sperm Motility and Sperm Kinematics

Figure 4.11 shows a comparative analysis of the total, progressive and non-progressive motility of *C. meridionalis*, *C. gigas* and *P. angulosus* sperm using the flush and swim-up techniques. These data establish the similarities and differences between the activity of testicular and dry-spawned sperm used directly during the flush technique and after sea water stimulation with the swim-up technique.

**Table 4.6:** Percentage of total sperm motility, progressive motility, and sperm subpopulation measurements (mean ± SD) of swim-up sperm samples of *D. serra*, *C. gigas*, *C. meridionalis* and *H. midae* (n = ≥10 animals per species), showing sperm activity over various time frames

| % | | *D. serra* | | | | *C. gigas* | | | | | *C. meridionalis* | | | | | *H. midae* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 5 min | 15 min | 30 min | 60 min | 5 min | 15 min | 30 min | 60 min | 24 hrs | 5 min | 15 min | 30 min | 60 min | 24 hrs | 2 min | 60 min |
| **MOT** | **Mean** | 53.6 | 52.2 | 59.2 | 53.3 | 24.1 | 17.1 | 15.5 | 15.5 | 11.9 | 59.0 | 53.4 | 58.6 | 47.3 | 45.2 | 88.8 | 95.1 |
| | **±SD** | 33.8 | 29.8 | 29.9 | 30.6 | 23.2 | 16.4 | 15.3 | 11.9 | 10.8 | 24.7 | 23.6 | 25.7 | 26.6 | 28.5 | 9.4 | 3.6 |
| **Prg Mot** | **Mean** | 2.9 | 2.5 | 2.8 | 10.2 | 4.5 | 3.5 | 3.6 | 3.3 | 2.5 | 6.6 | 4.5 | 6.1 | 4.6 | 6.8 | 8.3 | 10.1 |
| | **±SD** | 2.7 | 1.8 | 1.4 | 25.4 | 4.4 | 4.0 | 3.6 | 3.2 | 3.1 | 5.8 | 5.1 | 4.9 | 4.4 | 6.3 | 3.6 | 2.0 |
| **Rpd Mot** | **Mean** | 49.1 | 46.0 | 51.7 | 45.6 | 8.7 | 8.6 | 7.5 | 6.9 | 3.7 | 24.7 | 18.2 | 20.9 | 16.4 | 15.3 | 39.9 | 64.2 |
| | **±SD** | 33.9 | 29.7 | 32.7 | 32.3 | 9.2 | 10.3 | 9.8 | 7.0 | 4.8 | 25.3 | 18.6 | 17.6 | 21.9 | 21.0 | 29.2 | 13.0 |
| **Med Mot** | **Mean** | 1.9 | 2.9 | 3.0 | 3.1 | 8.3 | 6.3 | 5.7 | 5.9 | 5.5 | 23.6 | 23.2 | 25.4 | 18.1 | 22.0 | 36.9 | 20.8 |
| | **±SD** | 2.4 | 3.6 | 2.5 | 2.9 | 6.4 | 5.1 | 5.1 | 4.4 | 5.1 | 18.5 | 14.1 | 14.8 | 8.5 | 22.6 | 28.8 | 8.4 |
| **Non-Prog** | **Mean** | 2.5 | 3.2 | 4.6 | 4.5 | 1.8 | 2.1 | 2.2 | 2.6 | 2.5 | 10.6 | 12.6 | 12.2 | 12.7 | 7.7 | 11.9 | 10.2 |
| | **±SD** | 3.1 | 4.5 | 4.4 | 4.6 | 1.7 | 1.6 | 1.3 | 2.2 | 2.2 | 8.7 | 11.7 | 11.3 | 11.8 | 2.9 | 7.9 | 8.9 |

MOT = total sperm motility, Prg Mot = progressive sperm motility, Rpd Mot = rapid progressive motility, Med Mot = medium progressive motility, Non-Prog = non-progressive motility

**Table 4.7:** Sperm kinematic parameter measurements (mean ± SD) of diluted sperm samples of four marine invertebrates, *D. serra*, *C. gigas*, *C. meridionalis* and *H. midae* (n ≥ 10 animals per species), showing swim-up sperm activity over various time frames

| Parameters | | 5 min | 15 min | 30 min | 60 min | 5 min | 15 min | 30 min | 60 min | 24 hrs | 5 min | 15 min | 30 min | 60 min | 24 hrs | 2 min | 60 min |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| VCL (µm/s) | Mean | 253.5 | 247.2 | 252.8 | 231.5 | 109.9 | 112.3 | 105.6 | 105.1 | 99.3 | 113.2 | 104.0 | 104.2 | 94.8 | 109.2 | 96.8 | 113.9 |
| | ±SD | 101.0 | 84.6 | 86.8 | 82.3 | 17.1 | 18.0 | 21.1 | 16.2 | 27.5 | 45.1 | 35.2 | 27.3 | 37.1 | 58.6 | 29.1 | 14.9 |
| VSL (µm/s) | Mean | 44.0 | 44.2 | 49.4 | 53.5 | 21.1 | 21.5 | 20.1 | 20.8 | 17.5 | 26.4 | 23.3 | 26.1 | 24.1 | 27.3 | 29.5 | 36.2 |
| | ±SD | 18.9 | 12.1 | 14.8 | 19.8 | 5.3 | 4.1 | 4.3 | 5.2 | 4.1 | 12.4 | 6.8 | 7.6 | 9.7 | 14.2 | 9.8 | 6.3 |
| VAP (µm/s) | Mean | 187.0 | 193.7 | 201.6 | 191.2 | 44.9 | 45.6 | 43.4 | 43.1 | 40.9 | 76.3 | 71.7 | 68.3 | 63.1 | 73.5 | 86.3 | 102.2 |
| | ±SD | 54.8 | 60.1 | 62.6 | 71.2 | 6.7 | 5.4 | 6.7 | 5.8 | 9.5 | 30.6 | 27.4 | 18.2 | 25.5 | 43.9 | 31.3 | 15.3 |
| LIN (%) | Mean | 18.3 | 18.8 | 20.5 | 23.4 | 19.1 | 19.2 | 19.2 | 19.7 | 18.3 | 23.7 | 23.6 | 25.3 | 25.7 | 25.8 | 30.8 | 31.7 |
| | ±SD | 4.9 | 5.2 | 5.9 | 4.1 | 2.7 | 2.3 | 3.5 | 3.3 | 4.6 | 6.5 | 6.7 | 4.8 | 3.7 | 4.9 | 7.5 | 2.7 |
| STR (%) | Mean | 23.2 | 23.9 | 25.3 | 28.6 | 46.6 | 46.9 | 46.2 | 47.7 | 43.5 | 35.8 | 35.2 | 39.0 | 39.4 | 39.2 | 35.5 | 35.4 |
| | ±SD | 6.6 | 7.6 | 6.8 | 5.8 | 5.7 | 6.0 | 6.8 | 7.3 | 8.1 | 11.1 | 11.2 | 7.9 | 7.8 | 8.0 | 10.4 | 2.6 |
| WOB (%) | Mean | 79.8 | 79.3 | 80.8 | 82.3 | 40.9 | 41.1 | 41.7 | 41.3 | 42.0 | 67.1 | 68.5 | 65.6 | 66.3 | 66.3 | 87.7 | 89.6 |
| | ±SD | 10.3 | 8.2 | 6.6 | 5.6 | 2.3 | 5.0 | 5.3 | 4.3 | 5.1 | 8.2 | 9.3 | 8.4 | 9.8 | 8.0 | 7.0 | 2.8 |
| ALH (µm) | Mean | 1.5 | 1.8 | 2.1 | 2.1 | 3.1 | 3.3 | 3.2 | 3.5 | 2.5 | 2.3 | 1.9 | 2.4 | 2.3 | 1.9 | 1.4 | 1.8 |
| | ±SD | 1.1 | 0.8 | 0.9 | 0.5 | 1.3 | 1.0 | 0.7 | 0.9 | 1.6 | 0.8 | 1.0 | 0.7 | 1.0 | 0.8 | 0.5 | 0.2 |
| BCF (Hz) | Mean | 13.9 | 13.8 | 14.3 | 14.2 | 7.9 | 9.5 | 8.1 | 8.2 | 6.4 | 9.7 | 7.9 | 9.8 | 8.1 | 7.7 | 10.0 | 12.3 |
| | ±SD | 11.1 | 9.0 | 6.5 | 6.1 | 3.1 | 3.2 | 2.3 | 2.6 | 4.6 | 5.6 | 4.5 | 4.0 | 5.0 | 4.9 | 2.7 | 2.3 |

VCL = curviliear 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

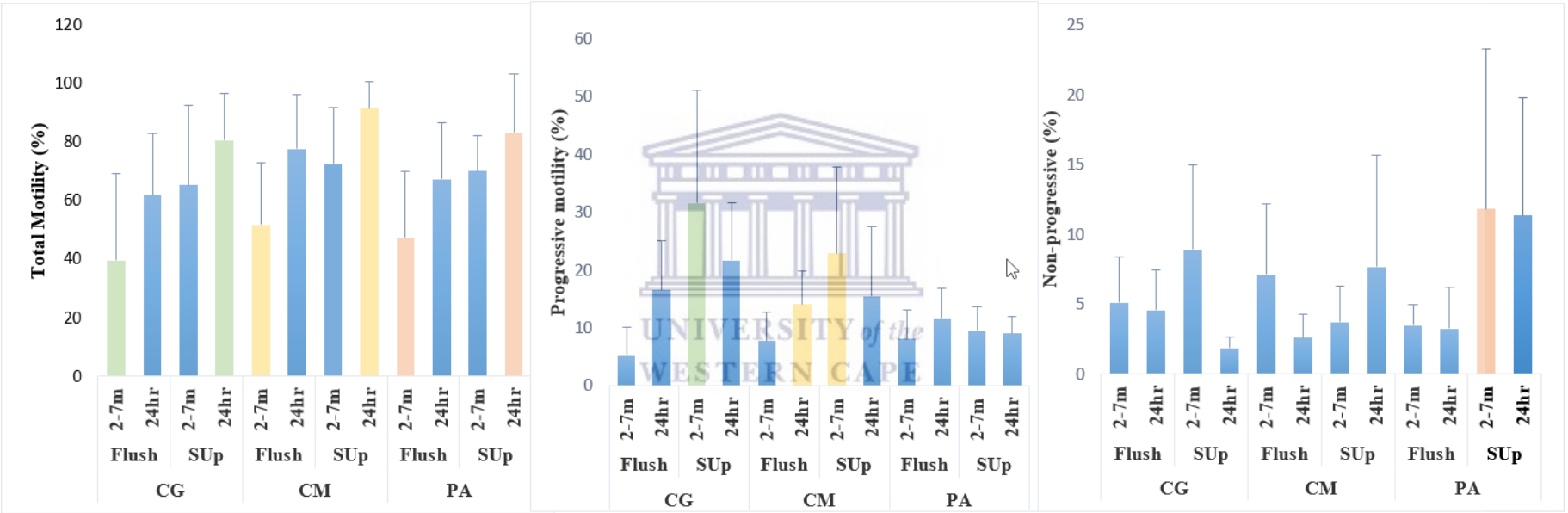



**Figure 4.11:** Total, progressive and non-progressive sperm motility differences between testicular-sperm or dry-spawned sperm and active swim-up sperm of three species, *C. gigas* (CG), *C. meridionalis* (CM) and *P. angulosus* (PA). Motility (mean ± SD) was captured immediately after removal from testis (CG, CM) and dry-spawning (PA) and again at 24 hrs after being stored as a pellet dry sperm at 4°C. Green, yellow and orange bars indicate significant differences ($P < 0.05$) within the individual groups of each species.

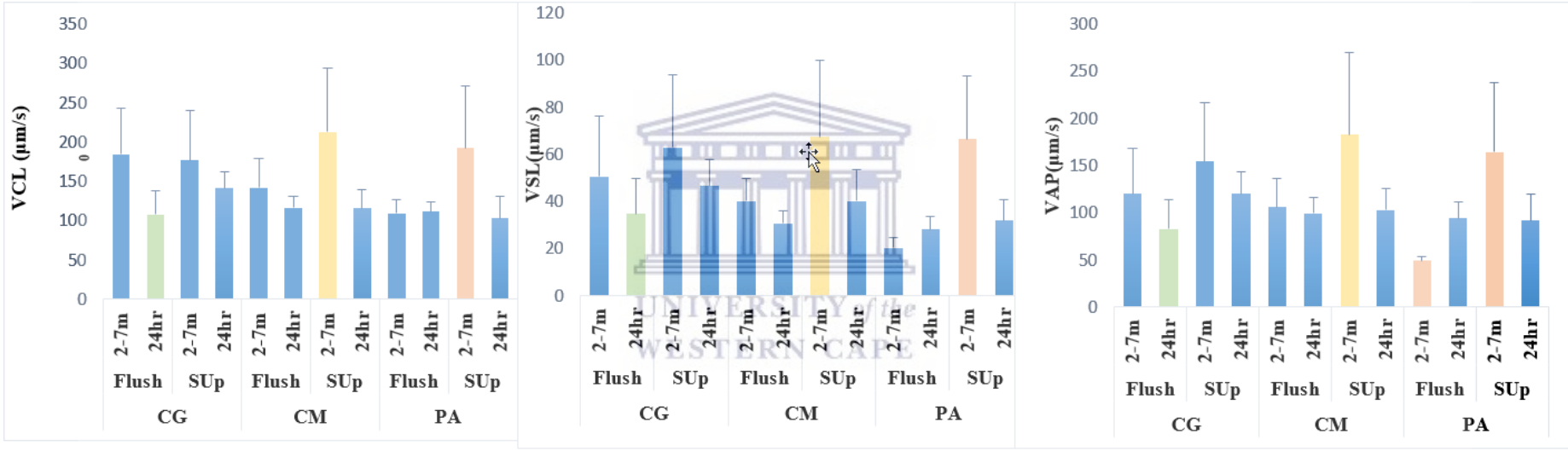



**Figure 4.12:** Comparison of sperm swimming speed kinematics values between testicular-sperm or dry-spawned sperm and active swim-up sperm of three species, *C. gigas* (CG), *C. meridionalis* (CM) and *P. angulosus* (PA). Kinematic values (mean ± SD) were captured immediately after removal from testis (CG, CM) and dry-spawned (PA) and again at 24 hrs after being stored as a pellet dry sperm at 4°C. Green, yellow and orange bars indicate significant differences ($P < 0.05$) within the individual groups of each species.

Sperm motility was measured at two time points, namely the initial activation time of 2-7 min for most species and after being stored at 4°C for approximately 24 hrs. For the swim-up technique, the sperm samples were allowed to incubate in the sea water filled Eppendorf tubes for 2-7 min before data were captured. The general trend among the three species showed significant changes ($P < 0.05$) between the initial (2-7 min total sperm motility and after storage, with an increase in the total percentage motility of flushed testicular and dry-spawned sperm after 24 hrs. Similarly, there was a significant increase ($P < 0.05$) in total sperm motility of swim-up sperm for all three species after being stored at 4°C for approximately 24 hrs as presented in Figure 4.11. Testicular flushed sperm for *C. gigas* and *C. meridionalis* showed a tendency to higher progressively motile sperm percentages after being stored at 4°C for approximately 24 hrs as presented in Figure 4.11.

*Parechinus angulosus* sperm showed no significant changes ($P > 0.05$) in the percentage progressive motility of sperm samples that underwent the flush and swim-up techniques over the two time points. However, the initial (2-7 min) *P. angulosus* swim-up samples had a significant increase ($P > 0.05$) in the percentage non-progressive sperm population compared to the dry-spawned sperm of the flush technique. Both *C. gigas* and *C. meridionalis* had higher ($P > 0.05$) percentage of non-progressively motile sperm during the first 2-7 min of the swim-up samples compared to the flushed testicular sperm samples.

Figure 4.12 shows the similarities of *P. angulosus* and *C. meridionalis* sperm in terms of the initial (2-7 min) swim-up technique samples that had significantly higher ($P < 0.05$) average VCL, VSL and VAP when compared to flushed testicular or dry-

spawned samples of both species. Flushed *C. gigas* testicular sperm had significantly lower ($P < 0.05$) VCL, VSL and VAP after being stored at 4°C for approximately 24 hrs. Similar lower values were found between the two time points in *C. merdionalis* flushed testicular sperm, though these changes were not significant ($P > 0.05$) as shown in Figure 4.12. As for the other kinematic parameters, *P. angulosus* flushed sperm samples were found to have a significant decrease ($P < 0.05$) for ALH and WOB after 24 hrs. No significant changes were found ($P > 0.05$) between flush and swim-up technique samples for STR and BCF of the *P. angulosus*. Crassostrea gigas and *C. merdionalis* sperm showed no significant changes ($P > 0.05$) for ALH, LIN, STR and WOB for both flush and swim-up technique sperm samples.

### 4.9.2 Detailed Comparisons of Swim-Up Sperm Motility Patterns and Individual Tracks

Figure 4.13 presents a collection of CASA-captured fields to indicate the similarities and differences observed in the swim-up sperm motility patterns of all five species, at 10 and 60 min after dilution with sea water. It is evident from the collection that all five species share a typical helical swimming pattern after 10 min activation in sea water. After 60 min dilution in sea water, *D. serra* sperm displayed a lower percentage of linear forward progressive sperm pattern, while *C. meridionalis* and *C. gigas* sperm had a distinctive change in pattern, with mostly linear forward progressive sperm pattern as shown in Figure 4.13. In contrast, *P. angulosus*, and *H. midae* sperm retained the helical swimming pattern after 60 min. While *P. angulosus* sperm displayed a larger helical diameter with higher individual sperm VCL of >400 µm/s, *C. gigas* sperm has a distinctive serrated helix with the smallest diameter and with individual sperm VCL values of >179 µm/s as presented in Figure 4.14.

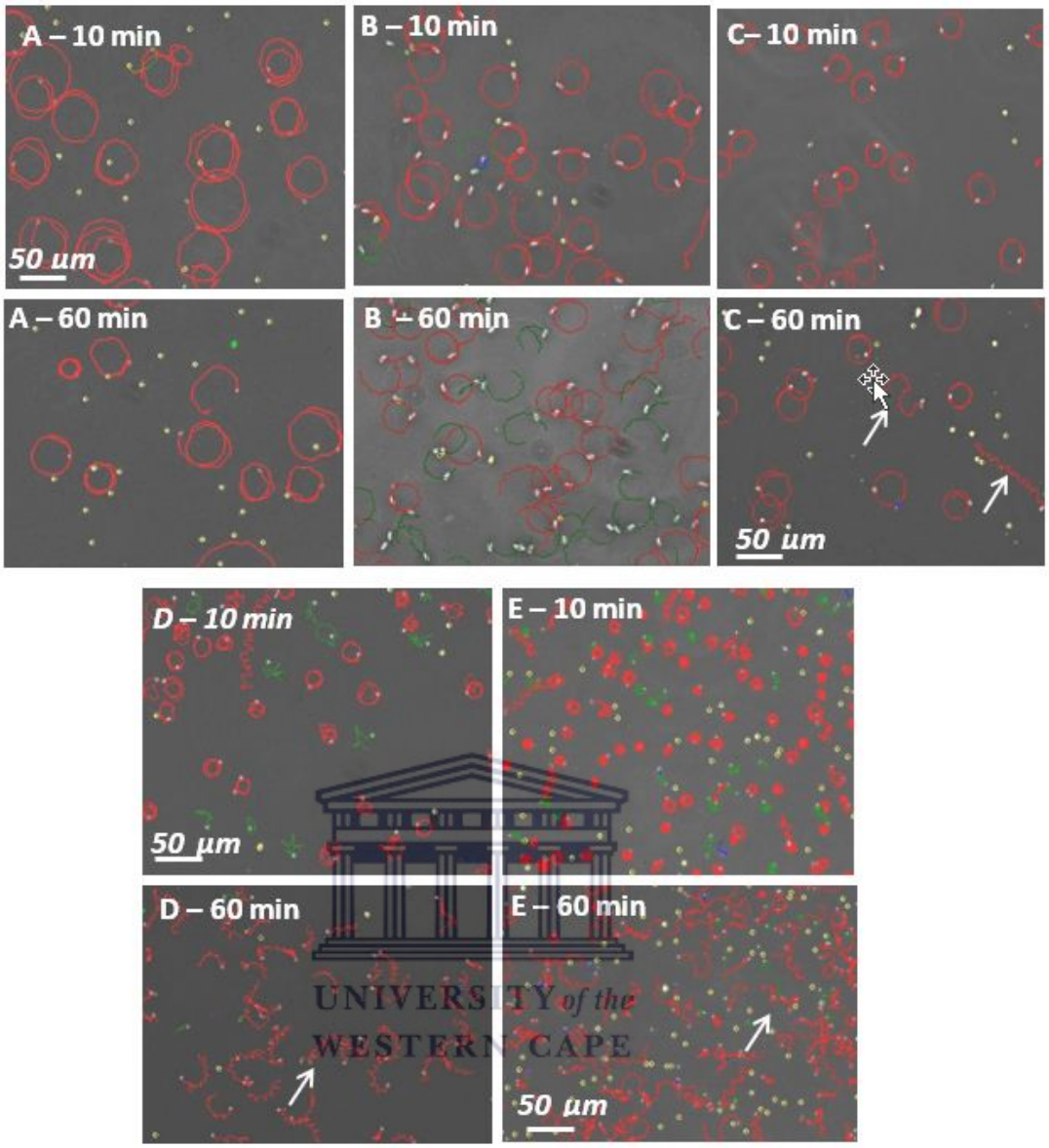





**Figure 4.13:** Characteristic sperm motility tracks of diluted sperm of five marine invertebrates (A = *P. angulosus*, B = *H. midae*, C = *C. gigas*, D = *C. meridionalis* and E = *D. serra*). Tracks of A-C remained circular after 60 min, while sperm tracks of D and E changed to a linear pattern after 60 min. Red tracks = rapid swimming sperm, green tracks = medium swimming sperm and blue tracks = non-progressive (slow) sperm. White arrows indicate sperm swimming progressively forward.

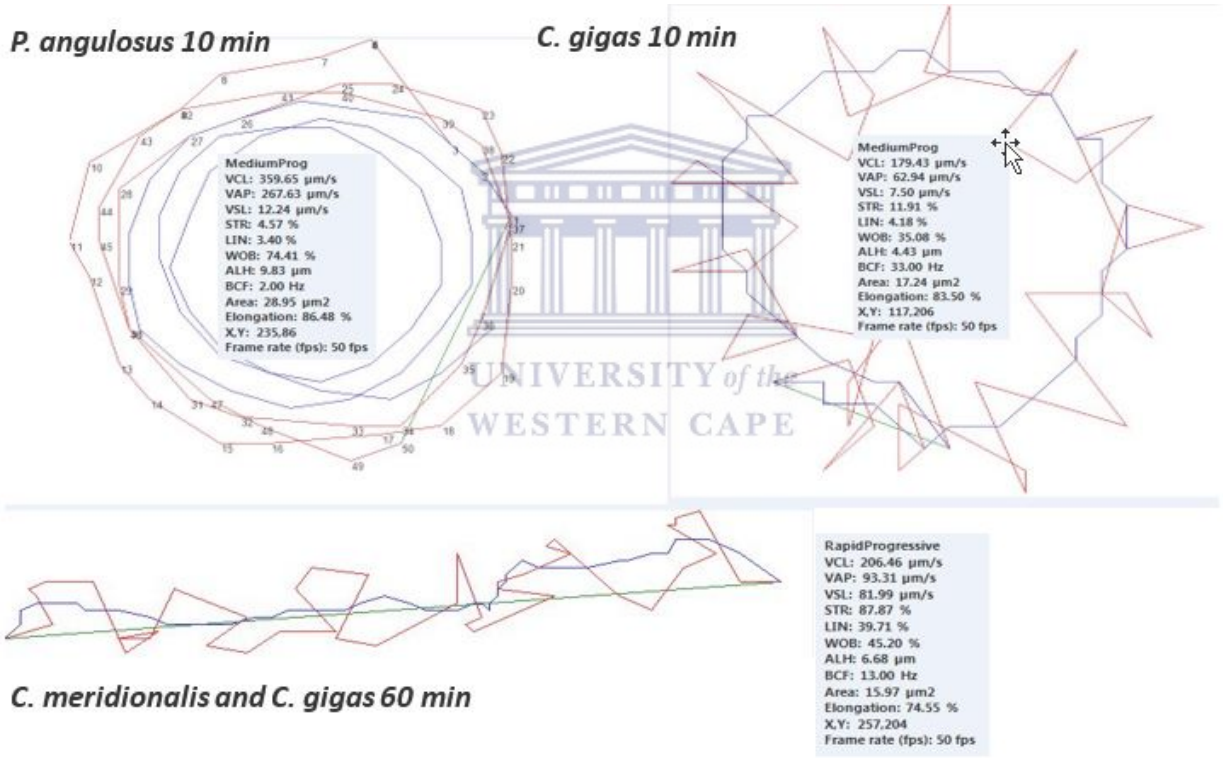



**Figure 4.14:** Individual sperm kinematic parameters of four species, showing characteristic differences between the motility patterns of tracks of these broadcast spawning species. The numbering in *P. angulosus* refers to the capturing points every 50th of a second over one second.

### 4.9.3 Comparison Between Sperm Kinematic Parameters and Helical Track Diameters

Individual helical tracks were analysed for all the study species by showing the relationship between the diameter, average VCL and DANCE as presented in Figure 4.15. All species had a similar basic helical form, with *P. angulosus* sperm speed and track diameter being significantly larger ($P < 0.05$). *Parechinus angulosus* helical diameter ranged from 30-70 µm compared to *C. gigas* sperm that had the smallest track diameter of 9-15 µm.

While *C. gigas*, *C. meridionalis* and *D. serra* sperm shared a similar range for VCL of 150-230 µm/s *P. angulosus* sperm had the fastest VCL (>400 µm/s) and *H. midae* had the slowest VCL as well as the smallest range for DANCE (space occupied) (Figure 4.15 D and E). Two-dimensional spaces occupied by individual helical tracks were similar for *C. merdinalis*, *D. serra* and *P. angulosus* sperm as shown in the DANCE (Figure 4.15 E).



Spearman rank correlations of the average populations of VCL, VAP, ALH, area, perimeter, diameter and DANCE of the total motile sperm helical tracks were further analysed using the combined diameters of all study species as presented in Table 4.8 and Table 4.9. The relationship between, motility, velocity and sperm area of movement within the population described both the sperm swimming velocity and the helical motion of the sperm tracks, which could be further related to sperm traits for egg-searching and sperm functionality.

These results are included in our publication (Appendix II). Table 4.8 presents the correlation of the combined mean populations for all species in relation to the helical

characteristics. For the mean populations, there were highly significant positive correlations between diameter and VCL, diameter and VAP and diameter and DANCE.

Accordingly, in general, when sperm swam faster, the diameter of the helix increased, as did DANCE. DANCE is a reflection of the two-dimensional space occupied by sperm during one second. Due to DANCE being a combination of ALH and VCL, the relationship between VCL and ALH was investigated in *P. angulosus*, *D. serra*, *H. midae* and *C. merdionalis* as shown in Table 4.9.

Similar high correlations (r = 0.6-0.91, P < 0.001, F = 9.3) were found between VCL and ALH for all four species. Accordingly, increases in both VCL and ALH contributed to an increased DANCE, relating to an increase in the 'sperm search area'.



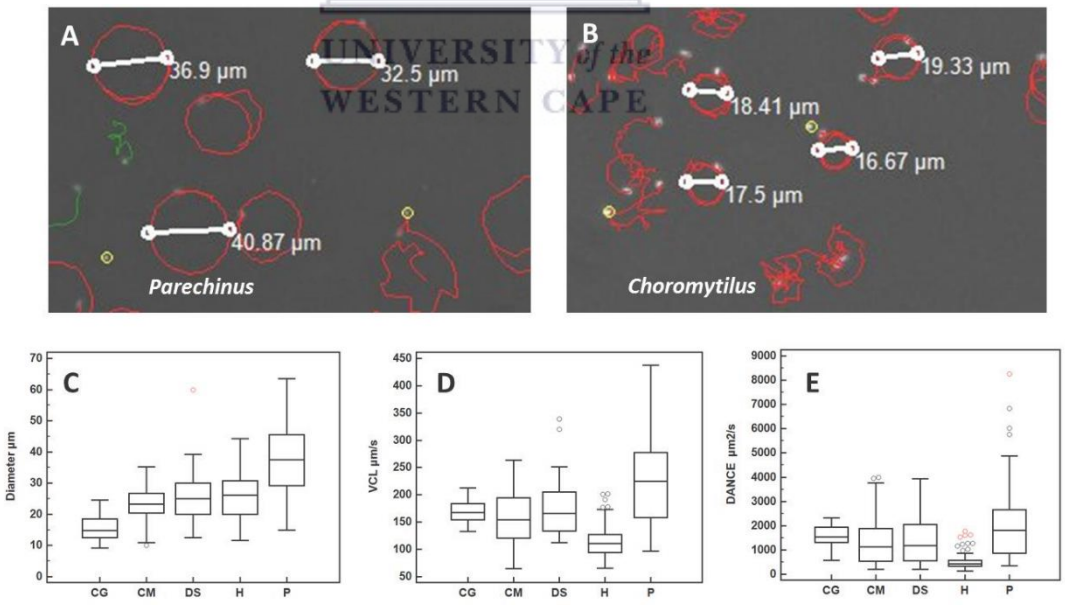



**Figure. 4.15:** A and B show measurement of the individual helical tracks diameters in two species and C, D and E show box and whisker plots for the diameter of the helical tracks, curvilinear velocity (VCL) and DANCE (VCL x

ALH) for these tracks in five species (CG = *C. gigas*, CM = *C. meridionalis*, DS = *D. serra*, H = *H. midae*, P = *P. angulosus*). In C and D, *P. angulosus* differed significantly from the other species ($P < 0.05$).

**Table 4.8:** Spearman rank Correlation of the average populations of VCL, VAP, DANCE, area, perimeter and diameters of the combined diameters of sperm helical track of all five study species

| Parameter | VCL | | VAP | | Diameter | | Perimeter | | Area | | DANCE | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | r | P-value | r | P-value | r | P-value | r | P-value | r | P-value | r | P-value |
| **VCL** | | | 0.89 | <0.0001 | 0.41 | <0.0001 | 0.3 | <0.0001 | 0.31 | <0.0001 | | |
| **VAP** | 0.89 | <0.0001 | | | 0.62 | <0.0001 | 0.62 | <0.0001 | 0.63 | <0.0001 | 0.68 | <0.0001 |
| **Diameter** | 0.41 | <0.0001 | 0.62 | <0.0001 | | | 0.41 | <0.0001 | | | 0.68 | <0.0001 |

n = 100 helical tracks per study animal. VCL = curviliear velocity (µm/s), VAP = average path velocity (µm/s), DANCE = two-dimensional space oocupied by a motile sperm during 1 sec (µm2/s), Area = size of the surface of circle (µm2), Perimeter = circumferance (µm), Diameter = linear measure across the helical swimming pattern (µm)

**Table 4.9:** Spearman rank correlations of VCL, VAP with ALH for the individual helical tracks of four representative species

| Parameter | ALH = Amplitude of Lateral Head Displacement | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *P. angulosus* | | *D. serra* | | *H. midae* | | *C. meridionalis* | |
| | r | P-value | r | P-value | r | P-value | r | P-value |
| **VCL** | 0.58 | <0.0001 | 0.8 | <0.0001 | 0.76 | <0.0001 | 0.81 | <0.0001 |
| **VAP** | 0.76 | <0.0001 | 0.315 | <0.0001 | 0.67 | <0.0001 | 0.315 | <0.0001 |

n = 100 helical tracks per study animal ALH = amplitude of lateral head displacement (µm), VCL = curviliear velocity (µm/s), VAP = average path velocity (µm/s)

## 4.10 Various Media Affecting Motility Patterns

The percentage total motility and sperm kinematic parameter measurements were captured for *P. angulosus* sperm activated in sea water, exposed to eggs only and to egg-water, presented in Table 4.10. Dry-spawned sperm was activated in sea water through flush, swim-up and mixed techniques. The motility of sperm was captured after 2-7 min for flush, eggs only, egg-water and mixed sperm samples. Swim-up sperm samples were assessed after a 10-15 min sea water incubation. This section establishes the similarities and differences found in the *P. angulosus* sperm (model species) activity and helical patterns after exposure to various media.

Data presented in Table 4.10 shows sperm exposed to egg-water had a significantly higher ($P < 0.05$, $F = 2.767$) total motility of 86.6% than sperm exposed to sea water. Dry-spawned, swim-up, eggs only sperm had a total motility rate that ranged between 61.5-64.8% and mixed sperm samples had the lowest total motility of 53.3%. Swim-up sperm had a higher ($P < 0.05$, $F = 9.569$) tendency for progressively motile sperm (34.7%) in comparison to dry-spawned (8.4%) and sperm in eggs only (16.2%) as shown in Table 4.10.



None of the media showed any significant influence on the motility within in the rapid and medium subpopulations. However, motility was higher ($P > 0.05$, $F = 2.131$) in the rapid subpopulations and ranged between 40–69% for sperm exposed to sea water, egg-water and eggs only. The dry-spawned sperm used in the flush technique had the highest ($P < 0.05$, $F = 4.159$) non-progressive/slow subpopulations compared to the swim-up, eggs only, egg-water and mixed sperm samples as shown in Figure 4.16.

**Table 4.10:** Sperm motility and kinematic parameter measurements (mean ± SD) of *P. angulosus* dry-spawned (DrySpm) and swim-up (SUp) sperm in sea–water, sperm exposed to eggs, egg–water (Egg-W) and mixed in sea–water

| Parameter | | SUp | DrySpm | Eggs | Egg-W | Mix |
|---|---|---|---|---|---|---|
| **Total Motility (%)** | **Mean** | 64.0 [b] | 64.8 | 61.5 | 86.6 [a] | 53.3 [b] |
| | **±SD** | 23.4 | 15.5 | 15.4 | 9.2 | 24.0 |
| **Prg Mot (%)** | **Mean** | 34.7 [a] | 8.4 [b] | 16.2 [b] | 25.1 | 19.5 |
| | **±SD** | 21.7 | 4.2 | 8.6 | 10.4 | 10.4 |
| **Non-Prg Mot (%)** | **Mean** | 6.4 [b] | 12.0 [a] | 2.8 [b] | 1.5 [b] | 4.9 |
| | **±SD** | 6.5 | 12.1 | 1.5 | 0.8 | 3.5 |
| **Rpd Prg Mot (%)** | **Mean** | 45.2 | 44.0 | 40.1 | 63.8 | 29.8 |
| | **±SD** | 22.9 | 25.5 | 10.3 | 15.2 | 30.5 |
| **Med Prg Mot (%)** | **Mean** | 12.4 | 8.9 | 18.6 | 21.3 | 18.7 |
| | **±SD** | 21.2 | 6.5 | 11.0 | 15.9 | 13.3 |
| **VCL (µm/s)** | **Mean** | 197.4 [a] | 185.2 | 134.6 | 140.5 | 114.6 [b] |
| | **±SD** | 66.2 | 75.0 | 17.5 | 18.7 | 42.1 |
| **VSL (µm/s)** | **Mean** | 66.6 [a] | 64.7 | 38.4 [b] | 50.1 | 44.0 |
| | **±SD** | 25.8 | 27.6 | 7.9 | 12.3 | 15.6 |
| **VAP (µm/s)** | **Mean** | 173.6 [a] | 157.1 | 103.5 [b] | 122.7 | 89.8 [b] |
| | **±SD** | 65.6 | 69.9 | 20.5 | 17.8 | 42.4 |
| **LIN (%)** | **Mean** | 34.0 | 34.8 | 29.0 | 35.7 | 38.8 |
| | **±SD** | 6.2 | 5.1 | 7.1 | 8.4 | 6.4 |
| **STR (%)** | **Mean** | 39.6 | 43.1 | 37.6 [b] | 40.9 | 51.5 [a] |
| | **±SD** | 7.2 | 7.7 | 8.0 | 9.5 | 9.5 |
| **WOB (%)** | **Mean** | 86.2 [a] | 77.6 [b] | 77.0 | 87.3 | 76.0 |
| | **±SD** | 60.9 | 18.6 | 11.4 | 2.6 | 8.5 |
| **ALH (µm)** | **Mean** | 2.2 | 2.3 | 2.7 | 2.5 | 2.9 |
| | **±SD** | 0.6 | 0.6 | 0.6 | 0.8 | 0.9 |
| **BCF (Hz)** | **Mean** | 10.8 | 12.2 | 10.8 | 9.9 | 9.3 |
| | **±SD** | 5.2 | 6.1 | 5.7 | 5.1 | 5.3 |

MOT = total percentage motility, Prg Mot = progressive motility, Rpd Mot = rapid progressive motility, Med Mot = medium progressive motility, Non-Prog = non-progressive motility, VCL = curviliear 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. a,b = values labelled with superscript letters in the same row are significantly different ($P < 0.05$).

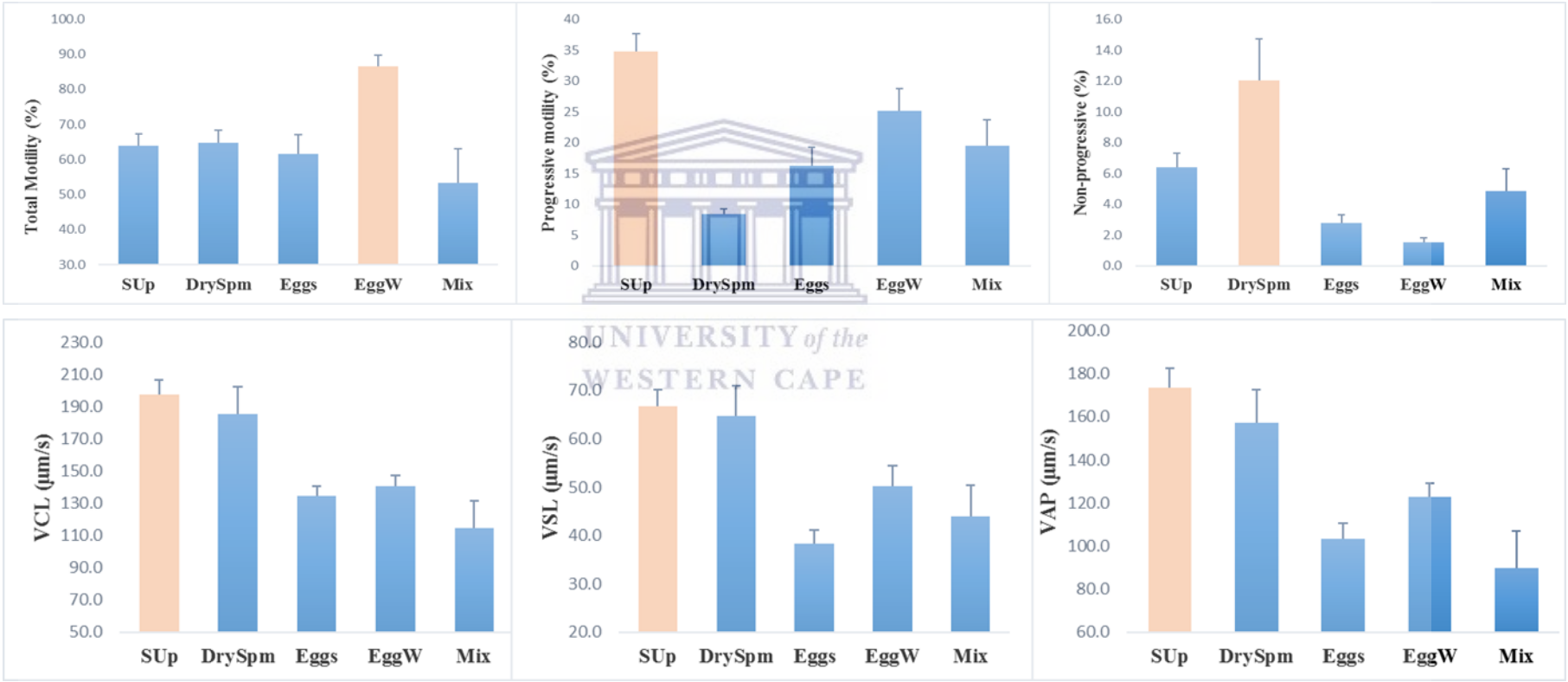





**Figure 4.16:** Effects of various media, namely sea–water (SUp, DrySpm and Mix), eggs and egg-water on *P. angulosus* total sperm motility and kinematic parameters. SUp = swim-up; DrySpm = dry-spawned sperm; EggW = egg–water. Orange bars indicate significant differences ($P < 0.05$)

Overall, the general trend showed sperm velocities were higher (P < 0 .05) in swim-up samples with VCL 197.4 ± 66.2 µm/s, VSL 66.2 ± 25.8 µm/s and VAP 173.5 ± 65.6 µm/s; compared to the other media shown in Table 4.10. Sperm egg samples had the second slowest (P < 0.05) sperm velocities with a VCL of 134.6 µm/s. Mixed sperm samples showed a significantly lower (P < 0.05) VCL and VAP (114.6 ± 42.1 and 89.8 ±42.4 µm/s, respectively). When arranging the activation media in order of fastest to slowest motile sperm, it would be arranged as follows: swim-up > flushed dry-spawn > egg-water > eggs only > mixed samples.

No significant differences (P > 0.05) were found for LIN, BCF and ALH values among the various mediums. The only significant differences (P < 0.05) were shown for STR. The eggs samples had the lowest (P < 0.05) STR while sea water mixed samples had the highest (P < 0.05). Swim-up sperm had a higher (P < 0.05) WOB (86.2 ± 60.9%) than dry-spawn flushed samples (77.5 ± 18.6%) (P < 0.05) (Table 4.10).



## 4.11 Correlations Among Selected Morphometric Parameters, Kinematics and Quantitative Sperm Track Characteristics

Pearson product-moment correlation coefficients were calculated from the swim-up sperm data of three of the study species, the *P. angulosus* model, and *C. meridionalis* and *D. serra* sperm. These were the species with a comprehensive set of parameters for morphometry, motility and quantitative sperm track characteristics. The three species were furthermore selected representing diverse “spawning” environments ranging from rock pools to high energy interface environments such as intertidal rocks. They accordingly represent the diversity of spawning environments and sperm functionality for the five species studied. Theoretically, it has been implied that there

would be a higher egg hit rate with helical swimming presumabily because of the larger search area per volume (Farley, 2002). These correlation comparisons provided an ideal basis for establishing potential diversity in sperm traits, sperm swimming behaviour and their helical tracks created. It showed how sperm form and function could be related to the spawning environment and the likelihood of colliding with an egg. Other than Lui *et al*. (2011) and Vogel *et al*. (1982) who referred to helical tracks as 'Don Giovanni' and the forward moving tracks as 'Don Ottavio', there are few detailed descriptions of the nature of the helical swimming patterns. Pearson correlation coefficients (r) determine the degree of association between two variables; whether they share variance and if the relationship is positive (+) or negative (-) and the degree to which they correlate (Cramer, 1998). Partial correlations measure the strength of the linear relationship between the variables having first adjusted for their relationship to other variables in the table. They are helpful in judging how useful one variable would be in improving the prediction of the second variable given that information from all the other variables has already. Table 4.11 shows the list of eleven selected parameters summarized for the correlations. The correlations of the eleven parameters are presented in Appendix III. Figure 4.17 present the correlations of the combined averages of four sperm morphometry features, four sperm kinematics and three sperm track characteristics (Table 4.11).

**Table 4.11:** The list of eleven sperm parameters used for correlation analysis

| Parameter | |
|---|---|
| ALH (µm) | Sperm kinematics |
| VCL (µm/s) | |
| VAP (µm/s) | |
| DANCE (µm²/s) | |
| Head Length (L) (µm) | Sperm morphometry |
| Head Width (W) (µm) | |
| Tail (µm) | |
| Total Length (µm) | |
| Area (µm²) | Sperm track characteristics |
| Circumference (µm) | |
| Diameter (µm) | |

### 4.11.1 Sperm Kinematic Correlations

ALH shows a significant positive correlation with VCL and VAP ($r = 0.52$, $P < 0.05$; $r = 0.15$, $P > 0.05$, respectively), but is highly positively correlated with DANCE ($r = 0.8$; $P < 0.05$). VCL shows a similar, highly positive correlation with DANCE ($r = 0.89$; $P < 0.0001$). However, the high correlation is biased as ALH and VCL are incorporated into the formula to calculate DANCE and cannot be considered. VCL and VAP show a highly positive correlation ($r = 0.88$, $P < 0.0001$), as the average path is a good representation of what happens with the curvilinear path.



### 4.11.2 Sperm Morphometry Correlations

The sperm morphometry correlations show a highly significant negative correlation among head length, tail and total sperm length ($r = -0.94$, $P < 0.0001$). This demonstrates that as the head length increases, the length of the tail shortens, resulting overall into a shorter total sperm length. In contrast, the positive correlation among the head width, tail length and total length ($r = 0.71$, $P < 0.05$), shows a direct relationship between these morphometric features. Therefore, a sperm with a thinner

head width would have a short tail and a shorter total sperm length. The negative correlations between sperm head length and head width ($r = -0.73$, $P < 0.05$), confirms this inverse relationship as the head length gets longer the sperm width narrows, providing a more streamlined head shape.

### 4.11.3 Sperm Track Correlations

Figure 4.17 presents highly significant positive correlations among the sperm track characteristics, namely area, circumference and diameter across the different species ($r = 0.98$, $P < 0.0001$).

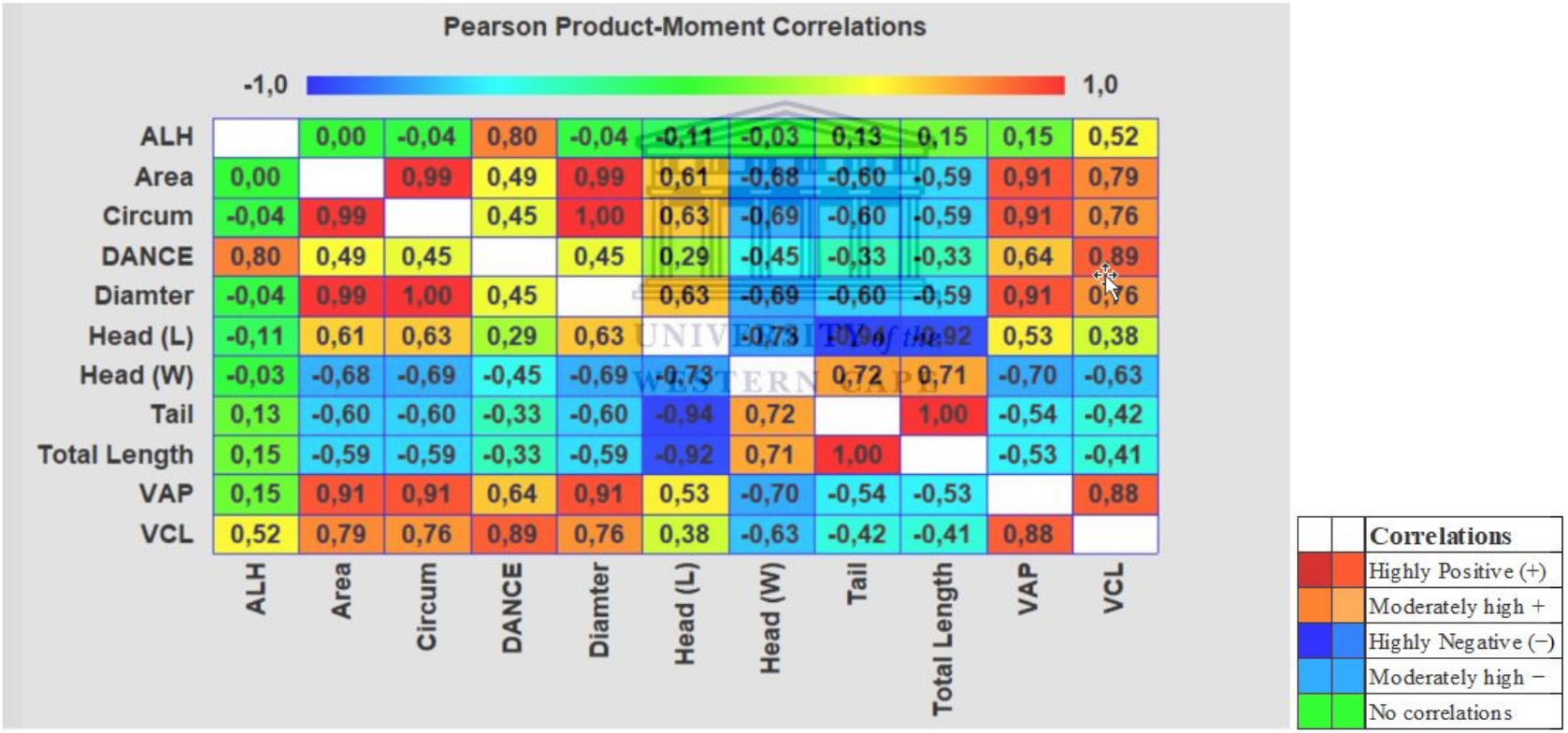



**Figure 4.17:** Correlation coefficient (r) for eleven parameters relating to four sperm kinematic parameters, four sperm morphometry features and three sperm track characteristics, of three species, *P. angulosus*, *C. meridionalis* and *D. serra*. ALH =amplitude of lateral head displacement, VCL= curvilinear velocity, VAP= average path velocity, DANCE = VCL x ALH, Circum = circumference, Head (L) = head length, Head (W) = head width, Tail = tail length, Total length = total sperm length.

These correlations signify that when the sperm swimming track pattern increases its circumference, the diameter across the track would naturally increase the area occupied by the sperm track. The correlation between the sperm kinematic and morphometry analysis shows no significant sperm head length correlation. Nevertheless, sperm head width, tail and total sperm length displayed a significant and highly negative correlation with VCL and VAP ($r = -0.63$, $P < 0.05$). These relationships demonstrate that faster swimming sperm have thinner heads with shorter tails compared to slower swimming sperm with longer tails. While sperm head length did not appear to correlate with the sperm kinematics, it did display a highly significant positive correlation with the sperm track characteristics (area, circumference and diameter) ($r = 0.63$, $P < 0.05$).

As a result, this means that a sperm with a longer head has a sperm track with a wider diameter. Highly positive correlations were also observed between sperm track characteristics and VCL and VAP ($r = 0.76$-$0.91$, $P < 0.05$). In contrast, ALH was not significantly correlated with any of the morphometry and sperm track characteristics. The positive association between sperm speed (VCL and VAP) and sperm track patterns shows that when sperm swim faster the sperm track pattern created increases in terms of diameter and surface area and circumference.



## 4.12 Multivariate Visualisations as a Further Aid to Describe Species Sperm Characteristics

Three multivariate visualizations were used for further species comparisons, namely Andrews plots, Star–sunray plots and Chernoff faces. The aim was to compare and enhance parameters of different species that may amplify small differences. It is

important to mention that all proposed associations between sperm morphometry, kinematic and sperm track traits as mentioned above in Pearson product-moment-correlations coefficient, were confirmed by the multivariate analysis. A combination of ten parameters were used from *P. angulosus*, *C. meridionalis* and *D. serra* swim-up data including sperm morphometric, kinematic and sperm track characteristics as mentioned above, excluding DANCE.

Figure 4.18 presents the Andrews plot, a powerful graphical technique for multivariate data, used for identifying differences and similarities among observed cases when the number of dimensions is too large to use in a scatterplot. Andrew plots are informative as they rely on a mathematical formula, $f_i(t) = \frac{X_{i1}}{\sqrt{2}} + X_{i2}\sin(t) + X_{i3}\cos(t) + X_{i4}\sin(2t) + X_{i5}\cos(2t) + \ldots$ to construct the graph and the parameters are combined to describe the data (Khatree and Naik, 2002). The plot shows some similarities between *P. angulosus*, *C. meridionalis* and *D. serra*, but overall, there are more distinctions between *P. angulosis* and the two mussel species sperm, while *C. meridionalis* and *D. serra* are more closely related.



Star-sunray plots in Figure 4.19, converts the smallest value of each parameter for the three species, to be one tenth of that of the largest parameter. In this ‘distortion’ it amplifies differences as can clearly be seen in Figure 4.19. Star-sunray plots consist of a sequence of spokes with each spoke representing one of the variables. The data length of a spoke is proportional to the magnitude of the variable for the data point relative to the maximum magnitude of the variable across all data points (Chambers *et al*., 1983; Klippel *et al*.,2009). A line is drawn connecting the data values for each

spoke, giving the plot a star-like appearance. The plots display the distinction of *P. angulosus* sperm with the large increase sperm motility and sperm helical swimming tracks in comparison to the *C. meridionalis* sperm.

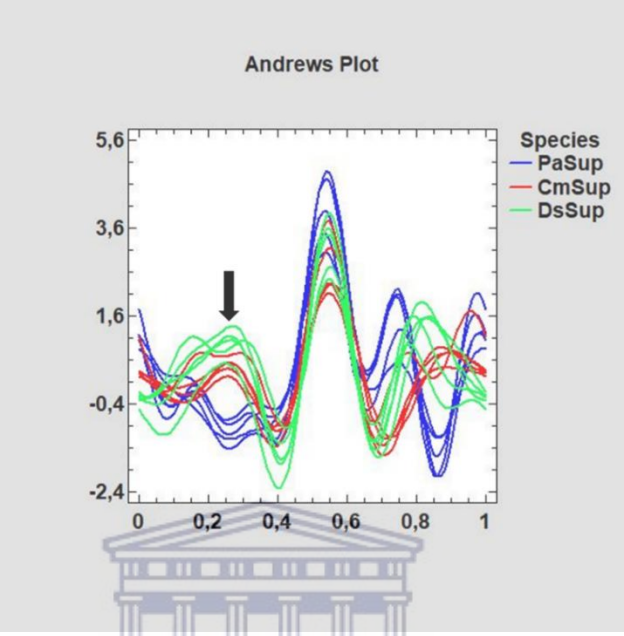



**Figure 4.18:** Andrews plot of ten parameters, including sperm morphometry, motility and sperm track patterns characteristics, of swim-up data of *P. angulosus* (PaSup), *C. meridionalis* (CmSup) and *D. serra* (DsSup). The plot clearly illustrates the distinction of *P. angulosus* from the Mollusca sperm, and a close relationship between the sperm parameters of *C. meridionalis* and *D. serra* (arrow).

Figure 4.20 presents Chernoff faces of the three species, where each facial feature relates to a specific parameter of the ten sperm parameter measured. This visualisation technique constructs cartoon faces in which various features, up to eighteen features, are scaled according to the values of different variables. Figure 4.20 clearly shows that for *P. angulosus* most parameters seem to appear at maximum and the other extreme is *C. meridionalis*, with faces depicting low sperm parameters.

Additionally, these faces exemplify the closeness between *C. meridionalis* and *D. serra*.

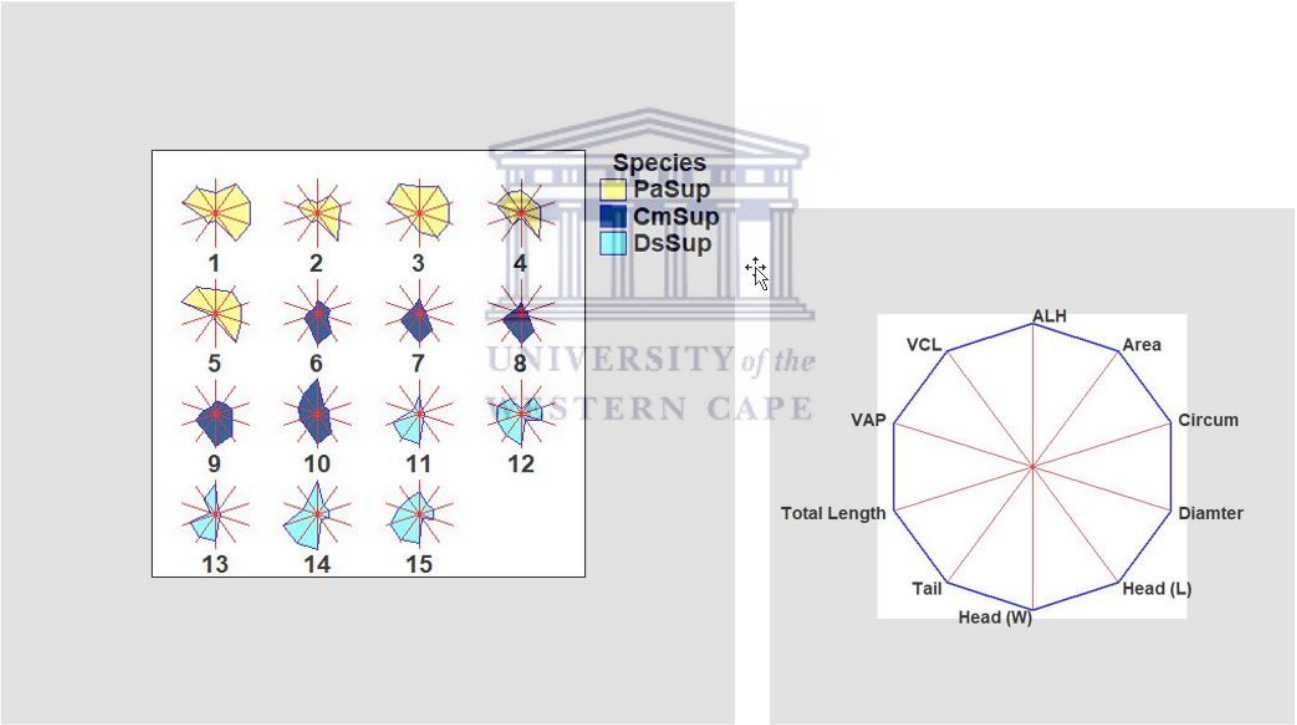



**Figure 4.19:** Star-sunray plots of ten parameters of *P. angulosus*, *C. meridionalis* and *D. serra*. *P. angulosus* plots shows the distinct difference of *P. angulosus* to that of the Mollusca species, with *C. meridionalis* displaying the smallest sperm motility and sperm track effects.

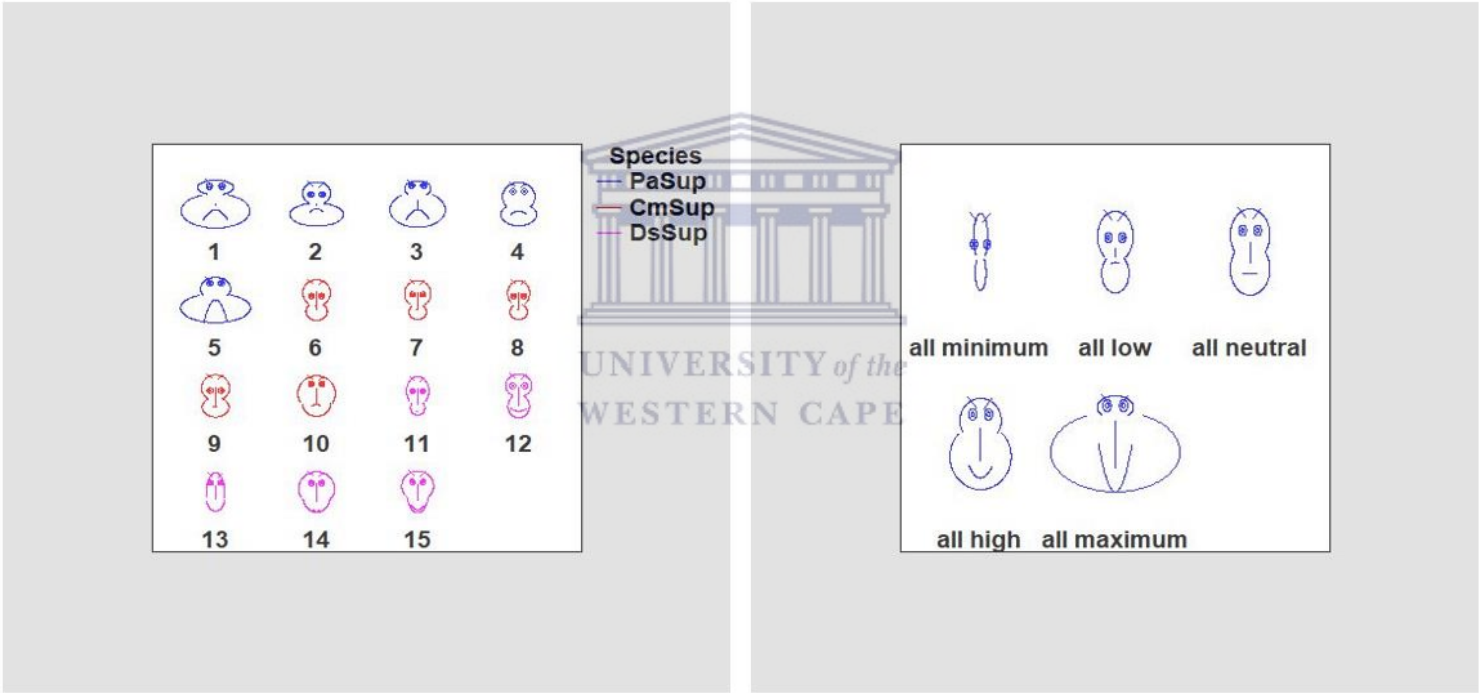





**Figure 4.20:** Chernoff faces showing the variations in ten parameters of the three species *P. angulosus*, *C. meridionalis* and *D. serra*. Cartoon faces for *P. angulosus* show the parameters are at maximum in comparison with the other two species. In contrast the cartoon faces of *C. meridionalis* and *D. serra* show a closer relationship.

# CHAPTER 5

# DISCUSSION AND CONCLUSION

## 5.1 Introduction

One of the major aims of this study was to establish baseline criteria/values for assessing sperm quality in *P. angulosus*, *C. meridionalis*, *C. gigas*, *D. serra* and *H. midae*. Sperm morphology, along with the various sperm traits, plays an important role in determining sperm quality as it relates to sperm functionality, fertilization efficiency and has been correlated with mating systems. The approach was to apply the required technology and develop methodology to quantitatively establish sperm quality in marine broadcast spawners. The assessment of sperm activity by CASA further assist to refine the definition for high quality sperm in a quantitative manner, which relates to the functionality of sperm.



In the study both testicular and spawned sperm traits were evaluated, to assess the possibility of a sperm 'maturation process' along the genital tract. A process important in mammalian sperm and is attained when sperm travel through the epididymis, although many aquatic species do not present with complex organ systems, like an epididymis (Suquet *et al*., 2012) as found in mammals. Obervations of sperm 'maturation processes' was reported in some aquatic species, including scallop and rainbow trout (Morisawa and Morisawa, 1990; Faure *et al.,* 1994). A study by Suquet *et al*. (2012) suggested a sperm 'maturation process' in Pacific oyster sperm, after the

flagellar beat frequency was found to be lower in testes sperm compared to sperm released from the gonopore after intragonadal serotonin injection.

Marine invertebrate spermatozoa typically rotate about their long axis, creating a helix as they swim (Gray, 1955; Jekeli *et al*., 2015). The helical pattern allows spermatozoa to sample the chemoattractant released from the spawned eggs either continuously or intermittently, and permits gradual adjustments to the swimming path (Jekeli *et al*., 2015; Guerrero *et al*., 2011). The helical nature of their sperm swimming patterns may indicate an efficient fertilization strategy, through allowing sperm to collide with eggs within its immediate vicinity (Farley, 2002). Sperm chemotaxis is believed to enhance sperm-egg fusion, but with the rapid in dilution in sea water, sperm must have a high level of sensitivity in order to be guided to the correct egg. The chemoattractant from eggs have the ability to increase both sperm velocity, sperm longevity and the forward progressive motility in external fertilizing species (Turner and Montgomerie, 2002; Shiba *et al*., 2006; Rosengrave *et al*., 2009). Understanding the mechanisms of sperm movement and behaviour in sea water and in egg water, particularly the changes occurring during the motile phase (Fabbrocini and D'Adamo, 2016), will assist to explain the sperm-egg fusion traits and fertilization strategies.



CASA analysis provides an objective and quantitative assessment of sperm, giving information on the motility parameters that are closely depended on the integrity of the sperm morphology and functionality. The velocity parameters are the most widely used in aquatic species (Liu *et al*., 2011). The kinematic parameters are used in combination to describe various facets of the sperm. Curvilinear velocity (VCL) and the amplitude of lateral head displacement (ALH) have been linked to active sperm

motility (Gallego *et al*., 2014). When including straight-line velocity (VSL), for free-spawning species, this combination of parameters is often shown to be possible predictors of sperm finding eggs (Levitan, 2000; Cabrita *et al*., 2014; Fabbrocini *et al*., 2016). Assessing sperm motility in marine invertebrate species is indeed an important factor in sperm-egg finding and fertilization success, as sperm cells need to swim to reach the egg.

## 5.2 Sperm Morphology

All four study species, viz., *P. angulosus*, *C. meridionalis*, *C. gigas* and *D. serra* used for morphology analysis have primitive sperm, also referred to as 'ectaquasperm' when fertilization is purely external or 'entaquasperm' when sperm are taken up almost 'internally' by the females for fertilization (Franżen, 1956; Rouse and Jamieson, 1987). According to Reunov (2005), the Franżen term 'primitive' relates to the sperms typical structure and Rouse and Jamieson terms refers more to the sperms' 'functionality'.



The sperm morphometric features of *P. angulosus*, *C. meridionalis*, *C. gigas*, *D. serra* and *H. midae* have previously been documented (Hodgson and Bernard, 1986; Hodgson *et al*., 1990; Hodgson and Foster, 1992; Reunov and Hodgson, 1994; Healy *et al*., 1998). The sperm taken directly from the gonad in this investigation had an ultrastructure similar to those described in previous studies, displaying similar primitive sperm head shapes with uniquely formed acrosomes and long sperm tails. All four species examined had shown no variation in the structure of the sperm tail, however species–specificity was displayed in the acrosome and mitochondria number and structure within the midpiece, including when these species sperm were exposed to

sea water and egg-water.

## 5.2.1 Echinodermata

### 5.2.1.1 *Parechinus angulosus*

*Echinoida* sperm morphology is well documented with the typical morphology of sea urchin sperm that has been previously described (Afzelius, 1955; Rothschild 1956; Fawett, 1970; Reunov and Hodgson, 1994). Among the study species, *Parechinus angulosus* were chosen as a model species to assess their sperm traits and the application of computerized sperm motility analyses on sperm activated in sea water and egg-water. Dry-spawned *P. angulosus* sperm collected during this study had a conical head, with an intact, apical and spherical acrosome located above the nuclear fossa containing the granular sub-acrosomal material. This conservative morphology had a simple, single and annular mitochondrion in the midpiece. The mitochondrion serves as the primary source of ATP needed for high energy motility, for reaction of the acrosome and for fusion with the egg during fertilization.



It was observed that activated *P. angulosus* sperm in the swim-up sea water and egg-water showed no structural changes. Dan and Wada, (1955) were the first to show structural changes found in the acrosome of sea urchin sperm when exposed to egg-water. Dan coined the term acrosome reaction (AR), describing the action that occurs when the acrosome is stimulated to release enzymes via exocytosis, with the release of the acrosomal process (Dan and Wada,1955; Austin, 1975, Hirohashi and Yanagimachi, 2018).

Although, AR is normally associated with the release of the acrosome process; this

process was not observed in *P. angulosus* sperm exposed to sea water and egg-water. It was suggested that the delicate acrosome process could be destroyed during fixative preparations (Dan and Wada, 1955). Also, the acrosome component of *P. angulosus* sperm is small and it is possible that the acrosome reaction had taken place but the acrosome process may have been too small to be visualized under a microscope.

Through the release of sea urchin sperm in sea water, motility is activated by the uptake of sodium ($Na^+$) and release of hydrogen ($H^+$) ions. (Schlegel *et al*., 2015). This initiates an increase in internal pH leading to the AR and induces mitochondrial activity. In contrast, egg-water contains the species-specific sulphated fucose homopolymers (FSP) found in the egg jelly coats of *P. angulosus* eggs (Hirohashi *et al*., 2002; Vacquier, 2011). Thus, sperm egg interaction in the presence of egg water, that contain species specific chemoattractants, would activate motility through chemotaxis, caused by species specific egg adhesion, acrosome reaction and egg membrane recognition (Suzuki, 1995; Kekäläinen *et al*., 2015). Indicating that egg-water acrosome reaction in *P. angulosus* may have promoted measurable changes in sperm behaviour and sperm motiliy, which could be better demonstrated through CASA sperm motility analysis. The quantitative measurements of the acrosome intactness and reaction could be a measure using some CASA systems and this should be carried out in future studies. This addition could possblity support the sperm motility analysis and verify the maturity of the testicular sperm cells, a tool that could assist with determining the gamete age or maturity in aquaculture.

## 5.2.2 Mollusca

### 5.2.2.1 *Choromytilus meridionalis*

The plasticity of the black mussel shell has caused confusion among the various species of black mussel along the rocky shore line. Since the shells alone are not reliable features for identification (Reunov *et al*., 2018), sperm ultrastructure traits have been considered good indicators to assess taxonomic and phylogenetic differences (Hodgson and Bernard, 1986; Reunov and Hodgson, 1994). Ultrastructural features of the sperm were successfully used to determine the difference between the endemic *C. meridionalis* and the invasive *M. galloprovincialis* that are from the subfamily *Mytilinae* (Hodgson and Bernard, 1986). In this study, ultrastructural features of testicular sperm *C. meridionalis* resembled those found in previous studies (Hodgson and Bernard, 1986). Compared to the long, narrow acrosome found in *M. galloprovincialis* sperm, *C. meridionalis* sperm had a short, thin acrosome. The more electron-dense acrosome of testicular *C. meridionalis* sperm had a bulging base located on the apical region of the spherical nucleus. Centrally the acrosome had a hollow vesicle filled with less electron-dense sub-acrosomal material. In comparison, the *C. meridionalis* swim-up sperm acrosome, did not appear to have two distinct electron-dense regions as described for the testicular sperm. However, acrosome reaction in sea water could not be confirmed in this study. Compared to Dan and Wada (1955) that observed partial acrosome reaction in Mytilus sperm exposed to low temperature sea water or sea water containing large amounts of $Ca^{2+}$.

The midpiece of testicular and swim-up *C. meridionalis* sperm both contained four round mitochondria, however, the swim-up mitochondria appeared more swollen and

some sperm appeared to have damaged mitochondria. The swollen or damaged mitochondria could be a result of the sea water entering the damaged plasma membrane of the sperm.

### 5.2.2.2 *Donax serra*

The morphology of white mussel sperm of testicular origin shared similar traits as described by Healy *et al*., (2008) and is important for all *Donax* species. *D. serra* testicular sperm has a typical primitive sperm structure composed of a spherical nucleus, with an apical acrosome and a midpiece containing four mitochondria. Testicular *D. serra* sperm ultrastructural features are similar to those previously described by Hodgson *et al*. (1990). The annular-shaped acrosome was electron-dense with a central less electron-lucent canal which contained the axial rod.

Although, in this study the axial rod was not clearly visible within the invagination of the unreacted testicular sperm acrosome. In contrast, the active *D. serra* sperm of the swim-up technique showed a less dense granular nucleus and the acrosome had an axial rod partially pushed into the apical region. This configuration created a conical elongated tip on the acrosome containing the homogeneous sub-acrosomal material. This feature was found in other mollusc acrosomes, that exhibited partially reacted acrosomes (Dan 1956; Hirohashi and Yanagimachi, 2018). However, not all sperm have displayed the axial rod lengthening and this reaction appears to be inconclusive.

### 5.2.2.3 *Crassostrea gigas*

Oyster sperm extracted directly from the testis, had morphological features that were almost identical to those previously described for this species by other authors

(Komaru *et al*., 1994; Drozdov *et al*., 2009; Reunov *et al*., 2018). *C. gigas* testicular sperm extracted in this study had a smaller peri-acrosomal space, with the posterior region of the nucleus had a flat bottom, in comparison to the *C. gigas* species grown in Taiwan and Vostok Bay in the Sea of Japan, where the posterior region of the nucleus was invaginated creating a fossa (Gwo *et al*., 1996; Drozdov *et al*., 2009). However, in this investigation oyster sperm resembled the sperm of the diploid *C. gigas* cultivated in Washington and Gokasho Bay, Japan (Komaru *et al*., 1994; Dong *et al*., 2005).

The sperm head length, mitochondria and acrosome structure of this study corresponded to the morphometric measurements of the diploid *C. gigas*. A difference in sperm width and length of the flagellum was observed. The Saldanha bay oyster sperm was narrower (1.8 µm ± 0.04 SD), compared to the diploid oyster sperm head width ranging between 2.29-2.32 µm (Komaru *et al*., 1994; Dong *et al*., 2005). The flagellum length of the diploid sperm was longer and varied from 34.2-41.1 µm (Komaru *et al*., 1994; Dong *et al*., 2005), compared to the 28.98 ±1.48 µm length recorded in the present study. However, Dong *et al*. (2005), indicated that the length of the live diploid sperm measured 37.3 µm compared to the fixed sperm flagellum length of 41.1 µm. The difficulty to measure sperm endpiece using light microscopy in the current study, could result in shorter measurements. The close proximity of the oyster sperm dimensions to that of the diploid oyster sperm could possibly be as a result of oyster spat being imported from overseas hatcheries for cultivation in South African waters (Britz and Venter, 2016). An ultrastructure study of *C. gigas* of four locations were found to have intraspecific variations in the sperm features based on

the habitat and it was suggested that environmental factors could also influence sperm morphology (Pitnick *et al*., 2009; Reunov *et al*., 2018).

### 5.2.3 Summary of Sperm Morphology of the Species Described in this Investigation

Free-spawning sperm experience two environments, while being produced they were stored within the father, paternal, and after being released they were immediately exposed to the aquatic environment (Marshall, 2015). Marshall (2015), also suggested that both these environments could generate intraspecific variation in the phenotype of sperm, that could possibly affect the offspring (Bonduriansky and Day, 2009). Although, the number of species were too few for any phenotypic assessments for this study. It could possibly be that for these species, no distinctive sperm morphological trait differences could suggest no visual intraspecific deformities or variation was observed between sperm of the two environments i.e., testicular sperm and sperm activated in sea water during swim-up.



Morphological studies would remain an important and cost-effective tool, when using light microscopy techniques, to ensure the integrity of the sperm form, which aids to distinguish sperm quality and sperm functionality. A reliable and standardized method for differential staining of marine invertebrate sperm would be beneficial for automated analysis morphometric studies for furure studies. As Gage *et al*. (2002) indicated that before understanding the adaptive variation in sperm morphometry, it would be important to determine how much of the variation in sperm morphometry would translate into sperm motility. The relationships between sperm form and function may be able to give insight into sperm competition, sperm limitation (Humphries *et al*.,

2008) within the various fertilization environments.

## 5.3 Sperm Motility

Sperm motility parameters are often used to determine the quality of sperm and generally this parameter usually has a positive correlation with fertilization success (Kime *et al*., 2001). Moreover, a number of sperm traits have been interconnected with sperm quality in relation to effective fertilization through optimal sperm motility (Snook, 2005; Levitan, 2018). Such common assessed sperm traits include sperm velocity and the population of progressively swimming sperm, the integrity of sperm components such as plasma membrane, flagellum, mitochondria and acrosome-intact sperm, chemotactic ability, as well as proper capacitation of sperm (Lewis and Ford, 2012; Riffell *et al*., 2004).



Since the sperm morphology of species is different, often related to their reproductive traits and fertilization strategies, this indicates that sperm motility among species may also be different and species specific (Gallego *et al*., 2014; Fabbrocini and D'Adamo, 2017). Aquatic broadcast spawners fertilize under various environmental conditions, from quiet shallow intertidal pools to wave-crashing rocks, indicating the possible adaption of certain sperm traits within each unique habitat (Levitan, 1998). Although, it has been suggested that species with similar reproductive strategies might share similar sperm traits, whereas those with radically different reproductive strategies might have sperm morphological and sperm kinematic differences (Gallego *et al*., 2014).

CASA was used in this study to make a distinction and determine similarities between

the various species-specific sperm traits such as the sperm kinematics, sperm concentration and sperm behaviour through the motility patterns of these broadcast spawners. This baseline study aims to explore the sperm traits that could be related to sperm quality and sperm functionality. Although, all sperm traits or kinematic parameters cannot be used, it provides a good background that one may rework and reinvestigate in the future. The developments in the applications of computerized sperm motility analyses to aquatic species have provided an objective assessment of sperm activity and is an easy tool to evaluate motility quantitatively. (Kime *et al*., 2001; Cosson, 2004; Fabbrocini *et al*., 2010).

### 5.3.1 *Parechinus angulosus*

In the present study, the sea urchin model was used to evaluate sperm quality using the flush technique where sperm was used 'dry' immediately after spawning and after being stored at 4°C for 24 hrs, swim-up measurements after activation over time and sperm exposed to eggs and egg water. Total motility and sperm kinematic evaluations recorded for sea urchin sperm fall within the ranges observed in previous studies. Nevertheless, values appear to vary among the species for example a total motility of 90% was observed in *P. lividus* sperm after being active for up to 24 hrs and *A. crassispina* reached total motility of approximately 80% after 2 min activation (Gallego *et al*., 2014; Fabbrocini *et al*., 2016). The latter value compared well to 70% total motility observed in freshly dry-spawned sperm after being active for 2 min. Investigators have indicated that dry-spawned sperm stored at high concentrations and at low temperatures slows down the metabolic activity of the sperm (Levitan 2000; Vacquier, 2011).

After dry-spawned sperm was stored for 24 hrs, the rapid progressive motility subpopulation was at 40% compared to the same subpopulation in freshly spawned sperm at 47%. Furthermore, all sperm kinematics had decreased after 24 hrs compared to the freshly dry spawned sperm. The mean VCL at 24 hrs (177.7µm/s), was still high in comparison to the freshly spawned sperm swimming at 192.6 µm/s. These results indicate the ability of *P. angulosus* sperm to maintain sperm cell integrity after 24 hrs storage at 4°C, including a total motility of over 50% and sperm with high swimming speeds.

The activation of sperm over 60 min using the swim-up technique shows the steady decrease in sperm motility and sperm kinematic parameters. Many investigators rely on mean values alone, however, it has been recommended that sperm subpopulations should rather be evaluated as subtle but important changes can be observed in such subpopulations (Abaigar *et al*., 1999; Martinez-Pastor *et al*., 2005; Fabbrocini and D'Adamo, 2017; van der Horst *et al* 2018). In this study, the subtle changes observed in the subpopulations confirm the importance of these evaluations. The initial swim-up measurement at 5 min showed a 78% total motile sperm population of which 26% displayed a progressive swimming pattern and 57% of this sperm subpopulation displayed rapid progressive motility. High VCL, VSL and VAP values corresponded with the rapid sperm subpopulation found. While the rapid sperm subpopulation decreased to 39.4% after sperm was active for 60 min, the progressively motile sperm increased to 35.7% compared to the first lower reading.

These results observed confirm the importance of including subpopulations in semen evaluation as mean values alone can be overly simplified. A previous study evaluating

the sperm motility of sea urchin sperm after being active for 24 hrs, considered changes to subpopulation values as a result of a decrease in VCL and deduced sublethal alterations in sperm physiology occurring in the period after activation (Fabbrocini and D'Adamo, 2017).

The decrease in sperm motility after activation has been studied in many aquatic species and the increase in energy expenditure leading to the exhaustion of energy reserves has been considered (Cosson, 2010; Suquet *et al*., 2010). Levitan (1991) explained that, dry-sperm or undiluted sperm retained viability for many hours whereas diluted sperm age rapidly. Rahman *et al*., (2001) confirmed that sperm in dense suspensions remain inactive as in the testis, but upon dilution the sperm become motile and are rapidly exhausted. This exhaustion stems from the fact that sperm respiration increases with increased energy expenditure when sperm become more active when diluted (Chia and Bickell, 1983). The limited longevity of diluted sperm has been shown to decrease in fertilization success (Rahman *et al*., 2001).



A field study by Pennington (1985) demonstrated a reduction in fertilization rates after male and female *S. droebachiensis* spawned a few meters away in comparison to individuals spawning in close proximity. *P. angulosus* egg-water was an excellent activator of sperm motility, displaying an increase in the total motility population up to 86% and a rapid progressive subpopulation of 63.8% compared to that of the swim-up procedure. However, VCL, VSL and VAP were all lower compared to measurements taken from the dry-spawned and activated swim-up sperm.

Chemotactic substances have been well documented in some marine invertebrates.

These substances are species-specific, with certain peptides assisting to stimulate motility and respiration levels needed for facilitating fertilization (Suarez and Ho, 2003). The chemotactic intracellular increase of $Ca^{2+}$ demonstrated in AR increases the flagellar bend asymmetry and swimming path curvature (Suarez and Ho, 2003; Gunaratne *et al*., 2006; Vacquier, 2011). The decrease in motility efficiency of sperm in egg-water over time may be the result of damage of the plasma membrane that decreases cell integrity, along with the exhaustion of ATP reserves (Suquet *et al.*, 2010) and the possible induction of hyperactivation. Hyperactivation of sperm is associated with vigorous motility (Yanagimachi, 1969), including rapid speed and whiplash tail movements (Mortimer *et al*., 2015), that signals sperm capacitation and ultimately acrosome reaction (van der Horst and du Plessis, 2017).

Other investigators suggest that an increase in BCF results in an increase in VCL (Boryshpolets *et al*., 2018). This dual increase was confirmed in the swim-up sperm with VCL and BCF (221.6 µm/s and 12.2 Hz, respectively). In contrast, a decrease was observed in the egg-water sperm VCL (140.5 µm/s) and BCF (9.9 Hz), suggesting alterations in the flagellar beat ability after exposure to the chemotactic substances. This result could align with damage to the plasma membrane and the energy expenditure of the mitochondria which is the source of energy for flagellar movement. The speed difference seen in swim-up and egg-water sperm could relate to the egg search strategy of broadcast spawners. Sperm released in sea water alone displays higher sperm velocity and is possibly related to intraspecific sperm competition, as several studies have indicated that the faster swimmers are more likely to collide with eggs (Rothchild and Swan 1951; Levitan *et al*., 1991; Levitan 1993). The pattern of

sperm movement changed once exposed to egg-water and sperm started to show hyperactivation patterns.

### 5.3.2 Mollusca

The motility of the testicular sperm of the three bivalves, *C. meridionalis*, *C. gigas* and *D. serra* was studied and compared to the motility of the spawned sperm of *H. midae*. The post-activation motility of the three bivalve species sperm was studied using the swim-up technique. Even though the Mollusca species have been used to study environmental contaminant exposure and toxicology assays; only few baseline Mollusca studies used CASA to understand their basic sperm motility patterns, except for the publication that emanated from this current study (van der Horst *et al*., 2018).

No major differences were found between the motility of spawned and testicular *C. meridionalis* sperm, except for a delay in the activation time, of testicular sperm and the fact that spawned sperm was active for longer than 60 min. The sperm 'maturation process' was first described in mammalian sperm, but this phenomenon was also reported in seabass, turbot and in Pacific oyster (Faure *et al*., 1994). These investigators reported a lower percentage of motile sperm from the testis in comparison to sperm from the gonopores, suggesting a sperm 'maturation process' along the genital tract of the fish species and Pacific oyster (Suquet *et al*., 2012). Another study on the spermcasting Australian flat oyster, *Ostrea angasi*, reported an initial low flagellar beating while sperm are attached to a spermatozeugmata and an increase of flagellar beating once individual sperm break-free from the group (Hassan *et al*., 2016). In the present study, the low sperm motility and the sperm 'clusters' found in testicular sperm of *C. meridionalis* could suggest a form of sperm 'maturation

process' required, as described for the fish and Pacific oyster sperm. Unfortunately, no information is available on the fertilization biology of *C. meridionalis*.

*C. meridionalis* inhabit a high-energy interface environment with large wave action and very high population densities. It is difficult to consider how gametes will be released and fertilized through the typical broadcast mode in a turbulent environment, even during synchronised spawning, as sperm dilute rapidly within a meter from the sperm source (Pennington 1985; Denny 1988; Denny and Shibata 1989; Yund 1990; Levitan 1991). This could imply that sperm may be deposited much closer to the eggs or even that sperm may be siphoned into the mantle cavity to assist with protected fertilization, as found in spermcasting?

Testicular sperm motility and kinematic results for *C. gigas* also showed no major differences between freshly spawned sperm and sperm stored at 4°C for 24 hrs. In contrast, *D. serra* sperm had no motile sperm after being stored. This could indicate that *D. serra* sperm have a short period for active sperm motility once removed from the testicular tissue. However, *D. serra* fresh sperm remained active for at least 60 min during swim-up. The duration of sperm motility varies within the aquatic species, with fish having some of the shortest-lived sperm ranging from seconds to minutes (Sansone *et al*., 2001).

After being active in sea water for a period of 60 min, species-specific sperm kinematic traits were found among *C. meridionalis*, *H. midae*, *C. gigas* and *D. serra*. Overall, these species exhibited a decrease in sperm activity over the 60 min activation period. Similar to the finding in *P. angulosus*, sperm dilution resulted in a decrease in sperm

viability. In contrast, the spawning *H. midae* exhibited a steady increase with up to 95% total motility with a rapid progression subpopuation of 64% and VCL and VAP also increasing after 60 min.

Although *H. midae* had the slowest sperm after the initial introduction to sea water, the increase in this animal's sperm velocities after 60 min was still lower than the initial values observed for the other species after initial activation. What cannot be ascertained is if the increase in *H. midae* sperm speed was created from the initial sperm which finally were fully activated or if newly released sperm were more active once in the presence of the sperm pool. This could possibly be related to sperm longevity and sperm competition. The increased swimming speeds of *H. midae* sperm after 60mins activation, noticed in this study, could be the effect of sperm competition among the sperm searching for gametes (Lymbery *et al*., 2017). However, Levitan (2000) suggested that fast swimming sperm are beneficial under conditions of sperm competition, while long-lived sperm are advantageous under conditions of sperm limitation, which is a likely suggestion for these benthic animals.



Information about the wave velocity and the corresponding frequency of the beating flagella has been directly related to the concentration of ATP within the flagellum (Gibbons and Gibbons, 1972). As the beat frequency increases so would the VCL of the active sperm (Boryshpolets *et al*., 2018), as well as the ALH, which is related to the level of bending in the proximal region of the tail (Su, *et al*., 2012). Taken together, these factors further assist to describe how motile sperm progress along their helical tracks (Fabbrocini and D'Amdamo, 2017). In this study some similar observations were found. There was an increase in both ALH and BCF and a slow decrease in VCL

over the 60 min post-activation time of swim-up sperm for all Mollusca, except in *C. meridionalis* where ALH and BCF decreased over the 60 min. *D. serra* stood out among the Mollusca species, with a high average VCL, of 231.4µm/s and BCF at 14.16Hz. This species also has the highest flagellum-to-head ratio, namely 15X, of the Mollusca species studied. *D. serra* are burrowing bivalves found on the wave exposed sandy beaches, the sperm morphology and motility characteristics could aid in the fertility strategy of the *D. serra*. The high speed and high BCF of *D. serra*, could demonstrate a possible whip-like action of the flagellum needed in that high-energy environment for successful tracking of an egg. A study by Fitzpatrick *et al*., (2017) found a high correlation between sperm flagellum and velocity within conspecific sea urchin sperm ejaculates. Other investigators have also shown flagellum length linked to faster velocities and shorter swimming periods (Levitan, 2000; Gallego *et al*., 2014). These results suggest that the possibility for producing faster swimming sperm would require the evolutionary adaptation of longer sperm (Fitzpatrick *et al*., 2017). This could be a possible sperm trait required by *D. serra* for the fertilization environment it inhabits.



## 5.4 Helical Swimming Pattern

The helical swimming pattern of sperm was a distinctive feature created once activated in sea water without the presence of chemoattractants. The testicular and swim-up sperm, responded with a similar helical pattern and variations could be seen in the size, shape and speed created by the helical track of individual species sperm (Fig. 5.1). The CASA helical sperm tracks found in this study were similar to those described in other studies on various external fertilizers (Levitan, 1993, 1995, 1998; Au *et al*., 2002; Farley, 2002; Rifell and Zimmer, 2007; Fitzpatrick *et al*., 2008; Liu *et*

*al*., 2011; Suquet *et al*., 2013; Fabbrocini *et al*., 2016). However, there have been only a few detailed studies on the nature of these patterns (Liu *et al*., 2011 and Vogel *et al*., 1982). Sperm velocity as a vital sperm trait has been the focus area in many studies without considering the sperm track created (Liu *et al*., 2011).

Farley (2002) indicated the importance of accounting for the helical motion created by the sperm, because without it there would be a higher possibility of inflating the velocity at which a sperm approaches an egg. This stems from the fact that the helical swimming pattern is used as a location method to collide with eggs and thereby assist with the fertilization approach. As the helical swimming pattern provides sperm with a faster way of colliding with eggs than sperm swimming in a straight or random pattern (Farley, 2002). Helical swimming sperm may ave a better chance of colliding with eggs that were within a 100-µm distance than straight swimming sperm? (Farley,2002).



The CASA evaluations for *H. midae* showed a lower sperm concentration and lower helical speed of all the study species, this could be related to better fertilization rates for this species. Based on the study by Roux *et al*., (2014) they found that the optimal fertilization and hatch rates of embryos of *H. midae* (>90%) were achieved when the sperm concentrations were between 5 x $10^3$/ml and 5 x $10^4$/ml and the egg density is approximately 50/ml. When sperm concentration increased to 5 x $10^5$/ml, embryo hatch rates decreased to <70% (Roux *et al*., 2014).

In this study the exposure of *P. angulosus* to egg-water showed a decreased speed and the typical helical track pattern changed to an uneven helical pattern, with the minority of sperm displaying hyperactivation patterns as seen in previous studies.

Helical motion is also believed to play a subsequent role in chemotaxis, whereby the difference in the concentration of sperm attractant on either side of the path creates a periodical signal used for orientation towards the source (Jikeli *et al*., 2015).

Certain spermcastors have relied on chemotactic swimming responses, as described for a species of hydroid, whereby sperm movement as plotted by non-computerised analysis of cine or multiple-exposure photomicrography. It was noted in Miller's (1973) studies that, when placed on a microscopic slide, hydroid sperm moved in a circular motion. This observation suggesting that, similar to broadcasters, a helical motion would occur in deeper water, because helical three-dimensional motion becomes circular in sperm constrained by a flat surface (Guerrero *et al*., 2011). The plausibility of a comparison by CASA of the characteristics of helical swimming patterns among broadcasters and spermcasters may prove informative in relation to their adaptation for their different mechanisms of achieving fertilization. Farley (2002) found helical swimming in general also accounts for higher fertilization rates in sea urchins. Levitan (2000) suggests that sperm traits mirror the interspecific differences in egg traits, Therefore the helical swimming patterns should be considered a focus area along with the sperm speed (Farley, 2002).



The present study found variations in the progressive helical swimming patterns of the study species, with a species-specific helical pattern of swimming possibly connected to the external fertilization strategy and species gamete traits. The helical track pattern, excluding diameter and kinematic differences, were the same for *H. midae*, *D. serra* and *P. angulosus*. The distinctive sperm swimming behaviour observed in *C. meridionalis* shows the spawned sperm swimming in a helical pattern, while gravid

testicular sperm displayed mostly straight-line progressive tracks suggesting species-specificity and a possible swimming pattern adaptation. Overall, *P. angulosus* had the largest helical diameter (30-70 µm) and *C. gigas* had the smallest diameter (9-15 µm) with a characteristic species-specific serrated helical swimming pattern.

However, *C. gigas* and *C. meridionalis* demonstrated a change in sperm swimming behaviour after being active for 60 min in sea water, as the initial fast helical sperm track patterns changed to slower, straight-line progressive swimming tracks. This change in sperm track pattern demonstrates the plausibility of sperm aging and slowing down after being active and diluted in sea water for an extended period of time. As broadcast spawners such as *P. angulosus*, displayed a helix diameter that positively correlated with a high VCL and VAP compared to the smaller diameter helix with a lower VCL and VAP. The change in swimming patterns could also be described by the “Don Giovanni” and “Don Ottavio” patterns, developed by Vogel *et al.*, (1982). They recognised the circular swimming patterns (Don Giovanni) may “kiss” many eggs before attachment whereas straight line swimming (Don Ottavio) may have a smaller chance to attach to many eggs but perhaps adhere to one. The differences found in sperm motility and kinematics among the species can thus be related to both the form and dimensions of the helical tracks, and the specific structure of the individual sperm.



It has been suggested that these patterns are an expression of the fertilization environment or it might be shaped by natural selection and/or it could assist to understand the reproductive strategies of external fertilizers. The considerable spread of variation in diameters of the helical paths presented among these broadcast species may presumably reflect differences among species in the exact

circumstances of spawning, chemoattraction and fertilization, although interpretation of the data in this way must await additional information.

## 5.5 Correlations Among Selected Morphometric Parameters, Kinematics and Quantitative Characteristics of Helical Swimming Patterns

Since the focus of the current investigation was also to determine the relationship among the sperm morphometry, sperm motility and helical swimming patterns of some broadcast spawners correlations and multivariate analysis were used to assist with exploring the finer details of these parameters. All differences and similarities found within the correlation analysis were supported by the multivariate analysis. The present study revealed significant correlations within the selected group of broadcast spawners of this study.



The findings show that head shape plays a significant role upon sperm hydrodynamics and how it could relate within the fertilization environment. This is based on the fact that sperm morphometry and kinematic correlations included the quantitative measurements of the helical tracks formed.

To date, no investigations of the relationships among CASA sperm morphometry, sperm motility and sperm swimming patterns were found, however, more studies on the correlations between sperm morphometry and sperm kinematics have been executed across taxa, including mammals, fish and marine broadcast spawners (Malo *et al*., 2006; Manier and Palumbi, 2008; Tuset *et al*., 2008; Fitzpatrick *et al*., 2010; Rezagholizadeh *et al*., 2015).

Previous studies have suggested that, in general, longer sperm with a longer midpiece benefit from higher flagellar thrust as larger amounts of energy are provided by the mitochondria present (Gomendio and Roldan, 1991; Cardullo and Baltz, 1991). However, sperm head morphology should also be considered as the thrust generated by the flagellum is opposed by the drag produced by the shape of the sperm head (Humphries *et al*., 2008). Most studies have indicated positive associations between the flagellar:head ratio and sperm swimming speed, especially among bird species (Humphries *et al*., 2008; Simpson *et al*., 2013).

In contrast, this investigation found a negative association between sperm head length and head width and a significant negative relationship among the sperm head width, tail and total sperm length and sperm speed (VCL and VAP). A study by Cramer *et al*. (2015) found similar results in Passer sparrows, which showed a negative correlation between flagellum:head ratio and sperm velocity.



Another investigation by Malo *et al*. (2006) suggested that sperm with shorter midpieces and elongated heads swim faster. The significant relationships in this investigation highlight the faster sperm as having a thinner head and shorter tail. This finding indicates that individually thinner sperm swim faster in comparison to fatter sperm, as portrayed in this study with the model *P. angulosus* compared to the primitive sperm with a more spherical structure as shown for *C. gigas*, *C. meridionalis* and *D. serra* in Figure 5.1.

The quantitative sperm tract features add another remarkable aspect in having a negative association with sperm morphology (head width, tail and total length) but has

a strong positively association with sperm speed (VCL and VAP). This finding suggests that a decrease in certain sperm morphometry parameters results in sperm swimming faster, creating a bigger area including diameter and circumference of the swimming track. *P. angulosus* is an excellent model and have very fast swimming sperm by broadcast spawner standards.

The super effective spear-shaped head with one mitochondrion is sufficient to push and with a short tail to cause a forward thrust of the whole sperm. A recent study has suggested that sperm with shorter midpieces may swim faster (Malo *et al*., 2006). Therefore, the one mitochondrion of *P. angulosus* could potentially make for a lighter sperm that provides enough energy to impinge on motility in order to move the whole sperm rapidly compared to sperm with a wider head and longer tail.

## 5.6 Conclusion on Sperm Morphology and Sperm Motility of Five Selected Broadcast Spawners and the Unanswered Question of the Role of Spermcasting



This study provides previously unknown information on the correlations among sperm traits, such as sperm morphology, sperm kinematics and the helical swimming track of selected species of marine invertebrates. This investigation found that a narrower sperm head with a shorter tail would be more hydrodynamically efficient by reducing drag for faster swimming. A narrower faster swimming sperm could also be more effective in penetrating the egg after the acrosome reaction.

Moreover, in this study it was determined that when sperm swim faster a larger helical pattern is formed, allowing sperm to cover a larger area to possibly be successful in finding an egg. The helical swimming pattern is evident of a good search strategy in

quickly locating eggs in comparison to straight-line swimming (Vogel *et al*., 1987). Swimming along helical paths seem to be a successful strategy during chemotaxis and it was proposed that this waveform is associated with $Ca^{2+}$ signaling and it determines the differences in swimming patterns (Alverez *et al*., 2014).

It was also suggested that longer sperm rapidly reach maximum speed at the start of motility and then rapidly decrease, whereas short sperm reach similar initial speeds and do not decline as rapidly (Tuset *et al*., 2008). Hence, our results are in agreement with the suggestion by Tuset *et al*., (2008) of sperm longevity among short sperm, as well as Malo *et al*. (2006) indicated that shorter midpieces provide sufficient energy for faster swimming sperm. Longevity of sperm motility is a good indictor of sperm functionality and could also be related to the morphological integrity of the sperm and thus a good indicator of sperm quality.

In the present investigation *D. serra* had the longest sperm among the Mollusca and on average had fast swimming sperm however, sperm longevity was short lived as the sperm were not motile after 24 hrs. This is in agreement with the theory that a trade-off is likely to be found between swimming fast and swimming for a long period as both require energy (Levitan, 2000). It does have an advantage during sperm competition in synchronous spawning species and possibliy in a high energy, wave action fertilization environments, where *D. serra* are located.

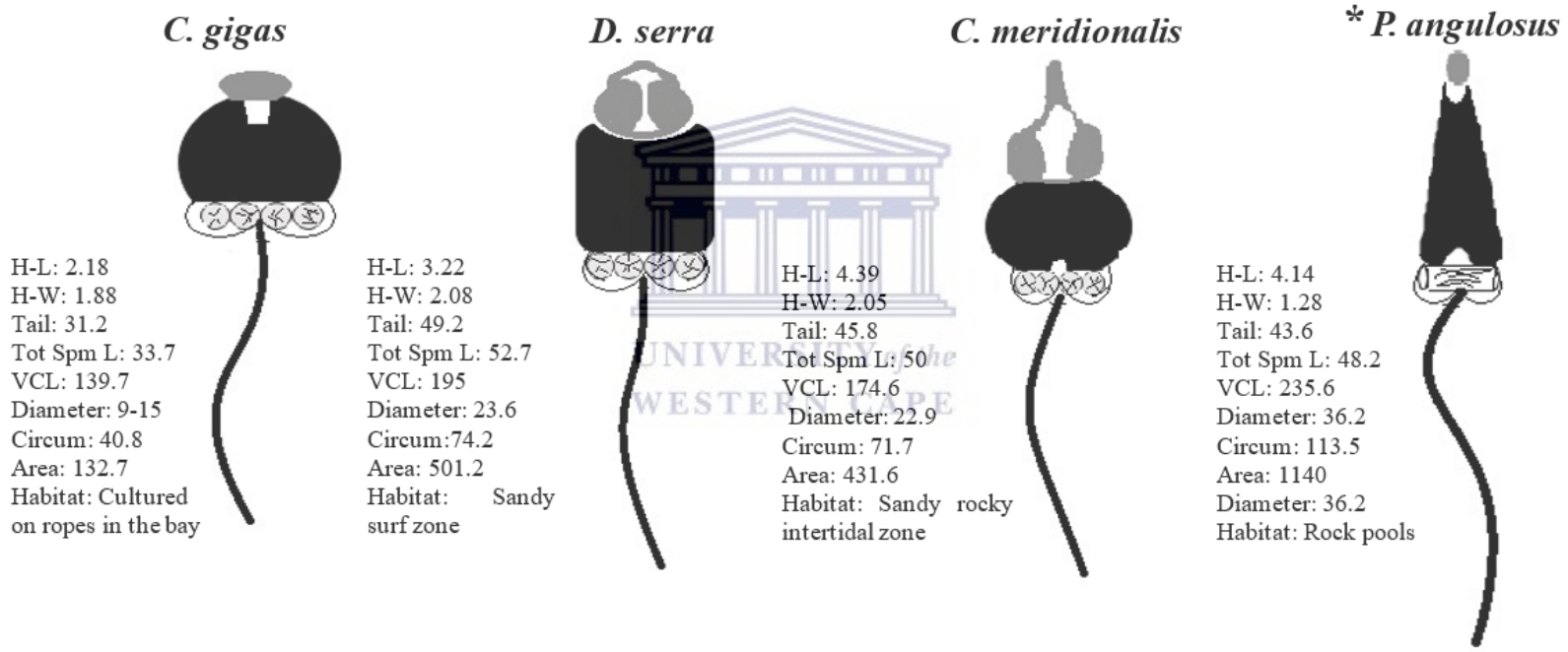



**Figure 5.1:** Summary of the sperm morphometric, sperm motility and sperm swimming track characteristics of the four study species. The *P. angulosus* sperm design relates to the Pearson correlation coefficient data by displaying faster sperm with the thinnest head with and shortest tail as shown in the diagram above (asterisks), in comparison to sperm with broader head widths, longer sperm tails and total sperm length. Area = $\mu m^2$; Circum = circumference, µm; Diameter = µm; H-L = head length, µm; H-W = head width, µm; Tail = tail length, µm; Tot Spm L = total sperm length, µm; VCL = curvilinear velocity, µm. *H. midae* not used in this comparison because of the lack of all the parameters and TEM indicated for the other species.

The optimal combination of sperm swimming speed and sperm endurance may influenece the chances of fertilization potential (Levitan, 2000). *Parechinus angulosus* was the only species among the study group that had sperm swimming at high speeds (VCL) after being incubated dry in a fridge for 24hrs. They also had a distinct subpopulation of rapidly swimming sperm and with individual sperm swimming faster (van der Horst, *et al*., 2018). Upon introduction to sea water there was a total sperm motility population of 70% (±12.1) with a rapid motile subpopulation of 48% (± 24.9). After 24hrs up to 60% (±17.3 SD) of the total population was motile with a slight decrease in the rapidly motile subpopulation of 40% (±24.7 SD). The results show that the total motile sperm of *P. anugulosus* only slightly decreased after 24hrs which shows the longevity of sperm of this species and is in agreement with other studies on the sea urchin, *P. lividus* (Fabbrocini and D'Adamo, 2017). These findings improve our basic understanding of *P. anugulosus* sperm behaviour which possibly relates to spawning dynamics within the spawning habitat. The relationship between speed and longevity may be related to the distance sperm have to travel under contant low energy wave motion within a spawning intertidal pool. After being spawned sperm are motile for longer which may increase the chances of colliding into available unfertilized eggs that could have drifted further from the spawning area. These sperm traits can be related to the sperm quality, sperm functionality and increases the fertilization potential. It is with great interest that, from the large body of information which has been sampled, this dissertation elucidates the immense contribution CASA has made to being able to correlate so many of these sperm traits and that these associations make biological sense.

Swimming patterns appear to be adapted to spawning habitat. In a sedentary intertidal pool, where sea urchins typical breed, one would assume that the release of gametes would perhaps take place during low tide, as the wave action is vigorous in those pools during high tide. In contrast, oysters inhabit a high-energy wave action zone, where their sperm swimming pattern change from a serrated helical track to a more forward progressive movement soon after sea water introduction.

The general reproductive patterns for the marine invertebrates studied have been indicated as broadcast spawning; however, based on previous spermcasting studies, this study suggests the possibility of *C. meridionalis* having spermcasting tendencies.

Three studies on bivalve filter feeders, including freshwater mussel (*Margaritifera margaritifera*), marine invertebrate mussels (*Nutricola confusa* and *N. tantilla*) and oysters (*Ostera angasi*), have shown spermcasting behaviour (Bauer, 1987; Falese *et al*., 2011; Hassan *et al*., 2016). Spermcast spawning patterns in aquatic invertebrates rely on the male to broadcast sperm which is carried by the water, where after the female would siphon the sperm into the female mantle cavity for fertilization (Bishop, 1998; Bishop and Pemberton, 2006; Hassan *et al*., 2016; van der Horst *et al*., 2018).

The success of the spermcasting process relies on several factors, such as the population density of the animals, a high concentration of sperm ejected and the formation of spermatozeugmata (Fretter, 1953). A spermatozeugmata is a non-encapsulated cluster of sperm, moving together as a group with sperm heads attached and tails extended (Falese *et al*., 2011; Hassan *et al*., 2016). In this study it was

observed that black mussel testicular sperm was extremely viscous and when used for flush analyses they formed 'sperm clusters' attached by the head with beating tails, similar to that described by spermcasting investigators. These previous studies indicated the dissociation of the gelatinous substance or mucous connecting the sperm once activated in sea water (Falese *et al*., 2011; Hassan *et al*., 2016). This mucous dissociation could be related to the slow activation time that was observed in this study when black mussel sperm was introduced to sea water. Their activation time ranged from 2-7 min before sperm were activated and released from the 'sperm cluster'. The reproductive strategy of other spermcast spawners allows one to consider *C. meridionalis* sperm displaying similar behaviours.

Additionally, it is difficult to imagine how gametes of broadcast spawners can be spawned for fertilization to take place in high-energy wave action zones, even when living in a high-density population, like *C. meridionalis*. To date no information concerning their fertilization biology is available. Based on this studies CASA sperm motility analysis and the sperm swimming patterns we would like to consider the possibility of spermcasting for *C. meridionalis*. *Choromytilus meridionalis* sperm create swimming patterns where they modulate between small helical sperm tracks, with a narrow diameter and a more forward progressing sperm track fairly soon after being diluted with sea water. This might imply that sperm may be deposited closer to the eggs or even that sperm are possibily taken into the mantle cavity to allow for fertilization. The possibility of another fertilization strategy for bivalves in high-energy zones may be difficulty to conceive but it should be considered.

Furthermore, this study adds meaningful insight into the sperm biology, sperm motility and helical track patterns of marine invertebrates along the West coast of southern Africa. Combined CASA data can be used to further test the correlation between the aspects of reproductive biology for novel methods of fertilization strategy by marine external fertilizers.

All the CASA parameters put together tells us about sperm functionality. It is not just the sperm concentration, percentage sperm motility and sperm kinematics that aids with sperm functionality but it includes sperm swimming patterns. In all species in this study, it was found they swim in very distinctive patterns that seems to be standard. In this context much work is needed to develop mathematical models that will use various sperm parameters and sperm functionality aspects to construct a greater likelihood for fertilization success than before (van der Horst and du Plessis, 2017).

Considering the results of this study, further research is required including many factors such as the association between egg-water fertilization rate and sperm motility, intraspecific sperm trait variation and the analysis of flagellar characteristics to provide insight into the mechanisms producing a helical swimming path. This form of computerized sperm developments would form a solid quantitative basis for applications in aquaculture and as a tool for investigating environmental problems such as ocean acidification, especially aquatic toxicology research.

# CHAPTER 6

# FUTURISTIC PERSPECTIVES ON SPERM MOTILITY ANALYSIS

## 6.1 Future Sperm Motility Analysis Developments: Some Preliminary Results

This chapter follows the final PhD Discussion which is unusual. It was considered very important to be included as an aftermath, since developments in CASA are rapid due to improved hardware and new software developments. In this last chapter some current problems in the field of CASA are emphasised and some preliminary solutions are presented. Accordingly, Chapter 6, in brief, has its own Introduction, Materials and Methods, and Results and Discussion. It may be questioned why this has not been pursued in more depth and incorporated as part of the results section of this dissertation? Most of these developments have taken place in the last three years and some have only recently been implemented and published during 2016 to 2019. These new developments represent highly sophisticated technologies and the author of the dissertation wrote this chapter in conjunction with one of the developers of the 2D to 3D programme, Prof Gerhard van der Horst, who assisted with the computer analysis using all CASA data that emanated from the present study.



## 6.2 Introduction

CASA has provided many new developments during the last three decades in the automated and objective quantification of sperm morphology, sperm vitality, sperm fragmentation, and the acrosome reaction, apart from the many facets of sperm moti-

lity (Maroto-Morales *et al*., 2016; Fabbrocini and D'Adamo 2016; Křížková *et al*., 2017; Daloglu *et al*., 2018). Despite the lack in CASA data among invertebrates, results from this PhD and other more recent publications have afforded a wealth of new information that assists to understand, among other, the basics of quantitative sperm motility. However, current CASA of sperm motility is based on head centroid movement and accordingly sperm track reconstruction is based on x and y coordinates over time which is two-dimensional and represent the movement of the sperm head in time.

Firstly, sperm movement is three-dimensional and how can the z-axis be determined to construct a more realistic three-dimensional sperm swimming track? Fortunately, van der Horst and Sanchez (2016, 2019), Soler *et al*. (2018) and several other investigators using laser and holographic technology provided methodology to (partly) overcome the z-axis problem.

Secondly, even if this is possible, it is the tail that produces the propulsive force to push the sperm forward and not the head pulling the tail. Accordingly, it should rather be the tail that needs to be tracked and over the last few years' substantial developments have been made in this context (Hansen *et al*., 2018; Gallagher *et al*., 2019).

The main aim of Chapter 6 is to present preliminary results of how three-dimensional reconstructions can be performed using the x and y coordinates and some selective kinematics of the five study species presented in this PhD. Secondly, in one species a preliminary attempt of the potential of flagellar tracking is demonstrated.

## 6.3 Materials and Methods

For all five species, namely *P. angulosus*, *C. meridionalis*, *C. gigas*, *D. serra* and *H. midae* the kinematic data with x and y coordinates were extracted from representative rapid progressive swimming sperm as a point of departure (Chapter 4). It is by no means a comprehensive set of reconstructed tracks and therefore must be considered as preliminary and a future approach to enhance 2D tracking. In one species, *C. meridionalis*, sperm flagellar analysis was performed using the Image-J plug-in programme SpermQ (Hansen *et al*.., 2018).

### 6.3.1 Three-Dimensional (3D) Tracking

Kinematic results from this investigation have been used to reconstruct 3D tracks according to van der Horst and Sanchez, (2016, 2019) and van der Horst *et al*. (2018). The programme used a specially designed Excel sheet for this 2D to 3D reconstruction and it requires input of VCL, ALH and BCF to calculate and graphically depict a sperm track in 3D from x and y coordinates. For the 3D reconstruction, van der Horst and Sanchez (2019) assumed that sperm of most species swim in a spherical helix which can be expressed as: x(t) = cos (t), y(t) = sin (t), z(t) = 1; VCL, ALH (max) and BCF kinematic parameters in their 2D to 3D program were used to calculate the 3D path (VCL = distance of detailed tracking along each x and y coordinate; ALH = width of helix (in SCA = 2 x ALH), BCF = number of times track crosses VAP; VCL/(BCF/2xALH) = distance between peaks of track). A smoothed VAP path was a pre-condition. This methodology allows visualization of sperm tracks in 3D but has the disadvantage that the z-axis is assumed to be harmonic, which is often not the case. The method is thus useful for visualizations, but it is strictly speaking not quantitative.

### 6.3.2 Flagellar Tracking

The developer of SpermQ, Jan Hansen, (Bonn University), assisted with the automated flagellar analysis. Jan Hansen *et al*. (2018) was provided with high quality *.avi files representing 100 fps analysis using negative phase contrast microscopy. The main aim here is to indicate that flagellar analysis can be performed in broadcast spawning species, rather than providing an array of quantitative data that can be extracted from this kind of analysis.

## 6.4 Results

Figure 6.1 shows a typical SCA CASA analysis field and then the selection of a sperm indicated in bold red (sperm number 531). In Chapter 4, it has been shown how tracks and their kinematic data can be exported as a report (track reconstruction in 2D and kinematic data). VCL, ALH (max) and BCF in conjunction with the 2D to 3D programme (van der Horst and Sanchez, 2016, 2019) were used in the reconstruction as shown in 3D.



Figure 6.2 shows part of the analyses fields in the five species investigated (Chapter 4). The particular sperm to be used is selected in bold red and finally the 3D reconstructions of those sperm tracks. These helical representations largely show some species specificity and give insight into the 3D pattern of rapid progressive swimming sperm in these species. Figure 6.3 shows an example of flagellar analysis of a single *C. meridionalis* sperm over one beat cycle. The local bending (A) for determination of curvature and the actual swimming path (arc length), the relative z-position (B) and the flagellar position based on A and B are shown.

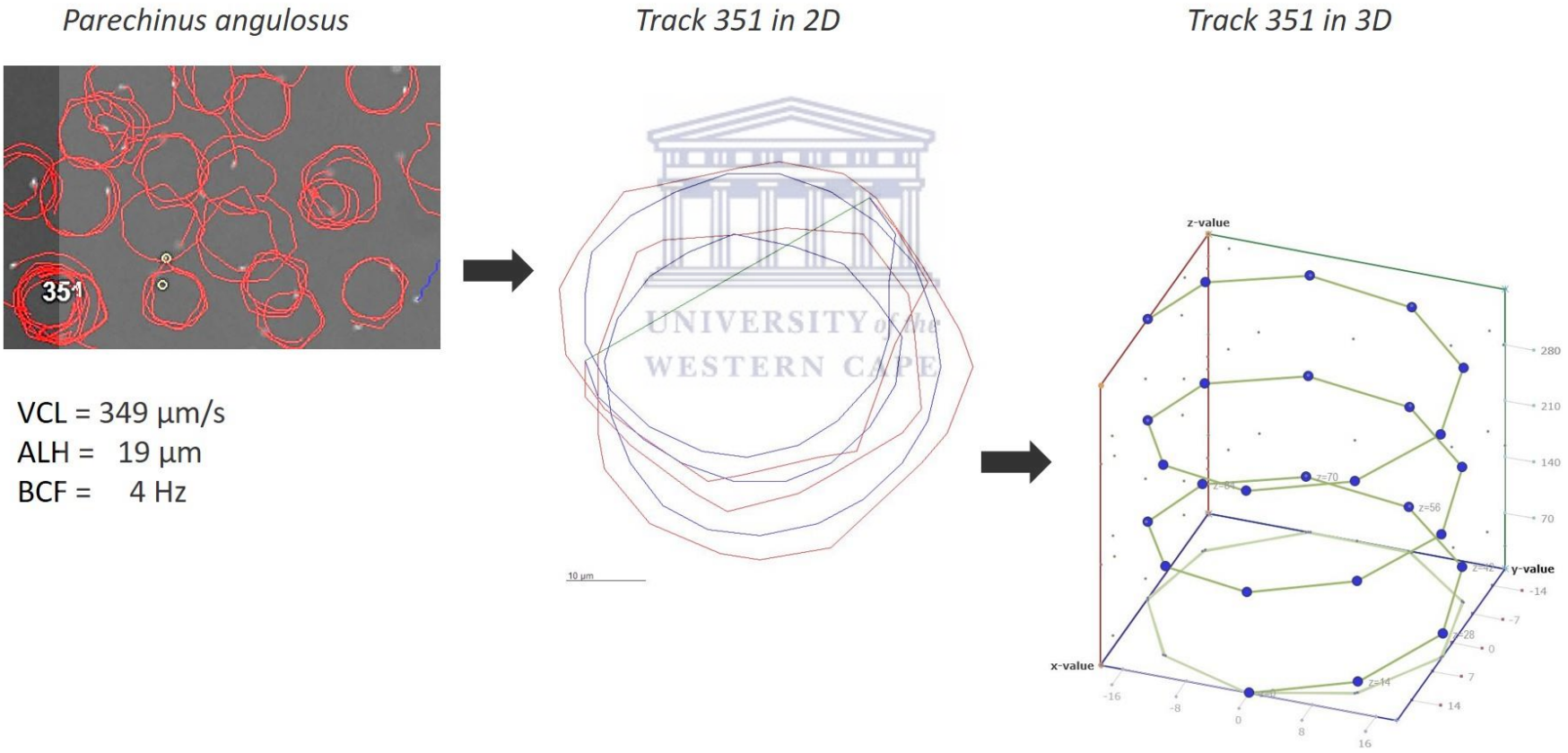



**Figure 6.1:** The stepwise process of three-dimensional tracking. At first, the SCA motility analysis is captured at 50 fps (one field on left), where after a particular sperm is selected (in bold red, e.g. 351). The actual reconstruction in 2D (middle) is extracted from SCA, including the three kinematic parameters required in the 3D reconstruction (right).

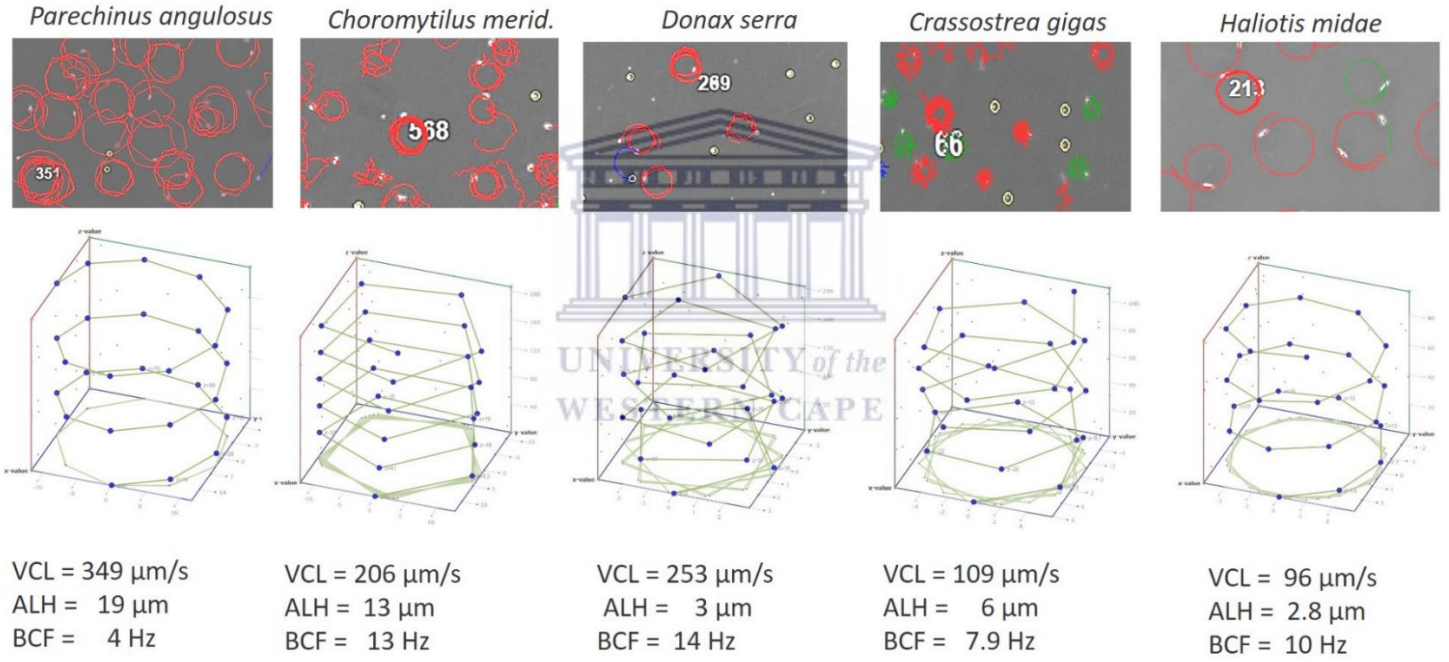



**Figure 6.2:** SCA CASA analysis field—Top row represents parts of an analysed field for the species indicated. The sperm highlighted in each case is shown as a 3D reconstruction with the relevant kinematics indicated below. Note that the 3D reconstruction are not all on the same scale as indicated by the x –axis: in *P. angulosus*-16 to +16; in *C. meridionalis*-10 to +10; in *D. serra*-2 to +2; *C. gigas*-4 to +4; H midae-2 to +2.

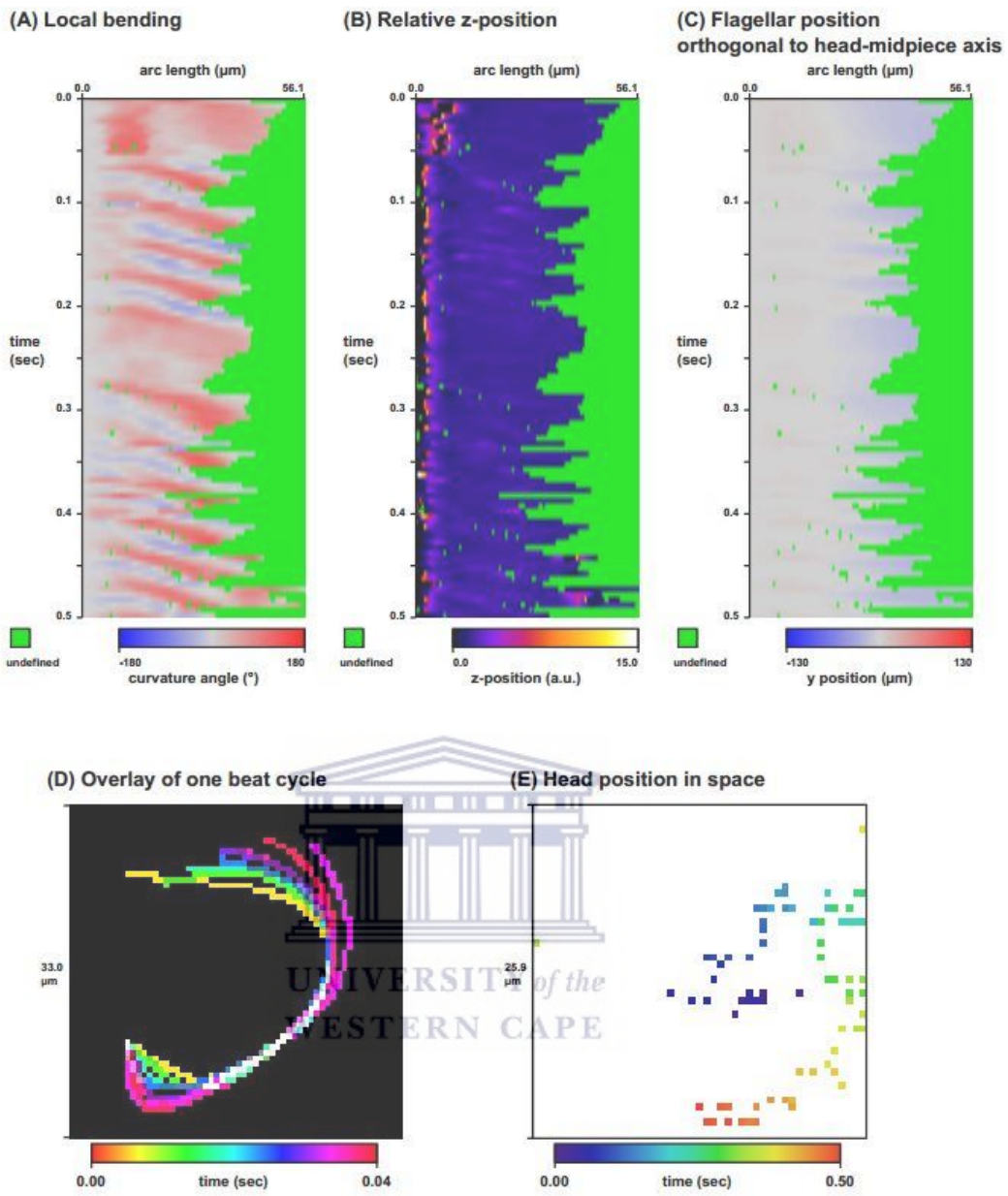



**Figure 6.3:** Details of flagellar analysis of *C. meridionalis* over one beat cycle using the SpermQ programme. This analysis include illustrations of the local bending (A) showing angle of curvature and the actual path (arc length), the relative z-position (B) and the flagellar position (C) based on A and B. Flagellar beat cycle (D) and sperm head position (E) are subsequently reconstructed.

Subsequently the reconstruction of one flagellar beat cycle (D) and sperm head position (E) were possible. This methodology and software (SpermQ) allow accurate

determination of the actual path of both the sperm flagellum and the sperm head. Accordingly, the real speed of the flagellar wave and sperm head can be established as well as a whole range of quantitative parameters that will not be included or discussed here. The main aim was to show that flagellar analysis in a broadcast spawner like *C. meridionalis* can be analysed.

## 6.5 Discussion and Conclusion

The motility section under Chapter 4 demonstrated that a high level of sophistication exists in CASA analysis of selected broadcast spawners. A vast range of quantitative data has been produced including some 50 motility parameters by using routine CASA. The data produced using centroid head movement are invaluable and have the potential to further quantify sperm motility when dealing with sperm competition, toxicology, establishment of the quality of a sperm sample and many other applications (Lu *et al*., 2014; Jekeli *et al*., 2015).



However, there is a need to further quantify and improve the above level of analyses and, in this chapter, information is provided on future prospects. In this context the 2D to 3D approach appears to be useful in constructing the potential path which helps to visualize the sperm swimming track. Care needs to be exercised with this approach as it is strictly speaking not quantitative but at least an attempt to determine the z-axis for 3D visual examination and comparison.

The only real disadvantage is that the 2D to 3D model of van der Horst and Sanchez (2019) assumes that sperm movement is harmonic and it is often not the case. The main advantage of this approach is that it greatly assists to visualize the actual helical

swimming pattern of sperm movement. Several laser-based studies (Alverez, 2017; Daloglu *et al*., 2018) strongly support the spherical helix model proposed by van der Horst and Sanchez (2019).

Flagellar analysis seems to be an essential component in future studies as it is the flagellum that determines the mode of movement and the sperm head structure may further influence the swimming pattern. Both these aspects need to be pursued to provide a more comprehensive relationship between swim motion and structure.

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# APPENDIX I

## PERMITS 2015-2019

agriculture,
forestry & fisheries
Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

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Fax: 021-4023639
E-mail : researchpermits@daff.gov.za

Prof. Mark J. Gibbons
Department of Biodiversity and Conservation Biology
University of the Western Cape
Private Bag X17
Bellville
7535

Attention Prof Mark Gibbons

**PERMIT FOR THE PURPOSES OF A SCIENTIFIC INVESTIGATION OR PRACTICAL EXPERIMENT IN TERMS OF SECTION 83 OF THE MARINE LIVING RESOURCES ACT, 1998 (ACT NO. 18 OF 1998)**

I, the undersigned, Chief Director: Fisheries Research and Development, Branch: Fisheries Management, Department of Agriculture, Forestry and Fisheries (the Chief Director) acting in pursuance of the delegated authority conferred upon me by the Honourable Minister of Agriculture, Forestry and Fisheries as contemplated in terms of Section 79 of the Marine Living Resources Act of 1998 (Act No. 18 of 1998) ("the Act") hereby permit, in terms of Section 83 of the Act, the following person(s)/institution to engage in the scientific investigation or practical experiment referred to below;

**PERMIT REFERENCE NUMBER:** RES2015/35
**PERSON(S)/ INSTITUTION:** Prof Mark Gibbons, Department of Biodiversity and Conservation Biology University of the Western Cape,
**SCIENTIFIC INVESTIGATION OR PRACTICAL EXPERIMENT:** Collection, possession, transportation and housing of marine species for research and educational purposes,

subject to the following conditions

**1. GENERAL CONDITIONS**

1.1. This permit is issued subject to the provisions and regulations of the following laws:
(a) The Marine Living Resources Act, 1998 (Act No. 18 of 1998) ("the Act"), and all regulations published in terms thereof;
(b) The National Environmental Management Act, 1998 (Act No. 107 of 1998) ("NEMA"), and in particular, the regulations that control vehicle use in the coastal zone (as amended;)
(c) The National Environmental Management Biodiversity Act, 2004 (Act No. 10 of 2004);
(d) The National Environmental Management Protected Areas Act, 2003 (Act No. 57 of 2003);
(e) The Sea Birds and Seals Protection Act, 1973 (Act No. 46 of 1973,) and
(f) The Prevention of Pollution from Ships Act (Act No. 2 of 1986).

1.2. This permit is intended to facilitate *bona fide* research, and should not be used for the purposes of financial gain by the Permit Holder.

1.3. If, in the opinion of the Chief Director there are sound reasons for doing so, the Chief Director may amend the conditions of the permit.

Page 1 of 4

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Department:
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REPUBLIC OF SOUTH AFRICA

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Prof. Mark J. Gibbons
Department of Biodiversity and Conservation Biology
University of the Western Cape
Private Bag X17
Bellville
7535



Attention: Prof. Mark Gibbons

**PERMIT FOR THE PURPOSE SOF A SCIENTIFIC INVESTIGATION OR PRACTICAL EXPERIMENT IN TERMS OF SECTION 83 OF THE MARINE LIVING RESOURCE S ACT, 1998 (ACT NO. 18 OF 1998).**

I, the undersigned, Chief Director: Fisheries Research and Development, Branch: Fisheries Management, Department of Agriculture, Forestry and Fisheries (the Chief Director) acting in pursuance of the delegated authority conferred upon me by the Honourable Minister of Agricultur, eForestry and Fisheries as contemplated in terms of Section 79 of the Marine Living Resources Act of 1998 (Act No. 18 of 1998) ("the Act") hereby permit, in terms of Section 83 of the Act, the following person(s)/institution to engage in the scientific investigation or practical experiment referred to below:

**PERMIT REFERENCE NUMBER:** RES2016/80
**PERSON(S)/INSTITUTION:** Prof. Mark Gibbons, Department of Biodiversity and Conservation Biology, University of the Western Cape,
**SCIENTIFIC INVESTIGATION OR PRACTICAL EXPERIMENT:** Collection, possession, transportation and housing of marine species for research and educational purposes,



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(c) The National Environmental Management Biodiversity Act, 2004 (Act No. 10 of 2004);
(d) The National Environmental Management Protected Areas Act, 2003 (Act No. 57 of 2003);
(e) The Sea Birds and Seals Protection Act, 1973 (Act No. 46 of 1973); and
(D The Prevention of Pollution from Ships Act (Act No. 2 of 1986).

1.2. This permit is intended to facilitate *bona fide* research, and should not be used for the purposes of financial gain by the Permit Holder.

1.3. If, in the opinion of the Chief Director there are sound reasons for doing so, the Chief Director may amend the conditions of the permit.

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I, the undersigned, Chief Director: Fisheries Research and Development, Branch: Fisheries Management, Department of Agriculture, Forestry and Fisheries (the Chief Director) acting in pursuance of the delegated authority conferred upon me by the Honourable Minister of Agriculture, Forestry and Fisheries as contemplated in terms of Section 79 of the Marine Living Resources Act of 1998 (Act No. 18 of 1998) ("the Act") hereby permit, in terms of Section 83 of the Act, the following person(s)/institution to engage in the scientific investigation or practical experiment referred to below:

**PERMIT REFERENCE NUMBER:** RES2017/58
**PERSON(S)/ INSTITUTION:** Prof. Mark Gibbons, Department of Biodiversity and Conservation Biology, University of the Western Cape,
**SCIENTIFIC INVESTIGATION OR PRACTICAL EXPERIMENT:** Collection, possession, transportation and housing of marine species for research and educational purposes,



subject to the following conditions:

**1. GENERAL CONDITIONS**

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(d) The National Environmental Management Protected Areas Act, 2003 (Act No. 57 of 2003);
(e) The Sea Birds and Seals Protection Act, 1973 (Act No. 46 of 1973); and
(f) The Prevention of Pollution from Ships Act (Act No. 2 of 1986.)

1.2. This permit is intended to facilitate *bona fide* research and should not be used for the purposes of financial gain by the Permit Holder.

1.3. If, in the opinion of the Chief Director there are sound reasons for doing so, the Chief Director may amend the conditions of the permit.

agriculture, forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

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Attention: Prof. Mark Gibbons

**PERMIT FOR THE PURPOSES OF A SCIENTIFIC INVESTIGATION OR PRACTICAL EXPERIMENT IN TERMS OF SECTION 83 OF THE MARINE LIVING RESOURCES ACT, 1998 (ACT NO. 18 OF 1998).**

I, the undersigned, Chief Director Fisheries Research and Development, Branch: Fisheries Management, Department of Agriculture, Forestry and Fisheries (the Chief Director) acting in pursuance of the delegated authority conferred upon me by the Honourable Minister of Agriculture, Forestry and Fisheries as contemplated in terms of Section 79 of the Marine Living Resources Act of 1998 (Act No. 18 of 1998) ("the Act") hereby permit, in terms of Section 83 of the Act, the following person(s)/institution to engage in the scientific investigation or practical experiment referred to below:

**PERMIT REFERENCE NUMBER:** RES2018/64
**PERSON(S)/ INSTITUTION:** Prof. Mark Gibbons, Department of Biodiversity and Conservation Biology, University of the Western Cape,
**SCIENTIFIC INVESTIGATION OR PRACTICAL EXPERIMENT:** Collection, possession, transportation and housing of marine species for research and educational purposes,

subject to the following conditions:



1. **GENERAL CONDITIONS**

1.1. This permit is issued subject to the provisions and regulations of the following laws:
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(b) The National Environmental Management Act, 1998 (Act No. 107 of 1998) ("NEMA"), and in particular, the regulation; .; .:1at control vehicle use in the coastal zone (as amended);
(c) The National Environmental Management Biodiversity Act, 2004 (Act No. 10 of 2004);
(d) The National Environmental Management Protected Areas Act, 2003 (Act No. 57 of 2003;)
(e) The Sea Birds and Seals Protection Act, 1973 (Act No. 46 of 1973); and
(n The Prevention of Pollution from Ships Act (Act No. 2 of 1986).

1.2. This permit is intended to facilitate *bona fide* research, and should not be used for the purposes of financial gain by the Permit Holder.

1.3. If, in the opinion of the Chief Director there are sound reasons for doing so, the Chief Director may amend the conditions of the permit.

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Department:
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REPUBLIC OF SOUTH AFRICA

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Attention: Pro.f Mark Gibbons

**PERMIT FOR THE PURPOSES OF A SCIENTIFIC INVESTIGATION OR PRACTICAL EXPERIMENT IN TERMS OF SECTION 83 OF THE MARINE LIVING RESOURCES ACT, 1998 (ACT N0.18 OF 1998).**

I, the undersigned, Chief Director: Fisheries Research and Development, Branch: Fisheries Management, Department of Agriculture, Forestry and Fisheries (the Chief Director) acting in pursuance of the delegated authority conferred upon me by the Honourable Minister of Agriculture, Forestry and Fisheries as contemplated in terms of Section 79 of the Marine Living Resources Act of 1998 (Act No. 18 of 1998) ("the Act") hereby permit, in terms of Section 83 of the Act, the following person(s)/institution to engage in the scientific investigation or practical experiment referred to below.

**PERMIT REFERENCE NUMBER:** RES2019/74
**PERSON(S)/INSTITUTION:** Prof. Mark Gibbons. Department of Biodiversity and Conservation Biology, University of the Western Cape,
**SCIENTIFIC INVESTIGATION OR PRACTICAL EXPERIMENT:** Collection, possession, transportation and housing of marine species for research and educational purposes,



subject to the following conditions:

**1. GENERAL CONDITIONS**

1.1. This permit is issued subject to the provisions and regulations of the following laws:
(a) The Marine Living Resources Act, 1998 (Act No. 18 of 1998) ("the Act"), and all regulations published in terms thereof;
(b) The National Environmental Management Act, 1998 (Act No. 107 of 1998) ("NEMA"), and in particular, the regulations that control vehicle use in the coastal zone (as amended);
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(d) The National Environmental Management Protected Areas Act, 2003 (Act No. 57 of 2003);
(e) The Sea Birds and Seals Protection Act, 1973 (Act No. 46 of 1973); and
( The Prevention of Pollution from Ships Act (Act No. 2 of 1986).

1.2. This permit is Intent to facilitate *bona fide* research, and should not fi&us tarf eS:eH mancial gain by the Permit Holde.

1.3. If, in the opinion of the Chief Director there are sound reasons fo:t 1r ;tile'CiteTe'r f tday amend · the conditions of the permit.

I CE RTIFY THIS TO BE A TRUE
Date: - 7= :29 !
Com DAWN LY N NE FAROE
m ss oner of Oaths, District of Bellville
CHIEF OFFICER
U nive rsity of the Western Cape, Bellville
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# APPENDIX II

## PUBLICATION FROM THIS THESIS

CSIRO PUBLISHING

*Reproduction, Fertility and Development*, 2018, **30**, 907–918
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### CASA in invertebrates

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Abstract. Sperm movement has been described in several phyla of invertebrates. Yet, sperm motility has only been quantified using computer-aided sperm analysis (CASA-Mot) in externally fertilising species (broadcast spawners) of two phyla, molluscs and echinoderms. In the present study we quantified in detail the nature of the sperm tracks, percentage motility groupings and detailed kinematics of rapid-, medium- and slow-swimming spermatozoa in the oyster *Crassostrea gigas* and four species never previously studied by CASA-Mot, namely the molluscs *Choromytilus meridionalis*, *Donax serra* and *Haliotis midae* and the echinoderm *Parechinus angulosus*. A feature common to all these species are the helical tracks, the diameter of which seems to be species specific. Using CASA-Mot, the behaviour of spermatozoa was also studied over time and in the presence of egg water and $Ca^{2+}$ modulators such as caffeine and procaine hydrochloride. For the first time, we show that hyperactivation can be induced in all species in the presence of egg water (sea water that was mixed with mature eggs and then centrifuged) and/or caffeine, and these hyperactivated sperm tracks were characterised using CASA-Mot. We relate the different patterns of sperm motility and behaviour to reproductive strategies such as broadcast spawning and spermcasting, and briefly review studies using CASA-Mot on other invertebrates.

Additional keywords: hyperactivation, motility, spermatozoa.

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#### Introduction

The invertebrates comprise over 30 phyla, plus the subphylum Tunicata within the Chordata. The Phylum Arthropoda is by far the most speciose, insects alone making up 95% of living animal species; insect fertilisation is internal, and the use of computer-aided sperm analysis (CASA) of insect spermatozoa has been very limited (but see Pearcy *et al.* 2014). Invertebrate diversity at the phylum level is greatest in the sea, and the variety of reproductive patterns in aquatic invertebrates is vast. Nevertheless, three main routes to fertilisation can be recognised: copulation, broadcast spawning and spermcasting; these correspond to functional categories of spermatozoa proposed by Rouse and Jamieson (1987) and Jamieson and Rouse (1989), namely introsperm, ect-aquasperm and ent-aquasperm respectively. Copulation, involving the direct transfer of an ejaculate into the female without external exposure, is also prevalent in terrestrial invertebrates. The remaining two mechanisms are exclusively aquatic.

Broadcast spawning is the release of both eggs and spermatozoa into the water for external fertilisation, whereas spermcasting involves the release of spermatozoa only, to disperse to recipients that have retained their eggs. Broadcast spawning is a long-recognised means of fertilisation in the sea, but spermcast mating was not overtly acknowledged as a distinct process until relatively recently (see Pemberton *et al.* 2003; Bishop and Pemberton 2006), although it was recognised and documented, for example by Daly and Golding (1977), and implicitly acknowledged in various other studies. As noted by Bishop and Pemberton (2006), it is possible to envisage a continuum between spermcast mating and broadcast spawning. This was re-emphasised by Havenhand and Styan (2010), noting that some species fertilise their retained ova in a seawater-filled concavity, such as the mantle of a flat (brooding) oyster (Ostreinae), through which water is pumped rather than in a true body cavity. There are few studies of the behaviour of spermcast spermatozoa (ent-aquasperm), even counting those on commercial flat oysters. In contrast, studies of the behaviour and performance of ect-aquasperm, including by CASA, are more numerous, although all concern species of two phyla, Mollusca and Echinodermata. In many cases these studies involve commercially exploited species such as cupped (non- brooding) oysters (Crassostreinae), mussels (Mytilidae) and abalone (*Haliotis* spp.).

Original CASA-generated data are presented in this paper on the sperm performance and behaviour of five marine invertebrates from broadcast-spawning taxa. The five species investigated are classified under Mollusca and Echinodermata, and we offer some additional observations on Insecta and Crustacea.

Globally, the research focus in CASA-Mot was unfortunately largely limited to the former two phyla, with many papers reporting on mussels and sea urchins (e.g. Au *et al.* 2001; Fitzpatrick *et al.* 2010; Fabbrocini *et al.* 2010, 2016; Basti *et al.* 2013; Suquet *et al.* 2013). Surprisingly, almost no detailed CASA research has been performed in other invertebrate phyla and accordingly our title falls short of expectations.

Previous surveys of the available data have suggested that spermcast spermatozoa are long lived compared with broadcast-spawned spermatozoa, perhaps because of quiescence following release until reaching a conspecific recipient (Bishop 1998; Manr´ıquez *et al.* 2001; Johnson and Yund 2004), although the spermatozoa of broadcast spawners may also be quiescent upon release, being activated by proximity to the egg or signals derived from it (e.g. Morita *et al.* 2006). In the present study we provide, in a systematic way, very detailed CASA-Mot analysis involving sperm concentration, percentage motility groupings and eight kinematic parameters of five broadcast spawners, some of which may show adaptations to highly wave- swept environments. The data provided is the first comprehen- sive comparison of CASA-Mot baseline data among these broadcast spawners. In addition, we analyse in greater depth the nature of the typical helical swimming tracks and the implications this may have for rates of encounter with oocytes. Furthermore, we provide results of CASA analysis of sperm motility in the presence of egg water in the Cape sea urchin *Parechinus angulosus* (see Table 1), with comments on two other species studied as well as the effect of classical hyper- activation stimulants on sperm motility in two species. Hyper- activation of spermatozoa has been considered as a landmark of capacitation and fertilisation success (Mortimer 1997; Mortimer *et al.* 2015). Finally, we assess the use of CASA-Mot in estimating the concentration of immotile spermatozoa in a prawn species and the potential movement of the spike attached to the acrosome in this species. We also review important aspects of subjective sperm motility assessment in other invertebrate phyla in the Results and Discussion sections.

## Materials and methods

### *Species studied*

Six marine invertebrates from three phyla were studied and are listed in Table 1. A permit for hand collection of species was obtained through the Department of Agriculture, Forestry and Fisheries, Cape Town, South Africa. Ten to twenty animals per species were used for the different motility studies, with the actual numbers indicated in the relevant tables.

### *Animal collection and maintenance*

Commercially grown oyster *Crassostrea gigas* (new accepted genus *Magellana*), black mussel (*Choromytilus meridionalis*), abalone (*Haliotis midae*) and tiger prawn (*Penaeus monodon*) were obtained from mariculture farms; sea urchins (*Parechinus angulosus*) were hand collected at monthly low tides, between 2014 and 2017 from intertidal rock pools at Bloubergstand, Cape Town. During spring tide lows, the burrowing white mussels (*Donax serra*) were dug out by hand along the mean tide level of the exposed sandy beach at Big Bay, Cape Town. All

**Table 1. The five marine broadcast spawners investigated in the present study, their common names, marine zone, the characteristics of that zone and suspected fertilisation strategy**
The number of animals used for each species is given in parentheses after the species name

| Phylum | Species (*n*) | Common name | Marine zone | Zone characteristics | Fertilisation strategy | Adult mobility |
|---|---|---|---|---|---|---|
| Echinodermata Echinoidea | *Parechinus angulosus* (14) | Cape sea urchin | Intertidal | Shallow protected pools during low tide | Broadcast spawner | Mobile |
| Mollusca Bivalvia | *Magallana gigas* (previously *Crassostrea*; 12) | Pacific oyster | Intertidal | Rocky shore, heavy wave action | Broadcast spawner? (see Discussion) | Not mobile |
| | *Choromytilus meridionalis* (13) | Black mussel | Intertidal | Rocky shore, heavy wave action | Broadcast spawner? (see Discussion) | Not mobile |
| | *Donax serra* (13) | White mussel | Intertidal | Sandy beach, under surface of sand, mild wave action, low tide | Broadcast spawner | Mobile |
| Gastropoda | *Haliotis midae* (16) | Abalone | Deeper water | Typically 5–10 m under water surface, mild to large currents | Broadcast spawner | Mobile |
| Arthropoda Crustacea | *Penaeus monodon* (18) | Tiger prawn | Nearshore | Bottom feeders | Internal fertilisation, spermatophores | Mobile, come to surface to mate |

animals were kept in aerated holders filled with sea water at 17–188C (sea water temperatures 12–198C).

### Gamete collection

Using a positive displacement pipette, spermatozoa were pipetted from the testes in the oyster and the two mussel species through an incision in the gonads and were dry stored in Eppendorf tubes. In *C. meridionalis*, we also tested the hypothesis that spermatozoa isolated from highly gravid testis for motility studies are similar to 'spawned' spermatozoa. Spawning was induced using a routine method (Stewart *et al.* 2012) and spermatozoa were immediately collected as close as possible to the mussel the moment it was ejected in the form of whitish clouds. This sperm–sea water suspension was placed in an Eppendorf tube and motility was studied by means of CASA-Mot for up to 90 min after spawning. These data were compared with the motility of spermatozoa isolated from gravid testes.

Gametes from sea urchin and abalone were obtained through chemically induced spawning. Sea urchins were given intracoelomic injections of 1–2 mL of 0.55 M KCl, which caused spawning to be initiated within seconds to 5 min, depending on the presence of gravid gonads. Female urchins, releasing orange eggs, were inverted onto a 100-mL beaker filled to the edge with filtered sea water, letting the aboral surface touch the water, allowing eggs to be freely ejected and settle at the bottom. This suspension was gently agitated and left to incubate for 20 min; a volume of this egg–sea water suspension was cen- trifuged at 11*g* for 5 min at 188C. The supernatant liquid, egg water, was decanted into a clean Eppendorf tube and kept as a diluting medium for motility evaluation later. Eggs and sur- rounding egg water obtained from the ovaries of *C. gigas* and *C. meridionalis* were treated in the same way as for *Parechinus* to obtain egg water for sperm exposure studies.

Milky white spermatozoa being released from gonopores of *P. angulosus* were harvested and stored as 'dry spermatozoa' (undiluted) in an Eppendorf tube. Abalone broodstocks underwent a two-step chemical induction procedure as described by Morse *et al.* (1977), with a few adjustments. Sodium hydroxide was initially added to the spawning tanks, followed by hydrogen peroxide 15 min later. After 1 h, these tanks were emptied, cleaned and refilled with aerated filtered sea water until males started spawning, generally 4 h after induction (Roux *et al.* 2014). Abalone spermatozoa were collected directly from the spawning tank, close to the respiratory pores located in the shell where the spermatozoa were ejected into sea water via the nephridiopore duct (Hahn 1989; and Purchon 1977), and stored in an Eppendorf tube.

### Preparation of motile spermatozoa

Microscope analysis included measurement of sperm concentration, total motility and progressive motility in motile populations. In all instances, a 20-mm deep two-chamber Leja slide (Leja Products) was used. The motility of spermatozoa was determined following two techniques, flush and swim-up. Dry spermatozoa (undiluted spawned or neat spermatozoa sampled from the testis) from *P. angulosus*, *C. meridionalis*, *D. serra* and *C. gigas* were used for both the swim-up and flush techniques; *H. midae* samples were evaluated using only the flush technique for the chemically induced spawned gametes.

### Flush technique

The flush technique evaluates sperm motility at the inception of exposure to sea water in a chambered slide and, for most of the species, the concentrated sperm columns created allow spermatozoa to swim out. In contrast, for *H. midae* sperm motility using the flush technique was assessed from spawned samples in sea water (already mixed) and stored in an Eppendorf tube. A 5-mL aliquot of this sperm–sea water suspension was flushed in a 20-mm deep Leja slide for subsequent CASA-Mot analysis.

For *P. angulosus*, the flush technique involved collecting approximately 0.5–1 mL undiluted spawned spermatozoa and transferring the sample to a 20-mm deep two-chamber Leja slide (Leja Products). Through capillary action, a slight sperm dome was created at the mouth of the chamber (Leja 20-mm slide); to this, 5 mL filtered sea water was pipetted or flushed into the chamber, producing columns of spermatozoa in the chambered slide, and activated spermatozoa were recorded immediately using Sperm Class Analyser (SCA; Microptic) CASA-Mot software (see below).

For *C. gigas*, *C. meridionalis* and *D. serra*, dry spermatozoa (undiluted) were obtained directly from the testis and the same procedure as described for *P. angulosus* above was followed. We believe this technique best mimics what happens during actual spawning.

### Swim-up technique

The swim-up technique was conducted individually for black mussel, white mussel, oyster and sea urchin dry spermatozoa using filtered sea water as a dilution medium. One part of dry spermatozoa from each of the samples was slowly pipetted, using a displacement pipette, forming a pellet at the bottom of an Eppendorf tube filled with five parts filtered sea water or egg water. Spermatozoa were usually inactive during the first 2 min upon dilution of testicular spermatozoa, but most spermatozoa became active after approximately 5 min. In contrast, spawned spermatozoa became immediately active after dilution with sea water. Accordingly, a 10-min period of incubation resulted in spermatozoa swimming from the pellet into the column of sea water or egg water. As a result of the spermatozoa swimming from the pellet, a cloud developed in the fluid column. By pipetting 5 mL motile spermatozoa, taken from above the sperm cloud of the pellet, a slide was prepared using a 20-mm deep two-chamber Leja slide. Sperm motility was recorded after 10 and 60 min.

### Effects of egg water and $Ca^{21}$ modulators

In three species (sea urchin, black mussel, Pacific oyster), egg water was also used as a diluting medium and sperm motility was accordingly measured after exposure to egg water using the flush technique.

The effects of two $Ca^{2þ}$ modulators that induce hyperactivation in many animal species, namely caffeine (10 mM) and procaine hydrochloride (5 mM), were tested on *C. meridionalis* and *C. gigas* spermatozoa and compared with egg water using

**Table 2. Mean (± s.d.) sperm concentration, percentage motility, percentage progressive motility and the percentage of non-progressive motility**
In all species, sperm concentration represents testicular samples, except in the case of *Parechinus* (collected at external gonopores), *Haliotis* (sperm collection took place during induced spawning) and *Penaeus* (collected from the spermatophore). Table 1 shows the numbers used for each species

| Species | Concentration ($\times 10^6$ spermatozoa $mL^{-1}$) | Total motility (%) | % Rapid progressive motility | % Medium progressive motility | % Non-progressive motility (slow) |
|---|---|---|---|---|---|
| *Parechinus angulosus* | 5057.2±1353.3 | 66.4±21.5 | 46.7±23.4 | 12.4±18.3 | 7.3±8.4 |
| *Crassostrea gigas* | 1544.3±1201.6 | 49.6±19.6 | 23.6±15.7 | 22.4±8.7 | 3.5±1.4 |
| *Choromytilus meridionalis* | 3247±2027 | 54.7±23.3 | 26.8±18.8 | 19.7±12.9 | 8.2±9.6 |
| *Donax serra* | 4642±3702 | 50.3±30.7 | 43.4±31.1 | 3.03±2.9 | 4.0±4.1 |
| *Haliotis midae* | 22.1±15.3 | 91.3±7.9 | 50.6±26 | 29.3±23.2 | 11.2±8.1 |
| *Penaeus monodon* | 2893.9±1031.4 | 0 | 0 | 0 | 0 |

the flush technique. Accordingly, 0.5–1 mL undiluted spermatozoa was flushed with 5 mL filtered sea water containing either egg water or the $Ca^{2+}$ modulators. Sperm motility was recorded after 10 and 60 min using the SCA CASA-Mot system (see below).

### *CASA equipment, acquisition properties and sperm functional parameters*

To divide spermatozoa into subpopulations based on swimming speed, default SCA settings (see below) for Fish/Invertebrate and curvilinear velocity (VCL) were used. The number of images captured and frame rate were 50 images and 100 images $s^{-1}$ respectively; VCL cut-off values of 38 „ 50 „ 120 mm $s^{-1}$ distinguish slow- (non-progressive), medium- and rapid-swimming spermatozoa. Sperm swimming „ 38 mm $s^{-1}$ were considered to be immotile (potential flow, Brownian movement and collisions of motile spermatozoa with immotile spermatozoa). All microscopic analysis measuring the motility populations was performed using SCA version 5.4.0.0 or 6.2.0.16 (Microptic S.L.). Motility parameters of flush slides were assessed at 10 min, whereas swim-up samples were assessed after incubation times of 10 and 60 min. 'Incubation' in this case refers to the time that swim-up spermatozoa were exposed to sea water after swim-up. The slides (Leja 20-mm deep chambered slides) were viewed using a Basler A312fc digital camera (Microptic), mounted (C-mount) on either a Nikon E50i microscope or an Olympus CH2 microscope, both equipped with negative phase contrast objectives used (Maree and van der Horst 2013). Two to five fields were captured randomly to eliminate bias towards best motility areas. The SCA systems automatically determine the sperm concentration of the fresh diluted sample using a Leja 20-mm deep slide (calibrated).

The following kinematic parameters were assessed: VCL, straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), wobble (WOB), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF). We also calculated DANCE as follows: DANCE ¼ VCL × ALH. Accordingly DANCE represents the two dimensional space occupied by a motile sperm during 1 s.

### *Statistical analyses*

MedCalc version 17.2 (MedCalc, Mariakerke, Belgium) was used for basic statistical analysis. Descriptive statistics were used to calculate the mean ± s.d. Comparisons of sperm motility parameters among the different groups were performed using either analysis of variance (ANOVA) or the Kruskal–Wallis test with appropriate post hoc tests, such as the Student–Newman–Keuls' and Tukey tests. Two-sided $P$ „ 0.05 was considered significant.

## Results

### *Visual, subjective description of sperm behaviour*

Swimming of spermatozoa in a typical compressed helix when diluted with sea water was observed after different time intervals in all five mollusc and echinoderm species. In Pacific oyster and white and black mussels, sperm activation was delayed after dilution with sea water (either flush technique or swim-up). Full activation was usually obtained 2–7 min after dilution with sea water. In contrast *P. angulosus* and *H. midae* spermatozoa were immediately activated with sea water. The helical sperm tracks for *P. angulosus*, *D. serra* and *H. midae* were essentially unchanged over 1 h, whereas in *C. gigas* and *C. meridionalis* most spermatozoa appeared to change their swimming behaviour from a helical progressive pattern to a more forward progressive pattern. The spermatozoa of tiger prawn were essentially immotile due to the absence of a tail or axoneme, but some kind of movement could be detected and is described later.

### *CASA analysis of sperm concentration and percentage and progressive sperm motility*

The sperm concentrations of dry spermatozoa and the percentage motility, progressive motility and non-progressive motility in the six species within the first 5–15 min after dilution and activation with sea water are given in Table 2. The sperm concentration of samples from testicular origin was extremely high (ranging from „ 1554.3 × $10^6$ $mL^{-1}$ in *C. gigas* to 5057.2 × $10^6$ $mL^{-1}$ in *P. angulosus* (gonopore collection)) compared with spermatozoa in general. *H. midae* samples had the lowest sperm concentration, but these samples are largely representative of spermatozoa spawned in sea water via the nephridiopore into surrounding sea water (i.e. spermatozoa collected while animals were spawning in tanks). After flush or swim-up, the sperm concentration in the other four species (excluding *H. midae*) varied from approximately 15 to 30 × $10^6$ $mL^{-1}$. Accordingly, sperm motility for all species was analysed at the same range of sperm concentrations. There was a large variation in the mean percentage sperm

motility, ranging from 54% to 91% among the broadcast spawning species.

### CASA analysis of sperm motility patterns

Fig. 1 shows a collage of the swimming patterns analysed by means of CASA in five species soon (5–15 min) after sperm activation, as well as the motility patterns after 60 min. The pattern for each species is shown at the same magnification, with *P. angulosus* clearly having the largest helical diameter for the sperm tracks (30–70 mm (50th percentile)) and *C. gigas* smallest (9–15 mm (50th percentile)) and the other species having helical tracks with diameters intermediate between these two above extremes (Fig. 2). Despite diameter and kinematic differences (see below) the actual pattern of the helix is quite similar in *P. angulosus*, *H. midae* and *D. serra*. However, in *C. gigas* there appears to be a distinctive species-specific track: a serrated helix with a very small diameter (Fig. 3).

Furthermore, after 60 min, the progressive helical patterns remained the same for swim-up spermatozoa of *P. angulosus*, *H. midae* and *D. serra*, but in *C. meridionalis* and *C. gigas* almost no helical progressive swimming spermatozoa were evident, with mostly straight line forward progressive spermatozoa (Fig. 1, white arrows). These forward progressively motile spermatozoa accounted for approximately 20% of the *D. serra* sample after 60 min (Fig. 1), but the helical tracks strongly prevailed in this species like in *P. angulosus* and *H. midae*. Spermatozoa collected from spawning *C. meridionalis* exhibited primarily helical swimming (5–60 min) in contrast with spermatozoa collected from the gravid testis, which primarily exhibited more forward progressive tracks after 60 min. However, sperm velocities (VCL, VAP and VSL) and most other kinetic parameters did not differ significantly between spermatozoa of testicular origin and 'spawned' spermatozoa. Fig. 3 shows a selection of the details of individual spermatozoa representing typical progressive helical patterns among the species, typical forward progressive-type spermatozoa and the relevant kinematics for each type.

### CASA sperm kinematic parameters

Fig. 4 compares the kinematic values of sperm subpopulations (rapid, medium, slow) for each species. There are significant differences in VCL and VAP between the rapid and medium sperm populations ($P$ „ 0.05, $F$/8.5), which emphasises the importance of the subpopulation approach when studying sperm kinematics. In *P. angulosus*, mean VCL and VAP values for the rapid sperm population are 240.7 ± 51.9 and 207.1 ± 52.1 mm $s^{-1}$ respectively (Fig. 4), and the VCL for individual spermatozoa can reach values ˛ 400 mm $s^{-1}$ at 50 frames $s^{-1}$ (Fig. 2). In contrast, *C. gigas*, which had the smallest-diameter helical swimming, mean VCL and VAP values were 149.4 ± 11.5 and VAP 60 ± 7 mm $s^{-1}$, with a maximum VCL of approximately 160 mm $s^{-1}$.

### More detailed comparisons of individual tracks in different species

The relationships among diameter, VCL, VAP and DANCE of the helical sperm tracks (Fig. 2) were further analysed by Spearman rank correlations and refer to the total motile sperm population. Some of these correlations are given in Tables 3 and 4. Correlations when population averages for all species were combined in relation to helix characteristics are given in Table 3. For the population averages, there were highly significant positive correlations between diameter and VCL, diameter and VAP, and diameter and DANCE. The greater the diameter, the higher the positive correlation with VCL, VAP and DANCE for all species together. Accordingly, in general, when spermatozoa swim faster, the diameter of the helix increases, as does DANCE, which is an expression of the surface area space occupied by the spermatozoa during 1 s. Because ALH × VCL ¼ DANCE, the relationship between VCL and ALH was investigated in four representative species (Table 4). Very high correlations ($r$ ¼ 0.6–0.91; $P$ „ 0.0001, $F$ ¼ 9.3) were established between VCL and ALH for the five species. Accordingly, increases in both VCL and ALH contribute to an increased DANCE and increased 'sperm search area'. The correlations for both population averages and individual species have implications in the spermatozoa finding an oocyte.

### Effects of egg water and hyperactivation stimulants on C. meridionalis, C. gigas and P. angulosus spermatozoa

Sperm motility patterns in *C. meridionalis*, *C. gigas* and *P. angulosus* changed when samples were diluted with egg water. In *Parechinus*, there was an initial (first 10 min) significant inhibition of VCL and VAP ($P$ „ 0.02, F 6.2) when samples were diluted in egg water compared with the swim-up procedure. In *Choromytilus* and *Crassostrea*, the sample size and thus the results for VCL and VAP were too variable to establish any differences. However, after 45–60 min exposure to egg water, there was an increase in what appears to be typically hyperactivated sperm motility patterns in *C. meridionalis* and *C. gigas* (Fig. 5*a*, *b*). In *P. angulosus*, the typical helical swimming pattern changed to an uneven helix pattern in egg water, with a minority of spermatozoa showing similar 'hyperactivation patterns' after 1 h as found in *C. meridionalis* and *C. gigas* from 5 to 60 min (Fig. 5*c*).

The effects of $Ca^{2+}$ modulators caffeine and procaine hydrochloride, which typically induce hyperactivation in many animal species, were tested on *C. meridionalis* and *C. gigas* spermatozoa. These two $Ca^{2+}$ modulators induced typical hyperactivation patterns (Fig. 5*d*, *e*). It appears that egg water induces similar changes in sperm motility patterns to the classical hyperactivation $Ca^{2+}$ modulators caffeine and procaine hydrochloride (Fig. 5).

### Special case of tiger prawn spermatozoa

It was possible to accurately determine prawn sperm concentration using CASA-Mot within 5 s. (Concentrations were measured in diluted sperm samples in a Leja slide using SCA software.) Because of the high sperm concentration, dilution with sea water was required. Sperm concentration in the tiger prawn was in the same concentration range as for broadcast spawners, namely ˛ 1500 × $10^6$ $mL^{-1}$. In the initial CASA-Mot studies, it was noted that there was approximately 10% 'motility', but these observations were discounted because of potential Brownian movement or flow. However, using

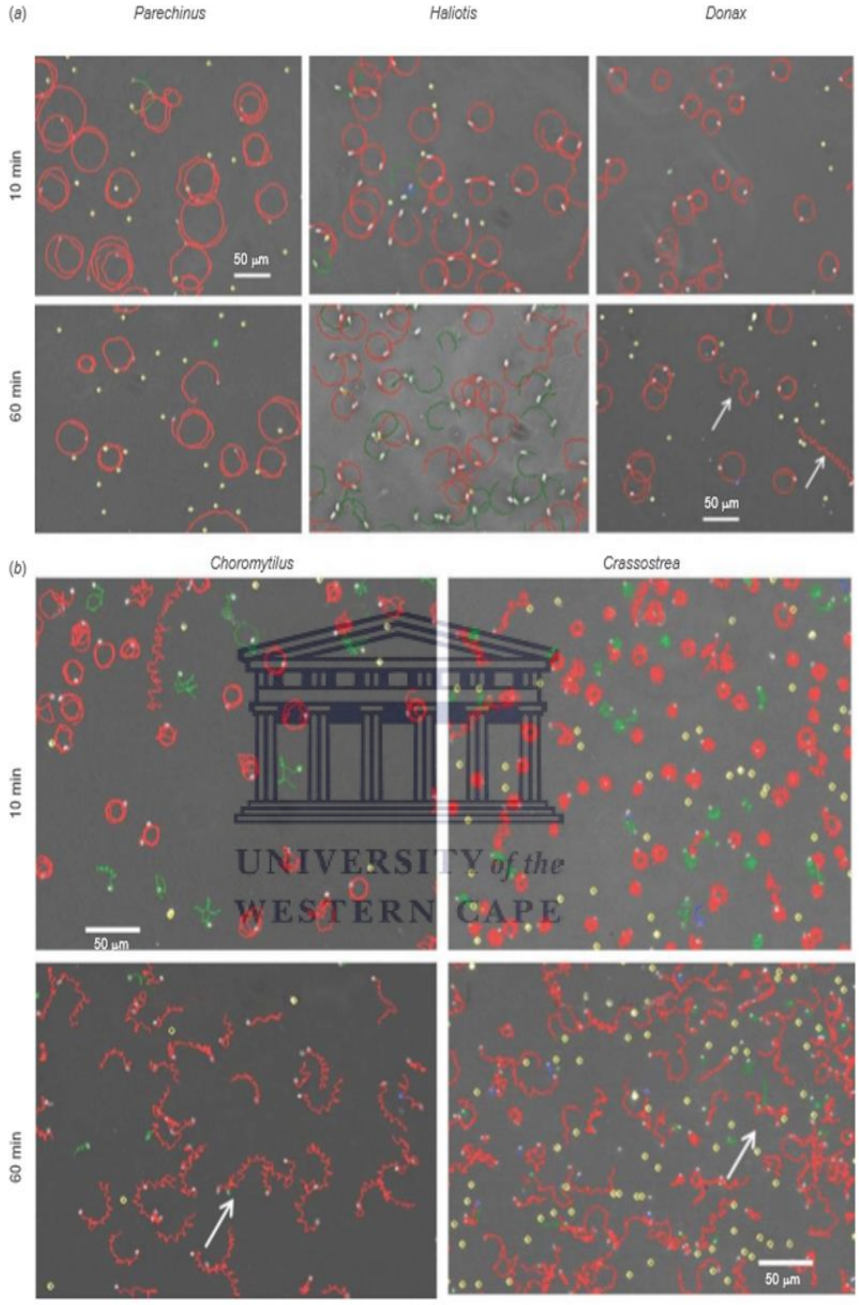




**Fig. 1.** (*a*) Characteristic sperm motility tracks of (*a*) *Parechinus*, *Haliotis* and *Donax* and (*b*) *Choromytilus* and *Crassostrea* 5–10 and 60 min after dilution. Scale bars apply to all figure panels. Red tracks, rapid swimming sperm; green tracks, medium swimming sperm. White arrows indicate sperm now swimming progressively forward.

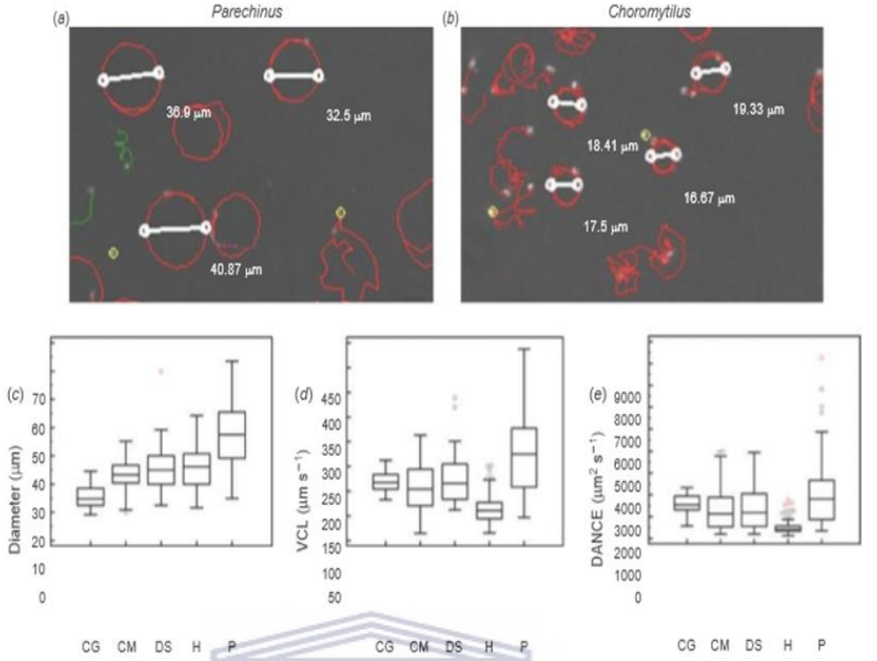


**Fig. 2.** (*a*, *b*) Actual measurement of the diameter of the helix in *Parechinus* (*a*) and *Choromytilus* (*b*). (*c*–*e*) Box plots of the diameter of the helical tracks (*c*) and the curvilinear velocity (VCL; *d*) and DANCE represents the two dimensional space occupied by a motile sperm during 1 s (VCL × ALH; *e*) for these tracks in the five species investigated in the present study. The boxes show the interquartile range, with the median value indicated by the horizontal line; whiskers show the range. Circles indicate outliers. CG, *Crassostrea gigas*; CM, *Choromytilus meridionalis*; DS, *Donax serra*; H, *Haliotis midae*; P, *Parechinus angulosus*. The diameter of the helical tracks and VCL of *Parechinus* differed significantly from those of the other four species ($P$ , 0.05).

high-resolution differential interference microscopy at a magnification of ×400, some movement of the flexible spike attached to the acrosome (Fig. 6) was documented. Fig. 6 shows scanning and transmission electron micrographs of tiger prawn spermatozoa. In Fig. 6*b*, the basic sperm components are shown, whereas Fig. 6*c* shows an enlarged view of the microtubule ring that seems to be able to provide movement of the acrosomal spike. It is unlikely that the spike can cause large displacement of the spermatozoa, but it may be a way of orientating spermatozoa to the eggs.

### *Special case of insects with motile spermatozoa: desert ant, honeybee and fruit fly spermatozoa*

The first author (GvdH) has access to video clips of desert ant spermatozoa (Pearcy *et al.* 2014), honeybee spermatozoa from Morocco (Microptic) and fruit fly spermatozoa (in the female reproductive system; Professor Scott Pitnick). Desert ant spermatozoa swim in groups of 50–100 with their heads glued together at the anterior end, with individual spermatozoa coming loose. In this ant species, as well as in honeybee and fruit fly, the spermatozoa seem to clearly exhibit fairly slow helical movement and progressive forward movement. The ant work has been published (Pearcy *et al.* 2014) and CASA-Mot findings of honeybee spermatozoa are in the process of being published by L. W. Simmons and C. Gasparini.

## Discussion

CASA-Mot analysis of five species in two invertebrate phyla of so-called broadcast spawners show helical sperm tracks similar to those described by many investigators (Levitan 1993, 1995, 1998; Au *et al.* 2001; Farley 2002; Riffell and Zimmer 2007; Fitzpatrick *et al.* 2010; Liu *et al.* 2011; Suquet *et al.* 2013; Fabbrocini *et al.* 2016) or at least analysed by CASA-Mot or objective sperm motility analysis using alternative methods (Basti *et al.* 2013; Lymbery *et al.* 2016).

There are few detailed descriptions of the nature of the patterns of these tracks, except for Liu *et al.* (2011) and Vogel *et al.* (1982), who referred to the helical tracks as 'Don Giovanni' and the forward moving tracks as 'Don Ottavio'. The implication is that theoretically there will be a bigger oocyte hit rate with helical swimming presumably because of the larger search area per volume. Farley (2002) correctly indicated that 'most investigators measure sperm swimming velocity without accounting for the helical motion of sperm, thereby obtaining an inflated estimate of the velocity with which sperm approach eggs'. During helical swimming, spermatozoa seem to collide more quickly with an oocyte provided oocytes are not further than 100 mm apart. This hypothetical distance of 100 mm is debatable because Roux *et al.* (2014) found that optimal fertilisation and hatch rates of embryos of *H. midae* ( 90%) under controlled aquaculture conditions are attained when the sperm concentration

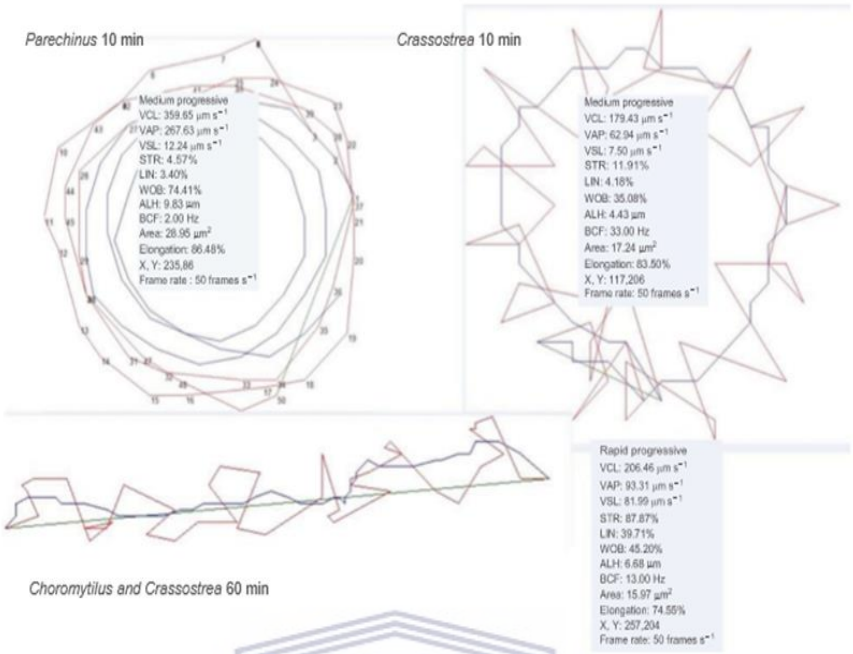


**Fig. 3.** Detail of characteristic sperm motility tracks in four species showing eight kinematic parameters. Red, curvilinear velocity (VCL); blue, average path velocity (VAP); green, linearity (LIN). Sperm were recorded at 50 frames per second. Accordingly, from one number to the next in the diagram for *Parechinus* represents one-fiftieth of a second. In all five species (four shown here), spermatozoa swim helically within minutes after dilution and retain helical swimming even up to 60 min, except in *Choromytilus* and *Crassostrea*, in which most spermatozoa start to swim fast progressively forward. VSL, straight line velocity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat-cross frequency.

is between $5 \times 10^3$ and $5 \times 10^4$ spermatozoa $mL^{-1}$ and the egg density is only approximately 50 eggs $mL^{-1}$. When the sperm concentration in *H. midae* reaches $5 \times 10^5$ spermatozoa $mL^{-1}$, hatch rates decreased to „ 70% (Roux *et al.* 2014). Under natural conditions these sperm and egg numbers may change, but at least the above data may still support the importance of helical swimming as well as chemoattraction rather than just mathematical extrapolation of helical tracks.

The helical swimming in general also accounts for higher fertilisation rates in sea urchins according to Farley (2002). Farley (2002) states that when selecting for gamete traits there should be consideration of the helical nature of sperm swimming and variations in helix characteristics, as well as variation in egg size and number. The present study shows that in broadcast spawners such as *P. angulosus*, helix diameter is positively correlated with high VCL and VAP compared with the smaller-diameter helix with lower VCL and VAP. The rapid sperm population may have a higher likelihood of encountering an oocyte and represents the actual fast swimmers with a large-diameter helix and, for a particular species, these spermatozoa may be those that preferentially fertilise the oocytes. This approach is important in assessing aspects such as sperm competition, sperm limitation, defining sperm quality and in toxicology.

We believe that the DANCE (VCL ALH) parameter, which provides an estimate of the space occupied by spermato- zoa over 1 s, may provide better estimates of sperm behaviour and the likelihood of reaching an oocyte than just the diameter of the helix or speed individually, and hope this is tested in future studies.

As suggested above, the characteristics of a spermatozoon's helical trajectory may affect the probability of its approach to an egg by chance. Helical motion is also believed to play a subsequent role in chemotaxis, whereby the difference in the concentration of sperm attractant on either side of the path creates a periodical signal used for orientation towards the source (e.g. Jikeli *et al.* 2015). The considerable spread of diameters of the helical paths reported here in a suite of broadcasting species may presumably reflect differences between the species in the exact circumstances of spawning, chemoattraction and fertilisation, although interpretation of the data in this way must await additional information. Chemotactic swimming responses to egg or tissue extracts have also been demonstrated in spermatozoa of spermcasting species of

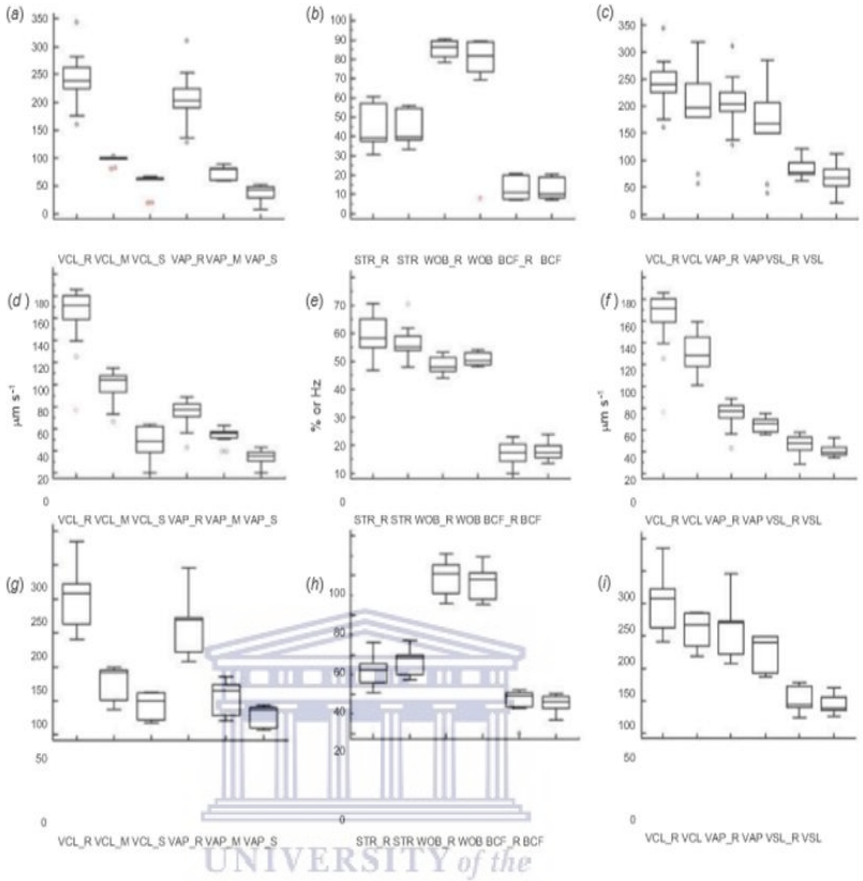




**Fig. 4.** Box plots showing kinematic parameters for rapid, medium and slow subpopulations of three representative species, namely (*a–c*) *Parechinus*, (*d–f*) *Crassostrea* and (*g–i*) *Donax*. The boxes show the interquartile range, with the median value indicated by the horizontal line; whiskers show the range. Circles indicate outliers. VCL_R, curvilinear velocity (VCL) in the rapid sperm subpopulation; VCL_M, VCL in the medium subpopulation; VCL_S, VCL in the slow subpopulation; VCL, mean VCL for the entire sperm population; VAP_R, average path velocity (VAP) for the rapid sperm subpopulation; VAP_M, VAP for the medium subpopulation; VAP_S, VAP for the slow sperm subpopulation; VSL_R, straight line velocity (VSL) in the rapid sperm subpopulation; VSL, mean VSL for the entire sperm population; STR_R, straightness (STR) in the rapid subpopulation; WOB_R, wobble (WOB) in the rapid subpopulation; BCF_R, beat cross frequency (BCF) in the rapid subpopulation; STR, mean STR in the entire population; WOB, mean WOB in the entire population; BCF, mean BCF in the entire population.

**Table 3. Spearman rank correlations of curvilinear velocity (VCL), average path velocity (VAP) and diameter of sperm helical tracks of all five species combined**

For each species, 100 helical tracks were measured using the Measure Tool of the Sperm Class Analyser (SCA; Microptic) CASA-Mot system. DANCE was calculated as VCL × ALH

| | VCL | | VAP | | Diameter | | Perimeter | | Area | | DANCE | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *r* | *P*-value | *r* | *P*-value | *r* | *P*-value | *r* | *P*-value | *r* | *P*-value | *r* | *P*-value |
| VCL | | – | 0.89 | „0.0001 | 0.41 | „0.0001 | 0.3 | „0.0001 | 0.31 | „0.0001 | | – |
| VAP | 0.89 | „0.0001 | | – | 0.62 | „0.0001 | 0.62 | „0.0001 | 0.63 | „0.0001 | 0.68 | „0.0001 |
| Diameter | 0.41 | „0.0001 | 0.62 | „0.0001 | | – | –0.41 | „0.0001 | | – | 0.68 | „0.0001 |

hydroid by Richard L. Miller (e.g. Miller 1973) in studies plotting sperm movements by non-computerised analysis of cine or multiple-exposure photomicrography. Hydroid spermatozoa on the surface of microscope slides moved in a circular motion, suggesting that, as with broadcasters, a helical motion would occur in deeper water, because helical three-dimensional motion becomes circular in spermatozoa constrained by a flat surface (Guerrero *et al.* 2011). This suggests that comparison by

**Table 4. Spearman rank correlations for curvilinear velocity (VCL) and average path velocity (VAP) with amplitude of lateral head displacement (ALH) in four representative species**

VCL is highly positively correlated with ALH in all species. For each species, 100 helical track diameters were measured, as indicated in Table 3

| | ALH | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Parechinus* | | *Donax* | | *Haliotis* | | *Choromytilus* | |
| | *r* | *P*-value | *r* | *P*-value | *r* | *P*-value | *r* | *P*-value |
| VCL | 0.58 | „0.0001 | 0.8 | „0.0001 | 0.76 | „0.0001 | 0.81 | „0.0001 |
| VAP | 0.76 | „0.0001 | 0.315 | „0.0001 | 0.67 | „0.0001 | 0.315 | „0.003 |

CASA-Mot of the characteristics of helical sperm motion between broadcasters and spermcasters may prove informative in relation to their adaptations for their different mechanisms of achieving fertilisation. Any distinctions may be related, in part, to the different size and nature of the 'target' to be found by spermatozoa: in broadcast spawning, this is typically an egg, whereas in spermcast spawning the spermatozoa have to reach a conspecific adult and, in many cases, will be drawn in by the feeding current.

Studies of sperm subpopulations, such as rapid sperm percentages and kinematics, to evaluate the effects of environmental factors have been rarely attempted, but Fabbrocini and

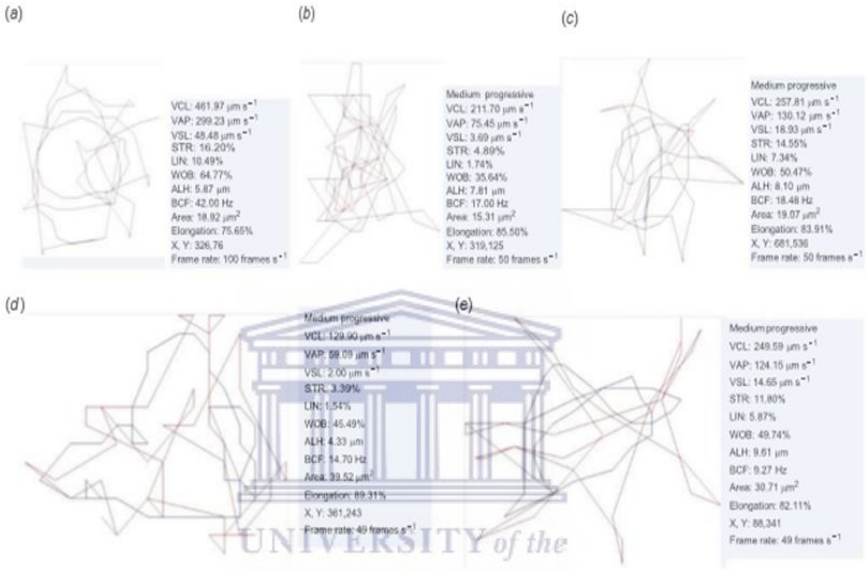


**Fig. 5.** Hyperactivated-like patterns of spermatozoa following activation of (*a*) *Choromytilus*, (*b*) *Crassostrea* and (*c*) *Parechinus* with egg water, (*d*) *Choromytilus* with 10 mM caffeine in sea water and (*e*) *Choromytilus* with 5 mM procaine hydrochloride and 10 mM caffeine in sea water. These tracks are characterised by rapid curvilinear velocity (VCL), very large amplitude of lateral head displacement (ALH) and small linearity (LIN). VAP, average path velocity; VSL, straight line velocity; STR, straightness; WOB, wobble; BCF, beat cross frequency.

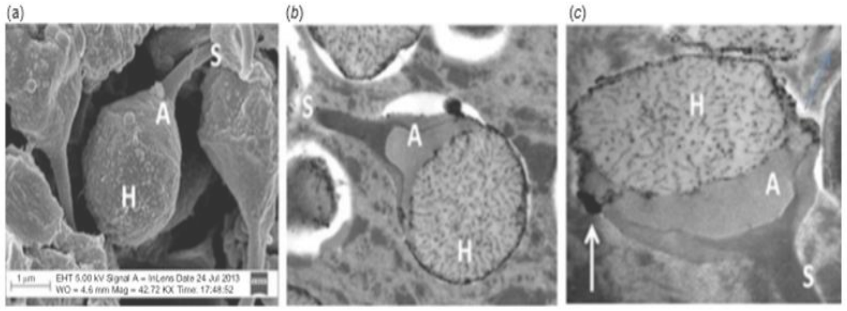


**Fig. 6.** (*a*) Scanning electron micrograph and (*b*, *c*) transmission electron micrographs of tiger prawn spermatozoa. The arrow indicates the microtubular apparatus that may be involved in spike mobility. A, acrosome; H, head; S, spike.

D'Adamo (2011) and Fabbrocini *et al.* (2016) have shown the value of studying subpopulations. Mortimer *et al.* (2015) emphasised that population averages for sperm kinematics in humans and animals are of limited use. Unfortunately, most papers using CASA-Mot in broadcast spawners considered only averages, and this may bias interpretation of data because of the very large ranges of values for most kinematic parameters. Stewart *et al.* (2012) showed in a very elegant experiment that there were no differences in sperm velocities in the presence or absence of eggs. However, averages were used and, in this instance, may have 'hidden' subtle but important differences in motility subpopulations. In the present study, the pattern of sperm movement changed when spermatozoa were exposed to egg water or eggs, and spermatozoa started to show hyperactive-like patterns that could also be induced with classical $Ca^{2+}$ modulators. The cation channels of spermatozoa (CatSper family of genes) responsible for hyperactivation is also present in most invertebrates, including cnidarians, and, accordingly, hyperactivation has been evolutionarily conserved for a long time (Cai and Clapham 2008).

Spermatozoa of testicular origin was used in the case of three species in the present study, in contrast with the use of spawned spermatozoa from *P. angulosus* and *H. midae*. However, we compared sperm motility from testicular origin versus spawned spermatozoa in *C. meridionalis* and, by and large, there were no differences except for activation time and the fact that helical sperm tracks were retained for longer (i.e. after 1 h) in samples of spawned spermatozoa. Furthermore, *D. serra* spermatozoa of testicular origin also showed predominantly helical tracks after 1 h. Therefore, it seems that testicular spermatozoa from gravid testes and spawned spermatozoa share similar characteristics. Care needs to be taken in terms of gonadal development and potential sperm maturation before spawning because there appear to be differences in CASA-Mot parameters in relation to testicular maturation and season (Fabbrocini *et al.* 2016).

Are there species-specific motility traits among broadcast spawners? The present study shows many differences in motility groupings (percentages and kinematics) among species, and most of these can be related to the form and dimensions of the helical track. Are these patterns an expression of the 'fertilisation environment' and/or do they assist in understanding reproductive strategies, such as pseudocopulation, spermcasting and broadcast spawning? *P. angulosus* occurs in rock pools in groups and as single individuals, but they are mobile. Rock pools are often isolated from the sea and wave action during low tide and, if spawning occurs in these isolated pools, it will largely represent a typical sperm broadcast scenario, as in *H. midae*, and the large helical swimming, very rapid hit-and-run, 'Don Giovanni' swimming spermatozoa may suggest sperm competition rather than sperm limitation. Levitan (2000) showed that fast swimming spermatozoa attain higher fertilisation rates than slow swimming spermatozoa, but different trade-offs exist between speed and longevity in some sea urchins.

*C. gigas* and, in particular, *C. meridionalis* inhabit a high-energy environment with large wave action. *C. meridionalis* is not mobile, but occurs at very high densities growing almost on top of each other. It is difficult to conceive how gametes will be released and fertilisation will take place in a typical broadcast mode in this turbulent environment despite the fact that the animals are closely situated. Unfortunately, no information is available on the fertilisation biology of *C. meridionalis*. All that we can currently rely on are the facts at hand in terms of sperm motility and sperm motility modulation for this species. *C. gigas* and *C. meridionalis* have very small helical sperm track diameters and apparently modulation of sperm motility from helical to more forward progressing can occur relatively soon after dilution with sea water. Does this perhaps imply that spermatozoa may be deposited much closer to the eggs or even that spermatozoa are somehow taken inside the mantle cavity to allow protected fertilisation? In such cases, some of the bivalves living in a high-energy environment may be making use of different ways to ensure fertilisation and present a scenario of fertilisation of non-dispersing eggs.

Many factors may affect sperm motion, reproductive strategy and fertilisation success, and among these are the role and size of oocytes, the morphology of spermatozoa (Fitzpatrick *et al.* 2010) and shear forces of the environment (Riffell and Zimmer 2007) to mention a few. Many theoretical models exist to describe sperm motion in external fertilisers (Levitan 1993, 1995, 1998, 2000) and, in the process, the different trade-offs between sperm velocity, sperm concentration and egg size, among others. However, the emphasis in the present study was to dissect sperm motion in great detail using CASA-Mot to provide basic but very detailed baseline information for future investigations.

## Conflicts of interest

G. van der Horst is a senior consultant for Microptic SL, manufacturers of the SCA CASA software used in the present study. However, all analyses were performed independently and the association had no bearing on the outcome.

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# APPENDIX III

## PEARSON PRODUCT MOMENT CORRELATIONS

| | ALH | Area | Circum | DANCE | Diamter | Head (L) | Head (W) | Tail | Total Length | VAP | VCL |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ALH | | -0,0040 | -0,0439 | 0,8002 | -0,0436 | -0,1087 | -0,0265 | 0,1343 | 0,1489 | 0,1482 | 0,5178 |
| | | (15) | (15) | (15) | (15) | (15) | (15) | (15) | (15) | (15) | (15) |
| | | 0,9886 | 0,8765 | 0,0003 | 0,8774 | 0,6998 | 0,9253 | 0,6332 | 0,5963 | 0,5981 | 0,0480 |
| Area | -0,0040 | | 0,9895 | 0,4929 | 0,9899 | 0,6140 | -0,6822 | -0,6040 | -0,5904 | 0,9076 | 0,7884 |
| | (15) | | (15) | (15) | (15) | (15) | (15) | (15) | (15) | (15) | (15) |
| | 0,9886 | | 0,0000 | 0,0619 | 0,0000 | 0,0149 | 0,0051 | 0,0171 | 0,0205 | 0,0000 | 0,0005 |
| Circum | -0,0439 | 0,9895 | | 0,4493 | 0,9980 | 0,6311 | -0,6861 | -0,6044 | -0,5870 | 0,9071 | 0,7561 |
| | (15) | (15) | | (15) | (15) | (15) | (15) | (15) | (15) | (15) | (15) |
| | 0,8765 | 0,0000 | | 0,0929 | 0,0000 | 0,0116 | 0,0047 | 0,0170 | 0,0214 | 0,0000 | 0,0011 |
| DANCE | 0,8002 | 0,4929 | 0,4493 | | 0,4545 | 0,2851 | -0,4536 | -0,3329 | -0,3306 | 0,6377 | 0,8893 |
| | (15) | (15) | (15) | | (15) | (15) | (15) | (15) | (15) | (15) | (15) |
| | 0,0003 | 0,0619 | 0,0929 | | 0,0888 | 0,3030 | 0,0895 | 0,2253 | 0,2287 | 0,0105 | 0,0000 |
| Diamter | -0,0436 | 0,9899 | 0,9980 | 0,4545 | | 0,6340 | -0,6934 | -0,6032 | -0,5854 | 0,9149 | 0,7647 |
| | (15) | (15) | (15) | (15) | | (15) | (15) | (15) | (15) | (15) | (15) |
| | 0,8774 | 0,0000 | 0,0000 | 0,0888 | | 0,0111 | 0,0042 | 0,0173 | 0,0219 | 0,0000 | 0,0009 |
| Head (L) | -0,1087 | 0,6140 | 0,6311 | 0,2851 | 0,6340 | | -0,7253 | -0,9432 | -0,9181 | 0,5316 | 0,3840 |
| | (15) | (15) | (15) | (15) | (15) | | (15) | (15) | (15) | (15) | (15) |
| | 0,6998 | 0,0149 | 0,0116 | 0,3030 | 0,0111 | | 0,0022 | 0,0000 | 0,0000 | 0,0414 | 0,1577 |
| Head (W) | -0,0265 | -0,6822 | -0,6861 | -0,4536 | -0,6934 | -0,7253 | | 0,7190 | 0,7134 | -0,6970 | -0,6345 |
| | (15) | (15) | (15) | (15) | (15) | (15) | | (15) | (15) | (15) | (15) |
| | 0,9253 | 0,0051 | 0,0047 | 0,0895 | 0,0042 | 0,0022 | | 0,0025 | 0,0028 | 0,0039 | 0,0111 |
| Tail | 0,1343 | -0,6040 | -0,6044 | -0,3329 | -0,6032 | -0,9432 | 0,7190 | | 0,9966 | -0,5361 | -0,4181 |
| | (15) | (15) | (15) | (15) | (15) | (15) | (15) | | (15) | (15) | (15) |
| | 0,6332 | 0,0171 | 0,0170 | 0,2253 | 0,0173 | 0,0000 | 0,0025 | | 0,0000 | 0,0394 | 0,1210 |
| Total Length | 0,1489 | -0,5904 | -0,5870 | -0,3306 | -0,5854 | -0,9181 | 0,7134 | 0,9966 | | -0,5281 | -0,4127 |
| | (15) | (15) | (15) | (15) | (15) | (15) | (15) | (15) | | (15) | (15) |
| | 0,5963 | 0,0205 | 0,0214 | 0,2287 | 0,0219 | 0,0000 | 0,0028 | 0,0000 | | 0,0430 | 0,1263 |
| VAP | 0,1482 | 0,9076 | 0,9071 | 0,6377 | 0,9149 | 0,5316 | -0,6970 | -0,5361 | -0,5281 | | 0,8844 |
| | (15) | (15) | (15) | (15) | (15) | (15) | (15) | (15) | (15) | | (15) |
| | 0,5981 | 0,0000 | 0,0000 | 0,0105 | 0,0000 | 0,0414 | 0,0039 | 0,0394 | 0,0430 | | 0,0000 |
| VCL | 0,5178 | 0,7884 | 0,7561 | 0,8893 | 0,7647 | 0,3840 | -0,6345 | -0,4181 | -0,4127 | 0,8844 | |
| | (15) | (15) | (15) | (15) | (15) | (15) | (15) | (15) | (15) | (15) | |
| | 0,0480 | 0,0005 | 0,0011 | 0,0000 | 0,0009 | 0,1577 | 0,0111 | 0,1210 | 0,1263 | 0,0000 | |