Aspects of the reproduction of male and female African penguins (*Spheniscus demersus*) with special reference to sperm biology and cryopreservation

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Sphenisciformes

Transmission electron microscopy

DECLARATION

I declare that "**Aspects of the reproduction of male and female African penguins (*Spheniscus demersus*) with special reference to sperm biology and cryopreservation"** is my own work, that has not been submitted before for any degree or assessment in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by means of complete references.

Patrick Siyambulela Mafunda August 2018

Signed

DEDICATION

This thesis is dedicated to Alondwe and Alwaze. The sky is the first step. Through it all learn to trust in Jesus. “Luke 23:43 ".

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First and foremost, I want to thank God for giving me the opportunity to gain more knowledge and understanding and being with me through the whole process.

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ABSTRACT

In the marine environment, penguins have been described as curators and serve a critical role in ecological balance. The African penguin (*Spheniscus demersus)* has undergone a rapid population decline, mainly due to disturbances in their natural habitat. The African penguin was up-listed from vulnerable to endangered on the IUCN Red List for Threatened Species in 2010 and thus urgent conservation action is required. Integral to long-term conservation action of any species is a basic knowledge of its reproductive biology, which is currently lacking for African penguins. The main aim of this investigation was to evaluate techniques for the collection of semen in African penguin and to determine sperm quality in order to cryopreserve sperm for in vitro fertilization (IVF) purposes of captive and wild populations. Semen was collected once a week during two breeding seasons from two captive African penguins. Ejaculates (*n=51*) were obtained over two breeding seasons (Jan-Feb and Jun-Oct) and evaluated for semen volume, sperm concentration, sperm vitality, sperm motility and sperm morphology. In addition twelve (six females and six males, *n=4* were breeding pairs) captive African penguins were monitored for hormone (estradiol, testosterone, progesterone) levels prior to and after the egg-laying period.

The testes were asymmetrical in adults, with the right testis on average shorter in length, width and volume respectively (16.80 ± 4.37 mm; 7.93 ± 2.63 mm; 0.75 mL) compared to the left testis (25.39 ± 5.85 mm; 11.48 ± 4.09 mm; 2.46 mL). The ovaries displayed variation in shape and size among the penguins, the adult ovary has a mean length of 25.72 ± 5.37 mm and a mean width of 9.02 ± 3.87 mm. The Follicles ranged from white small follicles with a diameter of <0.01 mm to mature, yellow large follicles which had a maximum diameter of 22 mm. The testis and ovary histological features such as

structure, weight and size, can give a clear indication of breeding status in African penguin. Estradiol levels showed a biphasic pattern in three of the four breeding females, whereas no clear pattern could be followed in other hormones investigated.

Semen volume ranged from 0.01 to 0.1 ml, sperm concentration from 802.6 to 7808.8 $x10^6$/ml and total number of sperm per sample ranged from 3.42 to 740.18 $x10^6$. The percentage total motility was between 40.1 and 87.1%. The recorded velocities was for curvilinear velocity (VCL 81.5 ± 10.2 µm/s), straight-line velocity (VSL 42.72 ± 7.3 µm/s) and average path velocity (VAP 59.4 ± 8.2 µm/s), and kinematics at straightness of track (STR 71.4 ± 8.9 %), linearity of track (LIN 52.4 ± 8.1 %), amplitude of lateral head displacement (ALH 2.3 ± 0.2 µm) and beat cross frequency (BCF 16.8 ± 3.8 Hz). Sperm quality and semen parameters were similar across all samples collected over breeding seasons. In comparison to fresh semen, percentage total motility of thawed semen decreased to 16.8% after two hours in liquid nitrogen. Since spermatozoa differ notably in their morphology within species, phase-contrast microscopy, scanning electron microscopy and transmission electron microscopy were used to examine structural abnormalities. The sperm morphology is almost identical and largely resembles that of non-passerine birds in terms of the filiform head, small acrosome and mid-piece containing 13 spherical mitochondria, arranged around the proximal and distal centrioles in a single helix. The ultrastructure of the sperm principal piece revealed the typical 9+2 microtubular arrangement without any outer dense fibres. An unusual feature was the occurrence of multiple axonemes contained in one plasmalemma in 4% of spermatozoa. Multiple axonemes found in penguin flagella could be an apomorphism that distinguish them from other bird spermatozoa. This research represents a critical step in the conservation and long-term survival of the African penguin by evaluating techniques for the collection and determination of sperm quality in order to cryopreserve sperm for IVF. August 2018

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LIST OF ABBREVIATIONS

°C	Degree Celsius
A	Acrosome
ALH	Amplitude of Lateral Head Displacement
An	Annulus
ANOVA	Analysis of Variance
APBMP	African Penguin Biodiversity Management Plan
ART	Assisted Reproductive Technology
ATP	Adenosine Triphosphate
BCF	Beat Cross Frequency
BL	Basal Lamina
BV	Blood Vessels

C	Capitulum
CASA	Computer-aided Sperm Analysis
CL	Corpus Luteum
CP	Central Pair
DHT	Dihydrotestosterone
EIA	Enzyme Immunoassays
ELISA	Enzyme-Linked Immunosorbent Assays
EP	End Piece
F	Female
FEM	Faecal Estrogen Metabolite
FF	Follicular Fluid
FPM	Faecal Progestogen Metabolite
GR	Granulosa Cells

H	Head
IUCN	International Union for Conservation of Nature
IVF	In Vitro Fertilization
L	Lumen
LC	Leydig Cells
LIN	Linearity
M	Male
mg	Milligram
ML	Milliliter
MP	Mid-piece
MT	Mitochondria
N	Nucleus
NRF	National Research Foundation of South Africa

NZG	National Zoological Gardens of South Africa
O	Oocyte
ODM	Outer Microtubule Doublet
P	Primary Spermatocyte
Pc	Proximal Centriole
PF	Primary Follicle
Pi	Leptotene
PM	Plasma Membrane
PP	Principal Piece
Ps	Secondary Spermatocyte
Pz	Pachytene
SANCCOB	The Southern African Foundation for the Conservation of Coastal Birds
SCA	Sperm Class Analyzer

SD	Standard Deviation
SER	Sertoli cells
SF	Secondary Follicle
Sg	Spermatogonia
SP	Spermatids
ST	Seminiferous Tubules
STR	Straightness
T	Testosterone
TE	Theca Interna
TEM	Transmission Electron Microscopy
TI	Theca Interna
TB	Toluidine blue
TM	Total Motility

UWC	University of the Western Cape
V	Vitelline Membrane
VAP	Average Path Velocity
VCL	Curvilinear Velocity
VSL	Straight-line Velocity
VD	Vas deferens
WOB	Wobble
µL	Microliter

CHAPTER 1

Introduction to the Study

1.1 General Introduction

The African penguin (*Spheniscus demersus*), Africa's only existent penguin, is endemic to southern Africa and classified as a non-passerine bird. In June 2010, the African penguin was uplisted from vulnerable to endangered on the IUCN Red List for Threatened Species because of the large, sustained decrease in their numbers in the 20th century (IUCN 2013; Borboroglu and Boersma, 2013). The breeding population has decreased by more than 50% in the three most recent generations and continues to decline. The African Penguin Biodiversity Management Plan (APBMP) (Crawford *et al*., 2010) recommended the development of conservation strategies for this penguin species both in the wild as well as in captive populations. One such strategy would be to understand the reproductive biology of *S. demersus*, since there is currently only limited information available for this species (O'Brien *et al*., 2015). Since species survival is related to reproduction performance, the development of techniques such as semen collection and cryopreservation with the ultimate goal for insemination can assist to preserve the genetic pool of this species. At the age of four years, Sphenisciformes are sexually mature and will breed for the first time in its natural habitat. However, in captivity these birds will select a partner and start breeding much earlier, usually at two years of age (Conway *et al*., 1999).

Aspects of reproductive biology that could enhance breeding success of both wild and captive penguin populations are obtaining basic physiological information on breeding

cycles as well as a full understanding of the normal semen parameters and subsequent assessment of semen quality. In order to achieve fertilization and sustain early embryonic development, it has been difficult to identify a single test capable of accurately predicting the fertility potential of an individual ejaculate (Møller *et al*., 2008). Instead, the evaluation of sperm motility, concentration, vitality and morphology are considered the four most important parameters in any semen analysis (Cooper *et a*l., 2002; Kuster *et al*., 2004; Łukaszewicz *et al*., 2008). These parameters are assumed to be under intense selection because of their close correlation with fertilization success (Møller *et al*., 2008).

Knowledge gained from this study of the African penguin population will contribute to the development of strategies or tools for the conservation of the species as well as the improvement of methodologies for enhancing reproduction.

1.2 Aims and Objectives

This study attempted to address one of the conservation strategies that can be developed in order to conserve the gene pool of African penguin considering the endangered status and the threats it faces as identified in the APBMP. The main aims of this investigation were to evaluate techniques for the collection of semen in African penguin and to define sperm quality in order to cryopreserve sperm for IVF purposes. An additional aim was to correlate the semen quality with hormone profiling in both captive and wild/free-ranging populations.

In order to achieve these aims, the following objectives were set:

- To describe the macro-anatomy and histology of the male and female reproductive systems of *Spheniscus demersus* and relate these aspects to

breeding status.

- To develop techniques to sample semen/sperm and determine parameters that defines high quality sperm.
- To collect semen, blood and faeces during the breeding season and correlate this with hormonal profiles.
- To develop cryopreservation procedures by testing specific extenders and cryoprotectants.

1.3 Outline of the study

Chapter 2: Literature review

Chapter 2 gives an overview of the literature relating to African penguin distribution, their anatomy and evolution. The reproductive biology and sperm biology of the African penguin are introduced in this chapter.

Chapter 3: Materials and Methods

This chapter gives more detailed information on the methodology and techniques used and modified in the study as well as the statistical analysis.

Chapter 4: Results

This chapter includes all the findings of the study and narrates them with avian species as a whole.

Chapter 5: Discussion and conclusion

This chapter emphasis on discussing the data from other chapters and explain the relationship between the micro and macro anatomy, hormone profiling, sperm characteristic and motility and cryopreservation. It further compares the findings of the study with what is known in the order Sphenisciformes, and the avian species as a whole. This chapter also provides the general conclusion or remarks of the study.

Chapter 6: Future studies

This chapter highlights future studies and provides suggestions on improvements which can contribute to the management of the species.

1.4 Research output

1.4.1 Publications

- Mafunda, .P.S., Kotze, A., Maree, L., Van der Host, G. (2017). Sperm structure and sperm motility of the African and Rockhopper penguins with special reference to multiple axonemes of the flagellum. *Theriogenology* 99:1-9.

1.4.2 Conference proceedings

- Mafunda, P.S., (05 - June -2015). Managing and conserving the biodiversity of African penguins. Oral presentation, Two Oceans Aquarium, Cape Town.

- Mafunda, P.S., Kotze, A., Maree, L., Van der Host, G. (19 – 20 November 2015). Aspects of the reproduction of male and female African penguins (*Spheniscus demersus*) with special reference to sperm biology and cryopreservation. Oral presentation at the 6th Annual Research Symposium of the National Zoological Gardens (NZG) & University of Pretoria – Faculty of Veterinary Sciences (UP-FVS), Pretoria.

- Mafunda, P.S., Kotze, A., Maree, L., Van der Host, G. (2015). Valuable study on the reproduction of African penguins. http://www.environmentmag.co.za/ebook/Env-21. Magazine news letter.

- Mafunda, P.S., Kotze, A., Maree, L., Van der Host, G. (4 September 2016). Aspects of the reproduction, endocrinology, semen characteristics and cryopreservation in African penguin (*Spheniscus demersus*). Oral presentation and the International Penguin Early Careers Workshop (IPECS), Cape Town.

- Mafunda, P.S., Kotze, A., Maree, L., Van der Host, G. (5 – 9 September 2016). Aspects of the reproduction, endocrinology, semen characteristics and cryopreservation in African penguin (*Spheniscus demersus*). Poster presentation at the International Penguin Congress 9 (IPC9), Cape Town.

- Mafunda, P.S., Kotze, A., Maree, L., Van der Host, G. (17 – 18 November 2016). Understanding African penguin reproductive biology: an essential step for conserving their genetic diversity. Oral presentation at the 7th Annual Research Symposium of the National Zoological Gardens (NZG), Pretoria.

CHAPTER 2

Literature Overview

2.1 Introduction

Penguins have been described as sentinels of the marine environment, observing their patterns we as researchers can have an insight into regional ocean productivity and long-term climate variation (Boersma, 2008). They are all biological contradiction; they are highly specialized species of birds but do not share their usual characteristics. The structure and physiology of penguins have been moulded both by their marine habitat and the climatological peculiarities of their environment. While other birds have wings for flying, penguins have small wings that are adapted as flippers to help them swim in water. Penguins share some of the characteristics of other birds, such as being warm-blooded with body temperatures that range between 37º- 40ºC. They also possess a layer of fat for body insulation. Penguins are wing-propelled diving homeotherms that search their prey in Southern Hemisphere oceans. Penguin physiological and behavioural adaptations help them forage in waters as warm as 28ºC and as cold as 0.6ºC, while maintaining a core body temperature of 38.5ºC (Thomasand Fordyce, 2012). Furthermore, they have a reduced reproductive system with females having one functional ovary (Crawford *et al*., 2011). They are athletic, interesting, and ancient (Borboroglu and Boersma, 2013). They live in different climate conditions, with some species living on the equator and others in Antarctica. Penguins communicate through vocalization and perform physical behaviours, body language and posturing.

2.2. Evolution, general distribution and taxonomic classification of penguins

Otago Peninsula during the 1930s and 1940s was the first to study a long-term penguin population. Between those years, New Zealand together with its southern island and Antarctic dependency was home to over half the world's species of penguin, and it was a centre of penguin research (Thomas and Fordyce, 2012). General insight regarding penguins is that they only exist in the icy parts of the Southern Hemisphere, but only two species of penguins are restricted to Antarctica: the Adélie (*Pygoscelis adeliae*) and the Emperor (*Aptenodytes forsteri*).The oldest penguin fossils date from 55 to 62 million years ago. It is suggested that their origins, may be rooted in the Cretaceous period, 140 to 65 million years ago, when their ancestor was a flying seabird (Borboroglu and Boersma, 2013). Whether that ancestral form was a Graviformes, a Procellariiformes or a Pelecaniformes is unclear.

To date, in the wild, discovery of all penguin fossil fragments have been found exclusively in the Southern hemisphere. Records show that prehistoric penguins were found within the range of present-day penguins. The earliest penguin fossil fragments were found in New Zealand in the mid-1800s, suggesting that penguins flourished between 10 and 40 million years ago (Thomas and Fordyce, 2012).

Penguin species live in environments ranging from the tropics to the South Pole, across the islands and on the continents of the Southern hemisphere. Two species of penguins can be found in extremely diverse climatic regions: in one of the warmest places on the planet, the equator, namely the Galapagos penguin, and the other penguin species can be found in the coldest place on the planet, Antarctica, namely the Emperor penguin (Thomas and Fordyce, 2012).

Penguins vary in size from the little penguin, which weighs just about one kilogram and

is a shallow diver, to the emperor penguin, which weighs 40 kilograms and can dive 500 meters holding its breath for 23 minutes (Borboroglu and Boersma, 2013). In comparison to humans, penguins can cover a fivefold deeper distance when diving. The record dive for human is 101 meters in 4.13 minutes (Borboroglu and Boersma, 2013).

Penguins belong to the Kingdom Animalia, Phylum Chordata, Subphylum Vertebrata, Class Aves and Order Sphenisciformes. Sphenisciformes is the only order within Class Aves that is both flightless and aquatic. Penguins share morphological and molecular characteristics with birds in the Orders Procellariiformes (albatrosses, shearwaters and petrels), Gaviiformes (loons and grebes), and Pelecaniformes (frigate birds). Order Sphenisciformes contains a single family, the Spheniscidea, six genera and 16 to 19 species of penguins, depending on the tools used in classification. The six genera of living penguins are: *Aptenodytes, Eudyptes, Eudyptula, Megadyptes, Pygoscelis* and *Spheniscus*. Eleven of the 19 penguin species are currently listed as threatened, with degradation of terrestrial habitats, pollution and fisheries activities representing primary causes of population decline (IUCN, 2013; O'Brien *et al*., 2015). Four species exist in the genus *Spheniscus* and all of those are classified as threatened with extinction (vulnerable or endangered categories) except the Magellanic penguin (*Spheniscus magellanicus*), which was up-listed to near-threatened status in 2000 in response to the declining population trends (IUCN, 2013).

In order to classify the 19 existing species; morphology and DNA evidence were used as an attempt to unravel the evolutionary relationship of the living penguin species. Both methods of evidence suggest that the genus *Spheniscus* and *Eudyptula* form one closely related group (Thomas and Fordyce, 2012). There are four living species in the genus *Spheniscus*, namely the South American Humboldt and Magellanic Penguins (*Spheniscus humboldti* and *Spheniscus magellanicus*), the African Penguin

(*Spheniscus demersus*) and the Galapagos Penguin (*Spheniscus mendiculus*). *Eudyptula* has only a single species, the Little Blue Penguin (*Eudyptula minor*). *Megadyptes* also has only one species, the Yellow-eyed Penguin (*Megadyptes antipodes*). The genus *Eudyptes* includes the eight species of Crested Penguins. *Pygoscelis* is the genus that includes the stiff-tailed penguins. There are three species, all of which favour cold environments. The popular Adélie Penguin (*Pygoscelis adelie*) is one of the world's most common penguins. Finally, the largest and most popular penguins belong to the genus *Aptenodytes*. Emperor Penguins (*Aptenodytes forsteri*) and King Penguins (*Aptenodytes patagonicus*) are tall, graceful birds with long, thin bills and striking orange colour patches on their necks and lower bills (see Fig 2.1) (Thomas and Fordyce, 2012).

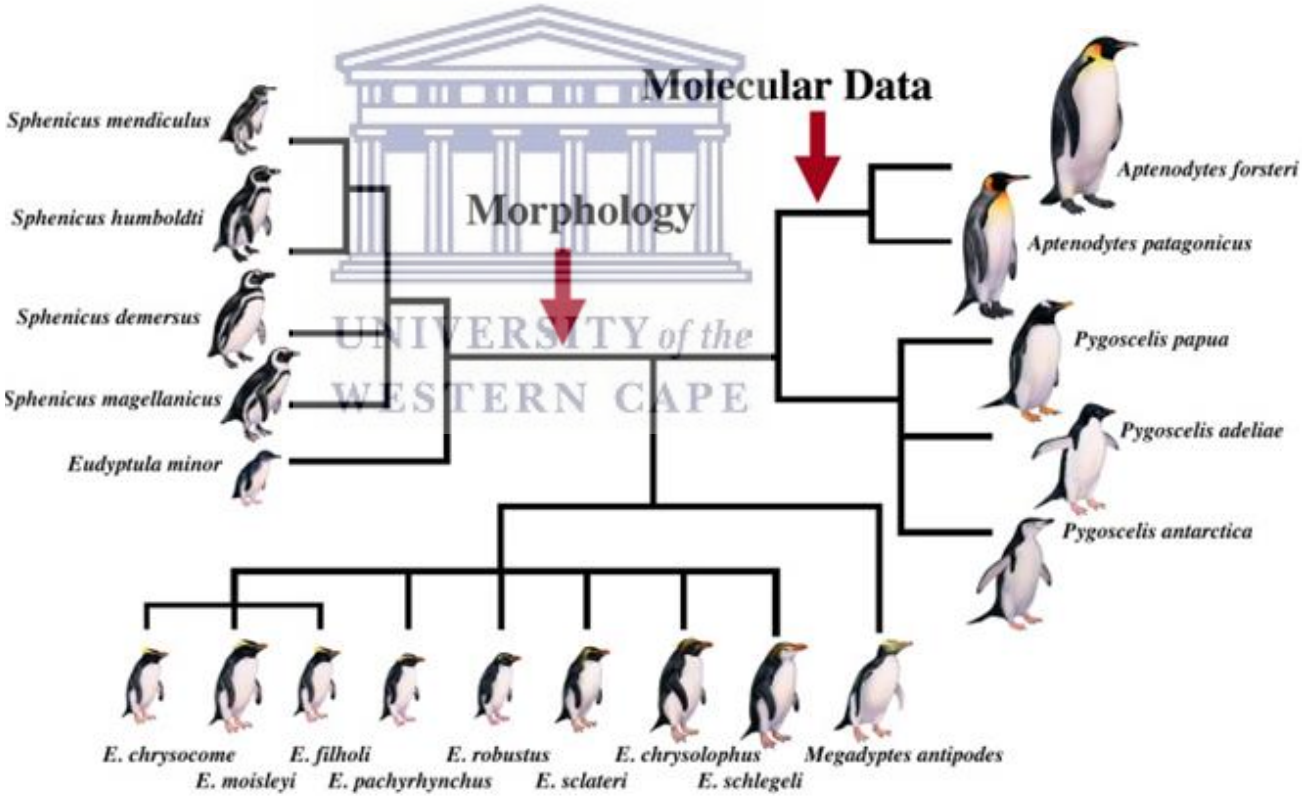

Figure 2.1: Penguin evolutionary relationships. The E.filhonli, although genetically different is still often considered a subspecies of the E. chryscocome. (Ksepka *et al*., 2010).

2.3. Distribution of African penguin

It is suggested that there were 1.5 to 3.0 million African Penguins in South Africa in the early 1900s (Crawford *et al*., 2010). However, factors like losses of breeding habitat, commercial harvesting of eggs (Frost *et al*., 1976), as well as a reduction in prey availability through industrial fishing and changes in the local marine environment (van der Lingen *et al*. 2006, Weller *et al*. 2014) have reduced the population by > 90% since the turn of the 20th century (Crawford *et al*., 2011).

The African penguin (Africa's only present penguin), is confined to southern Africa, where it breeds between central Namibia (Hollams Bird Island) and southern South Africa (Bird Island, Algoa Bay) (Fig 2.2) (Crawford *et al*., 2011). In South Africa, penguins breed in two groups of vicinities, one in the Western Cape Province and the other in the Eastern Cape Province (Fig 2.2). (Crawford *et al*. 2011).African Penguins breed in three regions separated by 600 km (Sherley *at al*., 2014). Comparing all the known breeding sites, 11 are found in Namibia, 11 are in the Western Cape and six in the Eastern Cape. However, just seven colonies held 80% of the global population of 26 000 pairs in 2010 (Crawford *et al*. 2011). Four of these were in the Western Cape and two of them, Robben and Dassen islands (Fig 1), held 60% of the global population in 2004 and 30% in 2009 (Crawford *et al*. 2011). Algoa Bay in the Eastern Cape is home to nearly half of the global population, with the largest colony residing at St. Croix Island.

It has been recorded that these travelling birds have been as high up as Sette Cama, Gabon, on the West African coast, and to the Limpopo River mouth and to Mozambique on the east coast (Borboroglu and Boersma, 2013). The travelling ranges of this species have been recorded up to 100 kilometres offshore, but most occur within 20 kilometres of the coast, except on the Agulhas Bank, where the distribution of their prey extends further offshore (Borboroglu and Boersma, 2013). Strong estimates of age-specific

survival and movement are not available because previous studies have focused on estimates for single colonies (Crawford *et al*. 2010; Crawford *et al*., 2011).

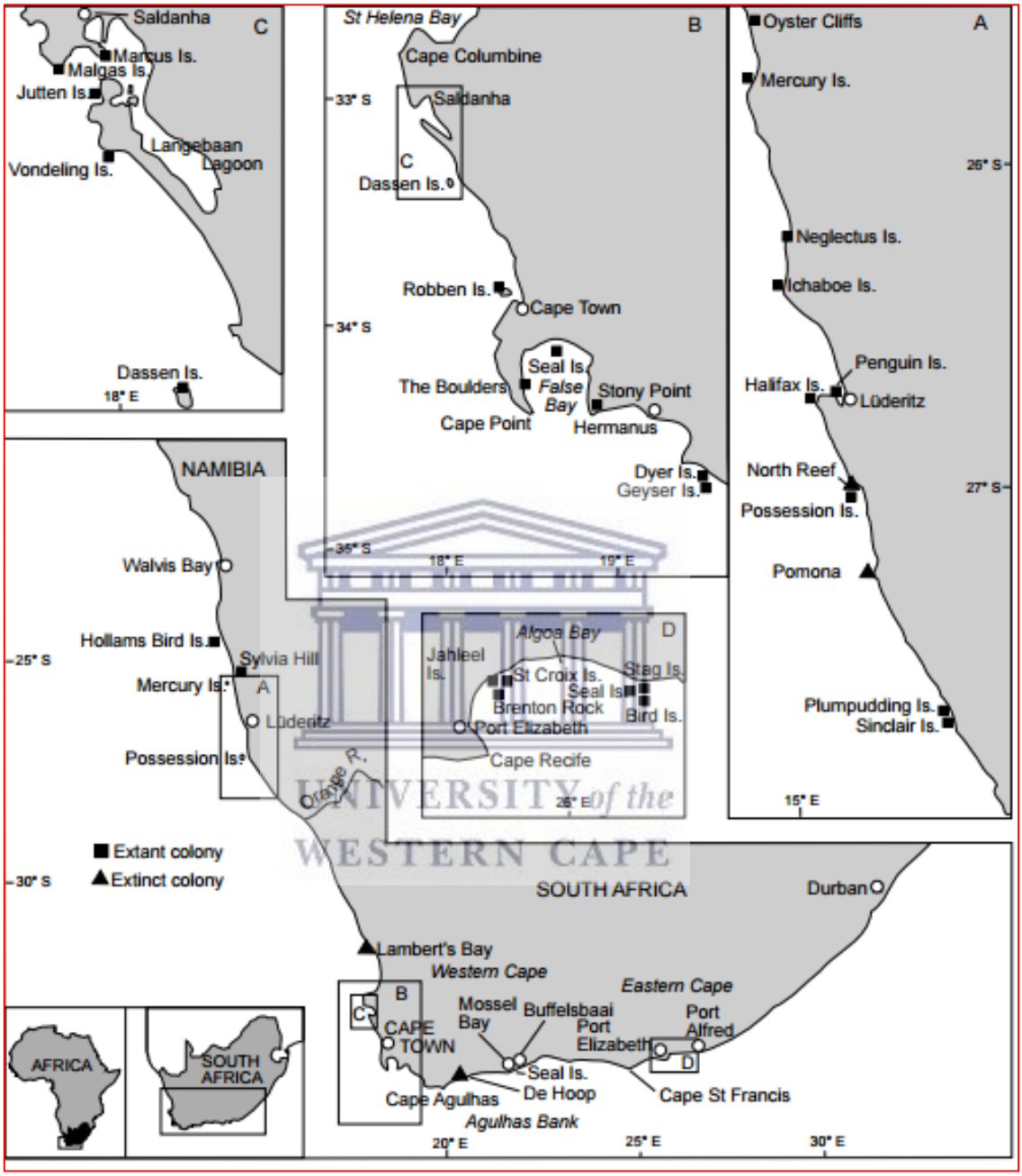

Figure 2.2: Current distribution, breeding localities and abundance of the African penguin (Crawford *et al*., 2011)

2.4. Conservation efforts of African penguins

The marked decrease in *Spheniscus demersus* through the 20th Century has led to essential conservational efforts. In early 1942, the penguin exclusion walls at Dassen Island were built (Price, 1942), this was to prevent the disturbance of penguin by humans. The effort to reduce the negative effect of fisheries on penguins, marine protected areas have been recommended as a valuable tool in an ecosystem approach to fisheries (Pauly *et al.*, 2002; Roberts *et al*., 2001). An ecosystem approach to fisheries aims to plan, develop and manage fisheries in a sustainable way by including the needs of the entire ecosystem, implying that fisheries effects on non-target species should be taken into account.

The dynamics of the African penguin population is dependent on the availability of food and suitable breeding habitat. Despite the enforced protection of numerous African penguin breeding colonies, industrial fisheries still have a major negative impact on the colonies at sea. Effects like changing the structure of marine communities, reducing stocks of fish species fed upon by penguins disturb the penguin population (Tasker *et al*., 2000). The other effort on the management of population decline was counteracted by a unique effort in 2009, involving two sets of islands: one set on the West Coast (the coastal area west of Cape Aghulas); the other in Algoa Bay on the East Coast (the coastal area east of Cape Aghulas). A zone of 20 km radius around one island in each pair was closed to small pelagic fishing while the other island remained open. Penguins in these areas were closely monitored to determine the effect of the closures on their foraging behavior and breeding success. After the pressure of fishing decreased, penguin populations in Algoa Bay began to level off. However, the implementation of stronger local fishery management measures is necessary to substantially increase food availability, and subsequently restore penguin population numbers (Durant *et al.,* 2010; Pichegru *et al*., 2010).

Shannon and Crawford, 1999, reported that the mean proportion of first-year birds in an African penguin population needs to be at least 0.51 in order to ensure the survival and equilibrium of the population. This suggests that increased in egg confiscation, mining of guano, oiling and competition for food over the last decades may have resulted in reduced chick production or reduced survival of juvenile African. The development of new models like the incorporation of density dependence may alter this outcome. Modelling density dependence will be the next step towards improved assessment of the impacts of oiling and disturbance on African Penguins (Shannon and Crawford, 1999). The development of conservational efforts aimed at improving methods for identification and collection of oiled birds should be implemented, thereby minimizing loss of clutches.

2.5. Influence of molting and fasting on reproduction

The working knowledge of penguin physiology is essential for the breeding success and well-being of this species. In general, penguins have a black and white appearance, however some may present with yellow and orange crests or bluish black feathers with a white chin and chest (Borboroglu and Boersma, 2013). The African Penguin (Fig 2.3) is thought to be most closely related to the Humboldt and Magellanic Penguins found in southern South America.

Figure 2.3: *Spheniscus demersus* (African penguin). Typical features: Dorsal above, with ventral below with variable amount of black spotting on breast and belly.

Penguins share all the other characteristics of birds, including being warm-blooded (ranging 37 - 40ºC) and having a reduced reproductive system (female with one functional ovary) (Crawford *et al*., 2011).

Molting is a process in birds that involves the removal of old feathers and replacing it with new feathers. This process is an important event in the life cycles of birds. As penguins are highly adapted to marine life, their survival depends on their feathers which wear with age and need to be regularly replaced (Otsuka *et al*., 1998). In penguins, the new feather grows under the rachis of the old one and forces it out, with the result that the old feathers are not totally discarded until the new ones are in place. Feathers serve as insulation and protect the skin against water.

In the pre-molt stage, the plumage is pale in comparison with the surrounding non-molting birds and the daily intake of food is increased. The pre-molting stage, last for

35 days (Borboroglu and Boersma, 2013). The actual molt is noticed when feathers begin to drop (Conway *et al*., 2005). This is rapidly followed by a complete pause of feeding. In the post-molt stage, the dark plumage is striking in comparison with the pre-molt condition. Indicative of this stage are the short tail and the feathered skin above the eye. During this last post-molt stage the bird continues to fast, while the new immature feathers grow and are preened to make them water-resistant. Molting usually starts with areas of friction, under the flippers and around the legs, extends to the sides of the body and then spreads to the higher parts of the body. The neck is the last region to molt and the belly is being molted before the back. When the molt is completed it takes two to three days before the plumage attains its typical sleek appearance, and the birds usually remain out of the water and continue to fast. However, after the first molt the chicks still differ in colour from the parents. The characteristic black and white stripe on the head and neck are absent and the colour is a uniform dark grey on the back and almost white on the front. Juveniles molt to adult plumage in the year following their year of birth (Conway *et al*., 2005).

During molt both waterproofing and insulation are deficient, so penguins are forced to remain on land they cannot enter the sea to forage. They will fast until their plumage returns to optimum condition. Thus it is necessary for penguins to spend some time at sea before commencing their molt in order to build up sufficient energy reserves. The necessary period of preparation varies from one species to another, ranging from about 10 to 70 days (Conway *et al.*, 2005). The daily expenditure of energy during molt is roughly double that used daily during incubation (Conway *et al.*, 2005). The whole molt-cycle takes approximately 20 days in Humboldt penguins and 21 days in African penguins. In South Africa, most adult African penguins molt between the September and January period, while at Namibia's Mercury Island, most penguins molt in April and May (Crawford *et al.*, 2011).

Penguins lose 47% body mass, 45% water, 56% fat and 43% protein during molt (Borboroglu and Boersma, 2013). *Spheniscus* penguins are capable of storing great reserves of energy in their layer of subcutaneous fat, which means that they can survive long periods without feeding at all. The length of time a bird can go without food depends basically on its weight at the beginning of the fasting period; the fatter the bird, the longer it can survive.

The behavior of African penguins during molt, is characterised by aggression where adults are significantly more aggressive. This behavior is more severe towards juveniles than towards other adults and head-molted juveniles receive significantly less violent action than non- molted bird (Borboroglu and Boersma, 2013).

2.6. Avian reproductive system

The reproductive system of birds replicates the basic vertebrate pattern of the connection between the central nervous system, anterior pituitary, gonads and other target organs. The avian reproductive system can be divided into the male and female reproductive systems. The primary sex organs, the generally unilateral ovary and testes, are involved in both gametogenesis and production of steroid and other hormones. In birds, the female represents the heterogametic sex, with sex chromosomes designated as ZW, while the male is homogametic (ZZ) (Smith and Sinclair, 2004). Studies on the development of the genital system and variation in their gonadal size from hatching to sexual maturity has greatly contributed to the understanding of the avian reproductive system (Parker *et al*., 1942; Bennet, 1947). The large, long-lived birds, which include albatrosses, condors and penguins, may take 4 to10 years to reach sexual maturity (Barrie, 2007). However, little information exists on the reproductive system of both male and female penguins.

An understanding of the avian reproductive system is useful for anyone who do wild life management on avian species. The testes in the male and ovary in the female are the sex organs where sperm and ova are produced, respectively. Macroscopically these two organs appear to be morphologically different, but due to their internal location identification is difficult. In order to minimize weight the gonads enlarge for the breeding season and become small at the end of the season. Development of successful reproductive procedures for visualization of these organs in African penguins is of increasing importance because penguins are considered endangered in the wild.

Seasonal reproduction in birds is marked by activation of the hypothalamus-pituitary-gonadal axis (Leska and Dusza, 2007). Ambient visual cues, such as daylight, activate photosensitive loci in the brain both indirectly, through the eyes, and directly, through the skull. The hypothalamus of the bird brain contains special cells that are sensitive to extremely low light levels, intensities comparable to the amount of light that can penetrate brain tissue (Akins, 2001). Testes and follicles increase dramatically in size as the breeding season approaches. As day length increases, photic stimulation of the hypothalamus results in the secretion of gonadotropin releasing hormone (GnRH). When activated by GnRH, the anterior pituitary secretes two gonadotropin hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH acts on sperm-producing structures in the testes, while in females it stimulates the growth of ovarian follicles in the ovary. LH acts on the interstitial cells of the testes causing them to secrete the steroid hormone testosterone and cause the egg to be release from the ovary in females. The pituitary gland monitors the amount of testosterone in the blood, thus creating a negative feedback loop to maintain hormone levels within a set range (Akins, 2001).

2.6.1 Macroscopic anatomy of the avian male reproductive tract

The male reproductive system is responsible for the continuous production, nourishment, and temporary storage of the male gamete, as well as the synthesis and secretion of male sex hormones. The main function of the testis is the production of spermatozoa via spermatogenesis and secretion of reproductive hormones, testosterone, the epididymis transport and that of the vas deferens, storage of sperm for maturation and use during threading. Testosterone in birds is responsible for germ-cell production, attributes of masculinity, such as deep voice (Nickel *et al*, 1977).

Like in mammals, the male bird's reproductive tract consists of the paired testes, which remain at the site of their development in the body cavity just under the vertebral column (Nickel *et al*., 1977), the epididymis, ductus (vas) deferens, ejaculatory duct, and mating organ, the phallus, while accessory sex glands are absent (Aire, 2000), (Fig 2.4). A body temperature of 39ºC to 40ºC is essential for normal spermatogenesis (Aire, 2000).

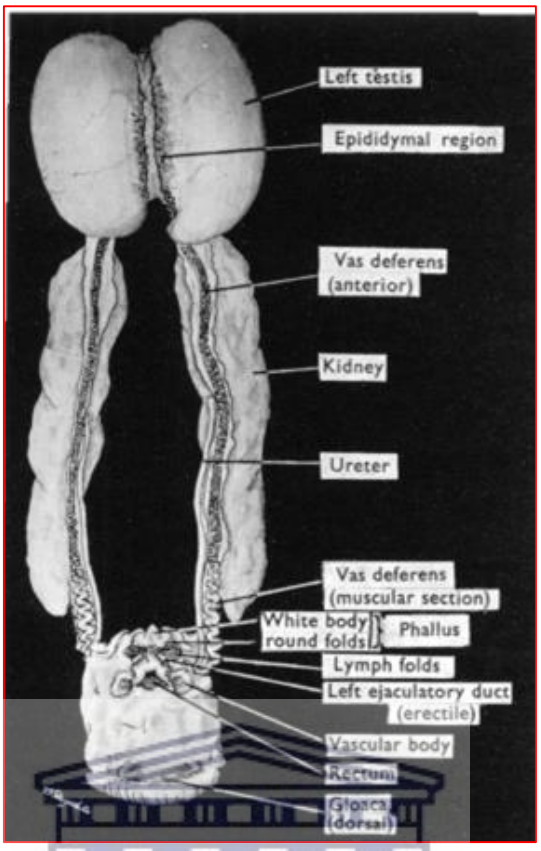

Figure 2.4: Avian male reproductive system of fowl (Lake, 1957).

The testes in avian species, are intra-abdominal. Unlike in most mammals, they do not migrate from the site of embryological origin as they retain their position of origin close to the kidney. Topographically, these organs are situated on the caudal aspect of the lungs, the ventral aspect of the right and left kidneys, as well as the visceral surfaces of the left and right liver lobes (Razi *et al.*, 2010). The right and left testes are oval in shape and loosely suspended from the dorsal body wall by short peritoneal folds, the mesorchia (Deviche *et al*., 2001; Jones *et al.*, 1993). The colour of the testes changes during a resting stage (yellowish) and is almost pure white during the breeding cycle (Nickel *et al.*, 1977). The colour change in testes is regulated by testosterone reducing melanin in testis. When gonads are fully developed their head pole reaches to the tail

of the ventral surface of the lungs and posterior they extend to the anterior lobe of the kidney. Medially the testes make contact with the aorta and inferior vena cava. The papillae serve as the mating organs.

Similar to mammals, each testis is contained within the connective tissue capsule made up of the tunica vaginalis, tunica albuginea and tunica vasculosa. The tunica albuginea is a solid capsule of dense irregular connective tissue. It consists predominantly of collagen fibers, a few elastic fibers, and myofibroblasts that meander along the branches of the testicular artery; a network of anastomosing veins constitutes the vascular layer of the tunica albuginea. In avian testis the seminiferous tubules frequently anastomise forming a complex network throughout the testis (Jones, 1993), and the testicular capsule does not give off septa to divide the testis into separate lobules as is the case in mammals. The seminiferous tubule contains germinal elements and supporting cells, providing a unique environment for production of germ cells. The germinal elements are made up of epithelial cells, including slowly dividing primitive stem cells and rapidly proliferating spermatogonia. Other germinal elements are spermatocytes undergoing meiosis and the metamorphosing spermatids.

The dorsal dorsomedial aspect of the testis attaches to the relatively small epididymis. The epididymis is not divided into clearly recognizable macro-anatomical parts. It is frontally attached to the corresponding testis on its dorsomedial aspect and it is continuous with the ductus deferens. The ductus deferens runs distally from the caudal border of the epididymis, towards the cloaca, into which it opens. Each vas deferens opens into a small bump, or papilla, on the back wall of the cloaca.

2.6.2 Macroscopic anatomy of the avian female reproductive tract

The female sex organ, like that of the male, has both gametogenic and endocrine

function. Functions of the ovary is to produce the ovum and the female hormones, estrogen and progesterone. Some testosterone is also produced by the ovary. In birds, the female reproductive system is unique. The female avian reproductive tract consists of the left ovary and the left oviduct (Fig 2.5). Although in the developmental stage of birds there are two gonads, in the majority of birds only the left ovary reaches full development (Nickel *et al*., 1977), except in birds of prey (order Falconiformes) including families Cathartidae (American vultures), Accipitridae (kites, eagles, hawks, and allies) and Falconidae (caracaras and falcons) (Barrie, 2007). By comparison, some species commonly maintain both gonads (ostriches, falcons, eagles, vultures, and the kiwi), yet these functional ovaries may be asymmetrical in size, the left being the larger. Kinsky (1971), reported that in various birds, including sparrows, gulls, doves and pigeons, a small percentage of individual specimens may maintain two active ovaries through adulthood. The reason for the unilateral development of female genital organs might be to reduce weight for flying (Barrie, 2007).

The ovary lies flat against the anterior lobe of the kidney, reaching the adrenal gland with its anterior border and the aorta medially. On the left, it is related to the ipsilateral abdominal air sacs and attaches anteriorly in close approximation to the stomach (Nickel *et al*., 1977). It is loosely attached to the peritoneal cavity by the mesovarian ligament at the cephalic end of the kidney, and is suspended from the dorsal body wall by a peritoneal fold, the mesovarium. As in mammals, the avian ovary is covered by a single layer of surface epithelium.

The ovary of a mature bird has a grape-like cluster of small follicles which are easy to identify. The grape-like cluster of follicles is the results of follicles beyond the primordial stage of development which project from the ovarian stromal tissue, unlike the mammalian ovary, where developing follicles largely remain fixed within the cortex. As

the breeding season approaches, several of the follicles enter a phase of rapid growth and maturation, becoming yellowish white and it has an elongated triangle shape, with the surface covered by germinal epithelium. The number of ovarian follicles has been estimated to be in the thousands or even millions.

The oviduct is the second major part of the avian reproductive system. This duct is a long convoluted tube which is divided into five major sections. The oviduct runs from the analogous of the ovary, where it is said to assist in the guidance of forcing out ova into the tube, to insertion on the virginal wall. Additional to forming and transporting the egg, the oviduct is the site of sperm storage, sperm transportation, fertilization and early embryonic development (Barrie, 2007). The oviduct surrounds the ovum with the albumen, the shell membranes, and the shell to form the characteristics avian egg. The first part of the oviduct is the infundibulum, which engulfs the ovum released from the ovary and is the site where fertilization take place (Fig 2.5). The magnum is the second section of the oviduct where the ovum remains for about three hours during the time the thick white albumen is added and the chalaza is formed. The section where the inner and outer shell membranes are added is called the isthmus. The fourth section of the oviduct is the uterus, the area where the shell is placed on the egg. To date, it is thought that the oviduct and its relationship with the ovary are uniform throughout Aves, presenting considerably less variation as seen in mammals (Marshall, 1961).When ovulation occurs, the mature follicular oocyte is released from the ovary and is received by the oviduct. The last part is the vagina, which does not play part in the formation of an egg, but assists with the oviposition of an egg.

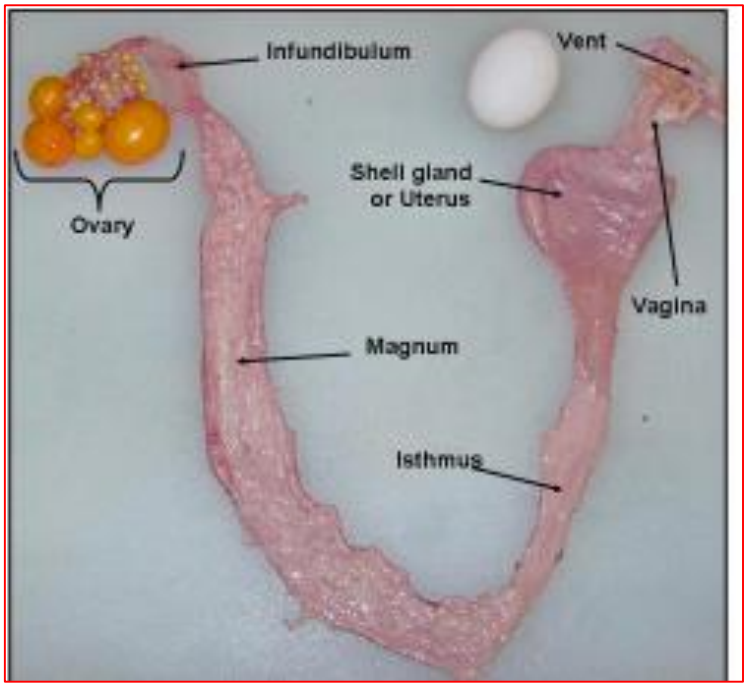

Figure 2.5: Female reproductive tract of chicken (Jacob, 2013)

2.7. Histology of male reproductive structures

2.7.1. Seminiferous tubules

The seminiferous tubules (ST) of birds are different to those of mammals by forming a highly non-blind-ending network of tubules (Lake, 1957; Barrie, 2007). The very twisted ST are embedded in the vascular interstitial connective tissue. These ST are enclosed by a thin tunica propria, which consists of collagen fibers and internal to this is the basal membrane on which lies the seminiferous epithelium. The seminiferous epithelium is made up of two functionally different cell types, fixed, somatic cells, represented by the supporting Sertoli cells (SER) and the temporary and mobile germinal cells including a series of distinguishing cells (spermatogonia, primary and secondary spermatocytes and spermatids). The SER broad base lies against the basal membrane and the slim part of their cytoplasm projects into the lumen of the tubules (Barrie, 2007). Between

these SER all the different developmental stages of the germ cells may be seen extending up to the lumen. The basal stem cell is the spermatogonium that divides repeatedly to form larger, upward-mobile, cells in successive stages of meiotic division and maturity.

2.7.2. Spermatogenesis

Spermatogenesis is the process in which spermatozoa are produced from spermatogonial stem cells by the way of separating chromosome into two new nuclei and reducing the chromosome number by half, creating four haploid spermatozoa. Spermatogenesis occurs in the seminiferous tubules and is a complicated process that encompasses a multitude of cell-to-cell interactions. These interactions includes sequential gene transcription and the control of cell function by endocrine, autocrine hormones, cytokines and growth factors (Thurston, 2000). The spermatogonial stem cells at the base of the seminiferous tubules divide and differentiate and ultimately give rise to spermatozoa at the luminal free space. In the testes of sexually mature and active birds, the seminiferous tubule contains a stratified epithelium comprising germ cells at various stage of meiotic division. It appears that the total sperm production is closely correlated with the number of Sertoli cells present in the testis (Meisami *et al*., 1994).

The purpose of spermatogenesis is to establish and maintain a daily output of fully deferential spermatozoa. In birds, the regulation of sperm growth and maturation are poorly understood, as the quality of sperm produced may be altered at any numerous points during spermatogenesis. Understating this process could have profound effects on bird's male reproduction system. The process of spermatogenesis in avian species appears to be of shorter duration than corresponding stages in mammals (Noirault *et al*., 2006). For example the Japanese quail it takes about 11 to 14 days as (Noirault *et al*., 2006; Lüpold et al., 2011) and 14 days in guinea-fowl (Marshall, 1961), while in

mammals like the mouse it takes about 26 days, 29.5 days in the ram and 37 days in the bull.

There are four stages of spermatogenesis which include spermatogonia, primary and secondary spermatocytes, spermatids and spermatozoa. These stages include the proliferation and renewal of spermatogonia, the meiotic events in primary spermatocytes and their morphological transformation during spermiogenesis. The literature indicates that there are fewer mitotic divisions during spermatogonial proliferation in birds than has been reported for mammals (Jones *et al*., 1993). The spermatogonia are found at the periphery of the seminiferous tubules, in contact with latter's basement membrane, containing larger and round nuclei. Spermatogonia sit next to the myoid tissue of the interstitium, progressing inward to spermatocytes and finally developing spermatids and sperm cells. Although cells are closely arranged, each cell is separated by a network of Sertoli cell cytoplasm.

The primary spermatocytes are the largest germ cells present in the testis and contain chromatin in the form of dispersed, thin filaments. They persist for a relatively long period during spermatogenesis and their development is characterized by a conspicuous nuclear structure in meiotic prophase (Jones *et al*., 1993).

The secondary spermatocytes are slightly larger than spermatogonia but smaller than primary spermatocytes. Their nuclei contain thick clumps of chromatin. The lifespan of the secondary spermatocyte is very short relative to primary spermatocytes so that it is difficult to observe the various phases of the second meiotic division (Jones *et al*., 1993).

This last part of spermatogenesis can be divided into spermiogenesis, or the spermatid elongation phase, during which the haploid spermatid generates sperm accessory structures, and spermiation, a process during which the remnants of germ cell

cytoplasm are rejected and the fully differentiated spermatozoa detach from the seminiferous epithelium. During spermiogenesis, a somatic cell-like, but haploid round spermatid transforms into a highly specialized spermatozoon capable of acquiring progressive motility after epididymal sperm maturation and fertilizing potential after interacting with oviductal fluid and epithelia. During spermiogenesis, many organelles and proteins are recycled to provide space and building blocks for the developing sperm accessory structures (Calvel *et al*., 2010). The spermatids, which arise after the second meiotic division are comparatively small cells with a small and spherical nucleus located above or between bundles of filiform spermatids. Fawcett, (1970), concluded that the process of spermiogenesis follows the basic pattern described in mammals. Classification of spermatid development is mainly based on the development of the acrosome and the changed structure of the nucleus (Jones *et al*., 1993).

2.7.3. The spermatozoa

The avian spermatozoon, like that of other vertebrates is composed of the sperm head, with an apical cap above the acrosome, and sperm tail or flagellum (Fawcett, 1975) (Fig 2.6). Morphologically the avian spermatozoon have been classified as of the "sauropsid" and "spiral-shaped" types (Korn *et al*., 2000). The main components of the head are the acrosome and the nucleus. Although all bird sperm heads are described to be filiform in shape (Santiago‐Moreno *et al*., 2016), the shape differs between avian orders, and there are visible differences in the shape of spermatozoa from Galliformes, Passeriformes, and Falconiformes. For example, a long and narrow head is typical of sperm in Galliformes, while wider sperm heads are apparent in Falconiformes (Barrie, 2007). Both the sperm head and sperm tail are covered by sperm plasma membrane. Progressing from inside-out, the sperm head is composed of a nucleus, containing the deoxyribonucleic acid (DNA) condensed core and protamines which have replaced the histones during spermiogenesis.

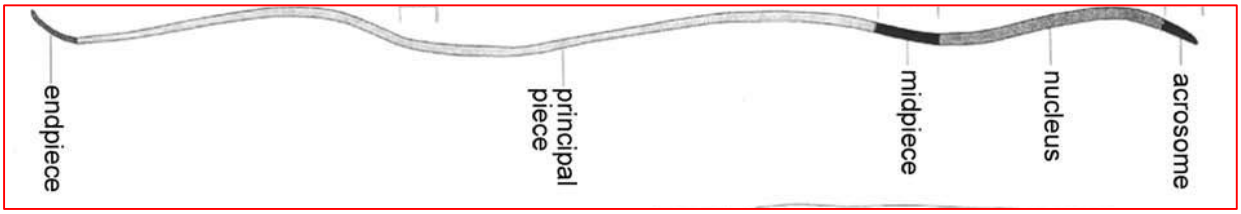

Figure 2.6: Schematic representation of a spermatozoon illustrating the various components of the head (acrosome and nucleus) and tail (midpiece, principal piece and endpiece) (Barrie, 2007).

The avian spermatozoa differ among different orders, families and class. Avian sperm morphology varies from the simple sauropsid form to a complex helical type with an exterior ribbon-like membrane and a long flagellum (du Plessis *et al*., 2014a). The nucleus is covered by a reduced nuclear envelop. The head shape of the avian spermatozoa diverges from each other from elongate cylinder, curved and narrowing, to filiform shape. The acrosome differ in size, from a small bump in the Ostrich to a substantial acrosome in the Rhea. The segments of the tail, in the order of their proximity to the head, are specified the neck, the mid-piece the principal piece and the end piece. The flagellum is long in non-passerine, although it is significant shorter than in passerine birds (Humphreys, 1972; Lake, 1957). Connecting piece is composed of nine striated or segmented columns that are a direct continuation of the outer dense fibers in the other flagella segments. Caged inside these nine columns is the dense mass of capitulum, which in most mammals, except rodents, contains the sperm (proximal) centriole, a remnant of the bi-centriolar centrosome found in early haploid spermatogenic cells.

Immediately posterior to the head is the mid-piece which is shorter than the head. The mid-piece is covered by the mitochondrial sheath in form of a helix, which generate the energy for sperm flagella motility. Each sperm mitochondrion carries multiple copies of

the paternal mitochondrial genome. Examination of sperm morphology of poultry semen indicated that the mid-piece is considerably longer than that of other bird species, approximately one quarter of the head length. In non-passerine birds the mid-piece is short, but longer than in the dove and pigeon. It is then followed by principal piece of the tail, tapering towards its posterior limit and followed in turn end piece. A raised hooplike ring at the junction of the mid-piece and principal piece is identified as the site of the annulus. A distinct annulus is situated posterior to the short mid-piece as in mammalian spermatozoa. The size of the different segments of spermatozoa differ even among the birds of the same phenotypical group. The mid-piece contains various number of mitochondria arranged in seven tiers with about five around the centriole (Jamieson, 2007).

Principal piece is separated from the mid-piece by the annulus or Jensen's ring, a traverse ring of dense material found distal to the mitochondrial sheath. The principal piece is covered by the protective scaffold of fibrous sheath, composed of two longitudinal columns running parallel to outer dense fibers three and eight, connected on both sides by a series of transverse ribs. The fibrous sheath provides support for the sperm axoneme. At the same time, proteins within the fibrous sheath seem to sequester protein kinases necessary for the process of sperm capacitation and hyperactivation prior to fertilization. The sperm tail or flagellum provides the motile force for the spermatozoon, which is based upon a unique 9 + 2 arrangement of microtubules within the sperm flagella axoneme. The 9 + 2 arrangement refers to nine peripheral, symmetrically arranged microtubule doublets connected doublet-to-doublet by dynein arms and to the sheath of central pair of microtubules by radial spokes. The outer doublets, but not the central pair, are paralleled by nine outer dense fibers that provide flexible, yet firm support during flagella movement. The sperm tail can be divided into four major segments that share a common innermost structure of the microtubule based

axoneme paralleled by nine outer dense fibers, but differ in their external substructure. The end piece contains axonemal doublets and the ends of outer dense fibers and fibrous sheath (Calvel *et al*., 2010).

2.8. Histology of female reproductive structures

The ovary consists essentially of an outer cortex containing ova which surrounds a highly vascular medulla composed primarily of connective tissue (Marshall, 1961). At the periphery of the cortex is the germinal epithelium which is made up of primordial germ cells that become enclosed in follicles: first primary follicles, secondary follicles then next mature follicle. The process of follicle growth can be divided into the organization of primordial follicles and the early growth of primary follicles. This is followed by the recruitment of primary follicles into the slow growth phase (the prehierarchal), lasting weeks to perhaps months, and the selection and rapid growth of preovulatory follicles which occurs during the final days preceding ovulation (Fig 2.7). When ovulation occurs, the mature follicular oocyte is released from the ovary and is received by the oviduct (Marshall, 1961).

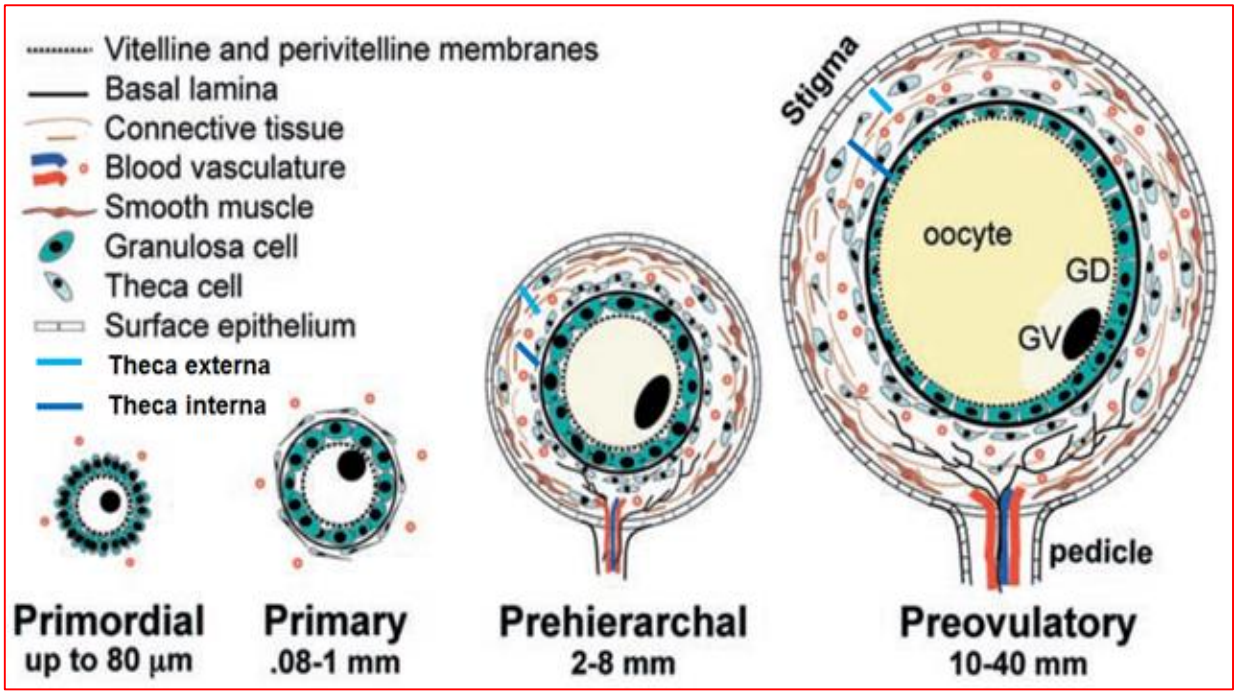

Figure 2.7: The diagram showing the organization of domestic hen ovarian follicles during development (Barrie, 2007).

Primary oocytes enclosed by the vitelline membrane become organized into a primordial follicle following the recruitment of presumptive granulosa cells, and the perivitelline membrane is subsequently formed by granulosa cells. The initiation of primordial follicle growth to the primary follicle stage is associated with the formation of the theca layer, which is separated from the granulosa layer by the basal lamina. Primary follicles have a vasculature and nervous innervation reaches the follicle through the pedicle and radiates through the theca layer. Following selection into the preovulatory hierarchy, preovulatory follicles grow rapidly. Granulosa cells from preovulatory follicles facilitate the uptake of large amounts of vitellogenin and very low density lipoprotein, except within the germinal disc region. Ovulation of the largest preovulatory follicle eventually occurs at the region of the comparatively avascular stigma region (Barrie, 2007).

2.9. The African penguin breeding cycle

Most penguins are monogamous; this means that male and female pairs will mate exclusively with each other for the duration of the mating season. It is observed that in many cases, the male and female will continue to mate with each other for most of their lives (Crawford *et al*., 2011). African penguins breed on islands off the south and southwest coast of southern Africa, from Hollams Bird Islands to Bird Island (Algoa Bay). There is no breeding along the coast of South Africa's Northern Cape Province, which lies between Namibia and Western Cape Province. The species nests on inshore islands or less frequently on mainland coast.

2.9.1. The Activation of breeding

Breeding occurs all year round depending on local variations. The peak laying of eggs is in November/January, May/July and April/June and the laying interval is on average three days. A clutch of two eggs are laid and the incubation starts after the first egg has been laid. The breeding interval for successful birds is 10.5 months and relaying occurs at 4.0 months after unsuccessful breeding. Mean incubation period for the first egg is 38.0 days, and the second egg is 37.2 days. Incubation is shared equally by both parents with shifts of 1.1 days on average in successful nests and 3.1 days in nests that were eventually deserted, with the last shift before desertion averaging 6.6 days. In sexually active birds, it takes one to four days for sperm to be formed and reach the ducts (Nickel *et al*., 1977). The environmental parameters, like temperature, humidity, photoperiod and diet, have an impact on reproductive success. Physiological and behavioural maturity probably determines the age at which individual first breed (Boswall *et al*., 1975), the mature the penguin then it breeds successful.

Breeding activity is very sensitive to environmental conditions, and therefore highly responsive to climate change (Boersma, 1978; Ancel *at al*., 2013). Boersam, (1978),

reported that for species like Galapagos penguin (*Spheniscus mendiculus*), the onset of breeding is closely linked to mean sea surface temperature (23.3°C).

The biggest problem for nesting *Spheniscus* penguins is the sun. They avoid the sun by nesting in sheltered sites like burrows, sea caves, and clefts or under rocks, and vegetation. Surface-nesting also occurs but only during winter or when there is a strong wind-chill. They tend to nest densely, to protect their eggs and young against aerial predators. The nearest nest distances are 4.3, 4.8 and 5.5 meters for nests in burrows / under rocks, under bushes or abutting bushes / rocks respectively and 0.86 m in open, surface nesting colonies. In Southern Africa, peaks in temperature are experienced during the summer period (January - March). This coincides with the first peak breeding attempt of penguins in the Eastern Cape (Shannon and Crawford, 1999).

At the age of four years, *Spheniscus demersus* is sexually mature and will breed for the first time in the wild. In captivity, usually after two years of age the birds are ready to begin breeding and to select a partner (Conway *et al*., 1999).

The annual cycle of birds is made up of a sequence of life-history stages: breeding, molt and migration. Each stage has evolved to occur at the optimum time and to last for the whole duration of time available. Photoperiod is the principal environmental cue used to time each stage, allowing birds to adapt their physiology in advance of predictable environmental changes. Penguins have a predictable breeding seasons, photoperiod is the principal environmental cue used to time each stage, allowing birds to adapt their physiology in advance of predictable environmental changes. The timing of gonadal regression affects the time of the start of molt, which in turn may affect the duration of the molt. Life-history strategies evolve in response to extrinsic (biotic and abiotic) constraints. Predictability of seasonal change is important because, in order to match their behaviour to the seasons, birds need to predict the optimal time for each life-history

stage to complete the necessary changes in physiology in advance (Degen *et al.*, 1994).

In the pituitary, cGnRH-I stimulates the synthesis of the two gonadotrophic hormones, luteinizing hormone and follicle stimulating hormone. These two hormones are secreted into the circulation and induce gonadal maturation. The activity of the cGnRH-I neurons is primarily controlled by photoperiodic information received from encephalic photoreceptors integrated in some way with a circadian clock. An increase in the photoperiod stimulates conversion of thyroxine to its active form, triiodothyronine (Yasuo *et al*., 2005), and this, in part at least (Takagi *et al*., 2008), leads to an increased secretion of cGnRH-I. If birds rely entirely on photoperiod to control the time of gonadal maturation and regression then these events would occur at the same time every year. The photoperiod alone controls the time of gonadal maturation and regression and so sets the limits to a physiological window within which breeding can occur. Flexibility exists within this window so that nonphotoperiodic cues can affect the exact timing of egg laying. Certainly, female birds often require a range of non-photoperiodic cues to initiate the latter stages of ovarian maturation and ovulation

2.9.2. Hormone levels

Field endocrinology techniques involve the collection of samples (i.e., blood, urine, faeces, and tissues) from free-living animals for analysis of hormones. Behavioural endocrinology examines hormonal influences on behaviour, and behavioural influences on the endocrine system (Ninnes, 2008). The first behavioural endocrinology study in avian species on how hormones influence an animal's behaviour, was conducted by Berthold in 1849 (Berthold, 1849). He discovered that the removal of the testes from a rooster caused it to cease displaying sexual or aggressive behaviours (Berthold, 1849). When a testis was re-implanted into the body cavity, the rooster again displayed sexual and aggressive behaviour. The group of steroid hormones which influence a bird's

behaviour is called androgens in males and estrogens and progestins in females. In males, androgens and estrogens activate masculine copulatory and aggressive behaviors, including song. In females, estrogens and progestins activate female receptive behaviors and parental care (Schlinger *et al*., 2001b).

In birds, LH stimulates steroidogenesis of estrogens, progestagens, and androgens in the ovaries of females, and androgens in the testes of males (Buckle, 1983; Harvey *et al*., 1987). FSH is important for maturation and yolk deposition of eggs in females, and for spermatogenesis in males (Blas *et al*., 2011). The increase in estrogen or androgen reduces the secretion of FSH and LH. The chief hormone in each of the estrogen, progestagen, and androgen groups is estradiol-17β, progesterone, and testosterone, respectively (Balthazart *et al*., 1976; Blas *et al*., 2011). Sex steroids regulate and synchronize fundamental aspects of reproduction in vertebrates, from gonadal reactivation and production of fertile gametes to the development of specific morphological and behavioural traits (Blas *et al*., 2011). It is well established that systemic changes in gonadal steroid levels regulate sexual function in a unique manner, adjusting the physiology, morphology and behaviour of the individuals as a function of the reproductive strategies. Seasonal breeding species typically show annual cycles of gonadal growth at the start of the breeding season, which are reflected by systemic elevations in testosterone and estradiol (Blas *et al*., 2011). After the breeding cycle, the avian species typically start gonadal regression. This process is accompanied by a progressive decrease in the levels of circulating testosterone and estradiol. Testosterone is expected to decrease soon after the sexual phase in monogamous species if this hormone inhibits parental behaviour. Therefore, as a monogamous species we expect male African penguin to show a decrease in testosterone as they perform parental activities. Although the above predictions regarding patterns of sex steroid levels are mainly related to testosterone and estradiol, progesterone may also

play a relevant role in physiology and behavior. However, the function of progesterone in avian reproduction remains poorly understood. While some studies report no significant changes in progesterone levels during the breeding season (Fivizzani *et al*, 1986; Hector *et al.*, 1985; Heath *et al*., 2003), others suggest that this hormone stimulates incubation (Thomas and Fordyce, 2012 *et al*., 1995; Fivizzani *et al*., 1986) or chick brooding (Hector *et al*., 1996). In seasonal breeding birds, testosterone (T) is elevated in the blood of males only during periods of reproduction, usually coincident with territorial, courtship, and copulatory behaviours. In adult females, estradiol (E2) is elevated when females are receptive to males and during laying, whereas progesterone (P) is elevated in some species during incubation and brooding (Schlinger *et al*., 2001b).

2.10. Semen evaluation

In order to achieve fertilization and sustain early embryonic development, it has been difficult to identify a single test capable of accurately predicting the fertility potential of an individual ejaculate (Møller *et al*., 2008). Instead, the evaluation of sperm motility, concentration, vitality and morphology are considered the four most important parameters in any semen analysis (Cooper *et al*., 2002.; Kuster *et al*., 2004; Łukaszewicz *et al*., 2008). These parameters are assumed to be under intense selection because of their close correlation with fertilization success (Møller *et al*., 2008).

Sperm motility is one of the principal parameters used to determine fertility as it supports the delivery of sperm to the site of fertilization. Motility is a common feature and an important characteristic of spermatozoa for both *in vivo* and in vitro fertilizing species. When spermatozoa are transported through the female reproductive tract, it display an activated motility pattern. The role of sperm motility is proposed as essential in driving the spermatozoa from the site of semen deposition to the oviduct. The importance of

sperm motility is highlighted by the fact that the measurement of this parameter forms an essential part of most semen or sperm evaluation criteria (Mocé *et al.*, 2008). There are various methods that are used to assess sperm motility, including both manual and automatic methods, which have been in existence since 1953 (Mortimer, 1992). These different methods of assessment do, however, not always result in comparable motility data. Techniques like time-lapse photomicrography and cinemicrography were used before the current Computer-aided Sperm Analysis (CASA) systems were developed.

Sperm morphology is thought to be involved in post-copulatory selection during sperm competition and fertilization (Møller *et al*., 2008). Numerous studies have found a close relationship between sperm morphology and fertility, with positive correlations reported between percentage normal sperm morphology or sperm size and fertilization success (Enginsu *et al*., 1991; Łukaszewicz *et al*., 2008, Møller *et al*., 2008). Spermatozoa with structural defects (i.e. head or tail) either do not reach the oocyte or, if they do, cannot penetrate the oocyte to complete fertilization. In support, Nothling *et al*., 2008 reported that spermatozoa with nuclear craters and otherwise normal heads reach the oocyte and bind to the zona pellucida as well as normal spermatozoa do, but result in lower embryo quality and fertility than morphologically normal spermatozoa (Nothling *et al*., 2008). Abnormal sperm morphology can also serve as an indicator of some disorders in spermatogenesis (Łukaszewicz *et al*., 2008). Such structural abnormalities may only be present during the early reproductive season in some birds. For instance, abnormal and immature sperm, including spermatogonia and spermatids, are more commonly observed in peregrine falcon ejaculates during the early stages of the breeding season (Łukaszewicz *et al*., 2008).

Across the animal kingdom the size, shape and ultrastructure of spermatozoa exhibit notable difference (Cohen, 1977). The ultrastructure of the spermatozoa of many

animals has been studied extensively in the last decade. Since the development of ultrastructural analysis of cell morphology there have been hundreds of studies involving the ultrastructure of spermatozoa and their development. Most of these studies have dealt with mammals. Among the vertebrates there have been very few studies of avian spermatozoa. Furthermore, those have focused primarily sperm cell function and to description problems can be gained from ultrastructural analysis of spermatozoa from a wider variety of avian species (Asa *et al*., 1986). Because of its superior resolution this technique has also been used to accurately determine the dimensions of the various components of sperm cells from some non-passerine birds.

The integrity of the sperm membrane is for cellular homeostasis, and it is vital for fertilization. The loss of sperm plasma membrane integrity will lead to cell death. It has been show that plasma membrane destabilization results to decline in sperm vitality and longevity and will lead to the loss of components enclosed by the membrane, such as cell enzyme (Ashworth *et al*., 1995). It has been reported that once the plasma membrane has been destabilized, the plasma membrane of the head region can fuse with the outer acrosomal membrane resulting in the release of acrosomal enzyme, called acrosome reaction (Rijsselaere *et al*., 2005). If the sperm acrosome is damaged early it will result in loss of sperm fertilization capacity.

CASA systems were developed which is software-hardware based used to automatically analyze the quality of a sample population by detecting individual sperm tracks. The usage of CASA is best for kinematic analysis of spermatozoa because of its ability to detect motile cells (Fig 2.8). A CASA system produce more information on sperm quality in functional terms than subjective methods and has been widely used, tested and modified for semen analysis of many species including birds (Farooq *et al*., 2016). When CASA systems are combined with a computer, sperm concentration and

motility is detected by a digital camera and converted to digital signal and subsequently processed with digitalization and automatically converted to corresponding semen parameters. CASA provides with several other option of measurements of sperm population, separating motile sperm into slow, medium and rapid sperm categories. Also it provides individual kinematic parameters, the percentage total motility, and progressive motility, rapid, medium and slow swimming spermatozoa. In addition, some CASA system have semi-automated morphology module as well as concentration detection ability (WHO, 2010). CASA systems recognize the sperm head by identifying the brightest point of the sperm head and thereafter the sperm trajectory can be determined.

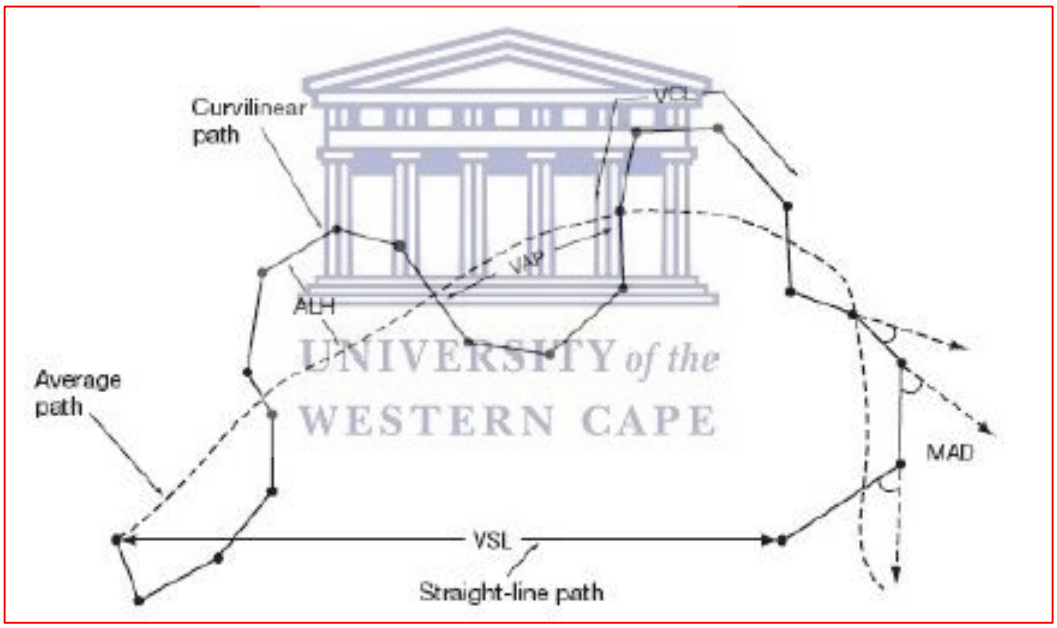

Figure 2.8: Diagrammatic presentation of kinematic parameters measured by CASA. ALH = amplitude of lateral head displacement, VAP = average path velocity, VCL = curvilinear velocity, VSL = straight-line velocity, MAD = mean angular displacement (not determined in this study). (From WHO, 2010)

2.11. Cryopreservation

Cryopreservation is a non-physiological method that involves a high level of adaptation of biological cells to the osmotic and thermic shocks that occur both during the cooling-freezing procedure and during the thawing procedure (Prieto *et al*., 2014). Semen cryopreservation is the process of cooling spermatozoa to very low sub-zero temperatures for prolonged storage. Damage occurring during the freezing–thawing procedures affect mainly cellular membranes (plasma and mitochondrial) and in the worst case, the nucleus. Two methods for gamete cryopreservation are currectly available: vitrification and slow freezing or cryopreservation (Arav *et al*., 2002). Vitrification is a process by which liquid turns into solid without the formation of ice crystals. Slow freezing has the advantage of using low concentrations of cryoprotectants which are associated with chemical toxicity and osmotic shock. During slow freezing, the cooling rate is thought to be the major factor that determines the survival of cells and tissue. It is suggested to freeze large volumes such as semen and tissue by means of slow cooling rate procedures (0.5–100 °C/min), because heat transfer in cells, tissues or organs, which has larger volumes (i.e. more than 0.1 ml), is too slow to allow vitrification without risk of 'solution effect' or crystallization (Arav *et al*., 2002). The cryoprotectant (glycerol) that achieved the first successful cryopreservation of sperm was discovered by Polge in the 20th century (Arav *et al*., 2002). The physiological actions of glycerol during the cryopreservation of spermatozoa take place by replacing intracellular water necessary for the maintenance of cellular volume, interaction with ions and macromolecules, and depressing the freezing point of water and the consequent lowering of electrolyte concentrations in the unfrozen fraction so that less ice forms at any given temperature (Arav *et al*., 2002). Biochemical characteristics of semen, buffer and permeable cryoprotectant are some of the factors affecting the

success of cryopreservation. Therefore, there is a need to develop biochemically defined extenders and cryogenic procedures that are species specific, and may result in the improvement of viability and fertility of frozen–thawed penguin spermatozoa.

Sperm cryopreservation, is an important component of genome resource banking, which offers tremendous potential for genetic management field. Semen preservation together with artificial insemination (AI) techniques may give benefits for conserving endangered African penguin. Reproductive research is becoming an essential component of long-term conservation programs for endangered species. Understanding of reproduction processes assists in ART and can maintain genetic diversity (O'Brien *et al*., 2016). Semen cryopreservation sustains the conservation of genetic biodiversity and improves the management of artificial insemination.

Intensive research on cryogenic preservation of semen from mammals and birds has been carried out since 1938 (Gee *et al*., 1990). The storage of frozen semen and embryos can be used to protect the gene pool of species, but semen preservation appears to be feasible with birds (Gee *et al*., 1990). With the increase of non-domestic birds being threatened with extinction in the recent years, more studies on semen preservation have been established (Gee *et al*., 1990). Semen cryopreservation in animal production and genetic improvement programs as well as for preserving genetic biodiversity has long been used (Phillips *et al*., 1996). Due to highly variable and low fertility levels achieved with frozen/thawed poultry semen, the poultry industry has been unable to take advantage opportunities of using frozen semen (Phillips *et al*., 1996). The information on cryogenic preservation of semen from chickens, turkeys, Goose and ducks is available with 65% level of fertility achieved through artificial insemination on chickens (Gee *et al*., 1990). Despite research being done on the application of avian sperm cryopreservation for commercial poultry production and conservation breeding

programs has been limited. This is largely the result of the so far unavoidable and severe loss in sperm viability upon thawing. It is predicted that more than 98% of frozen/thawed rooster sperm fail to reach the site of fertilization (Blanco *et al*., 2011).

The extinction rate is estimated to be up to 1,000 times higher than the natural, or background, extinction rate. It is estimated that in the next few decades, up to 30 % of species, including many mammals, birds, reptiles, fish, and amphibians, may go extinct (Prieto *et al*., 2014). Since spermatozoa from many mammals, birds, reptiles, fish, and amphibians, are usually more accessible and come in large numbers compared to oocytes and embryos, they are considered the primary cell type preserved. Semen from endangered species is currently cryopreserved to avail long term storage (Prieto *et al*., 2014). Frozen samples means that at least to some degree, the genetic diversity of a species is stocked away and can be reintroduced into the population at a later stage by thawing.

In combination with natural breeding efforts, assisted reproductive technologies (ART) such as AI and semen preservation have potential to maximize the genetic diversity of captive penguin populations. This also ensures that the sperm will remain healthy sustainable, and will be available for reviving wild populations if the need arises and a suitable habitat exists. Quite limited information about the commercial use of frozen penguin spermatozoa exists. The need for semen preservation of penguin is necessary because of the reduction in genetic variability of African penguin. A major goal of penguin semen cryopreservation is to preserve as much of the genetic pool as possible in order to guarantee healthy survival of penguins. The use of accessible penguin both captive and the wild, coupled with increased understanding of sperm cryobiology can help in the process of developing protocols to assist in the preservation of semen from endangered will penguin species.

2.12. Conclusion

The African Penguin *Spheniscus demersus* was abundant at the start of the 20th Century, but has since drastically decreased in numbers. Investigation of the biological aspects of both the male and female African penguin will highlight the relationships between the sperm characteristics in males and further shed light on the micro and macro anatomy of both males and females.

The current study will address the relationships between the testicular volume, size and weight, and further correlate the macro and microscopic observations with hormonal-sperm relationships in the male African penguin. The ovarian development, size and weight in females will be correlated with both macro and microscopic observations in addition to hormonal relationships.

Chapter 3:

Materials and Methods

3.1. Introduction

All semen/sperm analysis techniques applied in this study were previously reported as routine analysis techniques in the research laboratory (Maree *et al*., 2010). Due to species specificity, these techniques were revised to allow for optimization of all procedures to be used for the African penguin.

A general overview of all materials and methods used in this study is displayed Fig 3.1. Both live and accidental dead penguins were used in this study. Accidentally deceased penguins are those penguins that were euthanized or died because they did not respond to rehabilation. To study the macro- and microscopic structures of the reproductive system accidental dead penguins were used, while the live penguins were used to study the semen analysis and hormone profiling.

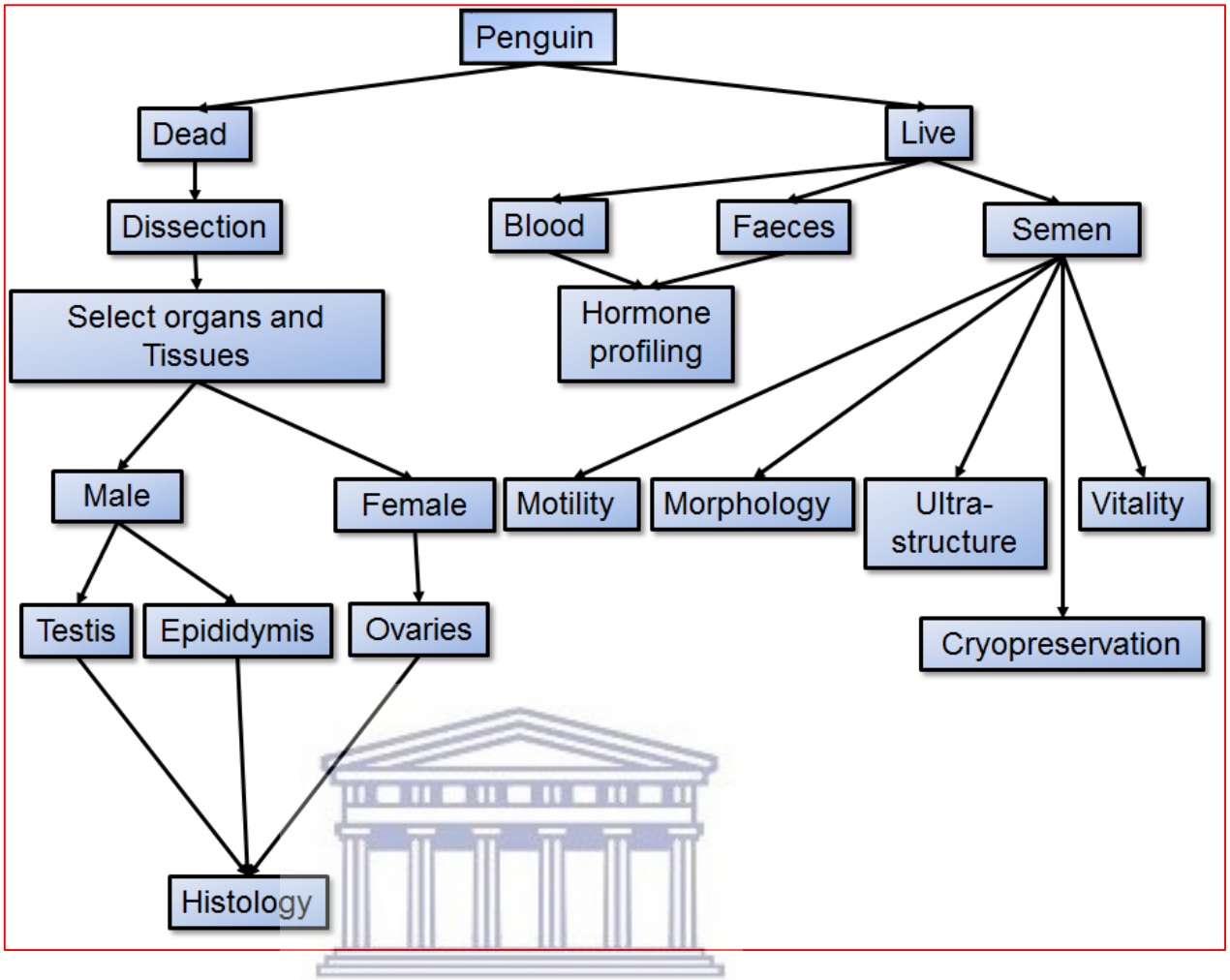

Figure 3.1: A simplified flow diagram to indicate all procedures undertaken during the study.

3.2. Ethical clearance

The study was conducted by collecting samples from organs, tissues, blood, faeces and semen from both healthy and unintended dead African penguins at SANCCOB and Two Oceans Aquarium, South Africa, using penguins housed in captive colonies. All procedures were in accordance with the ethical guidelines and approval of the University of the Western Cape (ScPGC2013/06/10), Cape Nature (RES201/41) and the National Zoological Gardens of South Africa (NZG/P13/07), documents attached as appendix.

3.3. Macro- and Micro-anatomy

3.3.1. Study animals

The penguins and tissues used in this part of the study were collected at The Southern African Foundation for the Conservation of Coastal Birds (SANCCOB) rehabilitation centre in Cape Town (Table View). This centre mainly focuses on the rehabilitation (treatment and release) of sea birds, but individual birds that are incapable to return to the wild are kept in the centre's home pens. African penguins (*Spheniscus demersus*) which were euthanized or accidentally deceased were dissected within fifteen minutes after the penguin died. Both the right and left testis and epididymis for males and the ovary for females were removed and fixed in Bouin's solution and in formaldehyde solution. Care should be taken when removing and identifying each testis, especially when the carcass of a penguin is placed in a dorsal resting position, with the left side of the specimen being on the right of the operator. Tissues from a total of 36 male (31 adults and 5 juveniles) and 29 female (23 adults and 6 juveniles) African penguins were examined and used for macro-anatomical descriptions and histology.

3.3.2. Description of macro-anatomy

For the macroscopic observations of reproductive structures, dissected organs were used. The size of the epididymis is very small and it was difficult to describe the macro-anatomy of this structure. The testis macroscopic structure was described, taking into consideration the following parameters: shape, position and dimensions (length and width) using a Digital Calliper KTV150 (Lasec, South Africa). The testes were removed and weighed using an analytical scale. The testes are smooth organs that are parallel displaced at the side of the body median line, showing smoothed surface, however, with different shape (Fig 3.2A and Fig 3.3). Testes are supplied by testicular arteries from the abdomen (Fig 3.2B), (Bull *et al*., 2007). The testis volume was measured using the

formula V= L x W^2 x 0.59 (Lin *et al*., 2009), used for small testis.

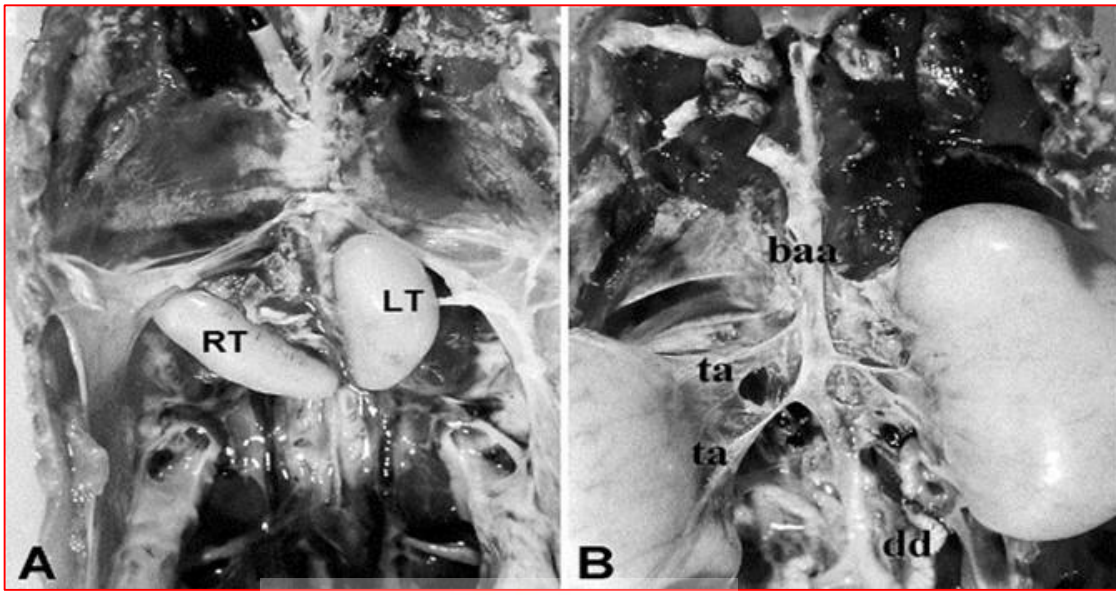

Figure 3.2: A) Fowl testes (RT and LT) 21 weeks of age. A) Right testes (RT) and the Left testes (LT). B) Observe: testicular arteries (ta), abdominal aorta artery (baa) and ductus deferens (dd) (Bull *et al*., 2007).

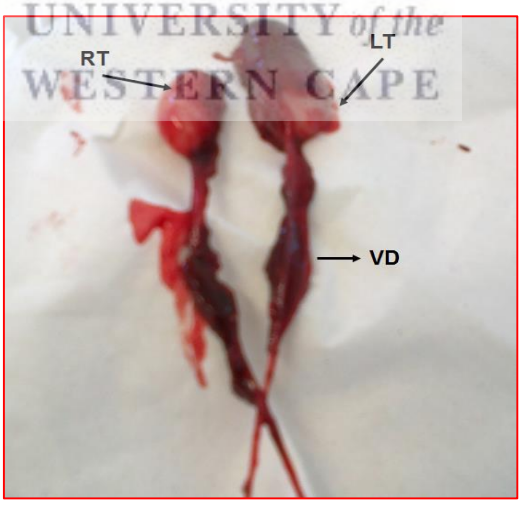

Figure 3.3: The topography of the dissected male reproductive tract of the African penguin indicating right testis (RT), left testis (LT), and vas deferens (VD).

The macroscopic assessment of ovaries included observations of the location, colour, number and the size of the ovary and follicles surrounding the ovary (Fig 3.4). The ovaries were weighed after fixation and the length and width of the ovaries were measured using a Digital Calliper KTV150 (Lasec, South Africa).

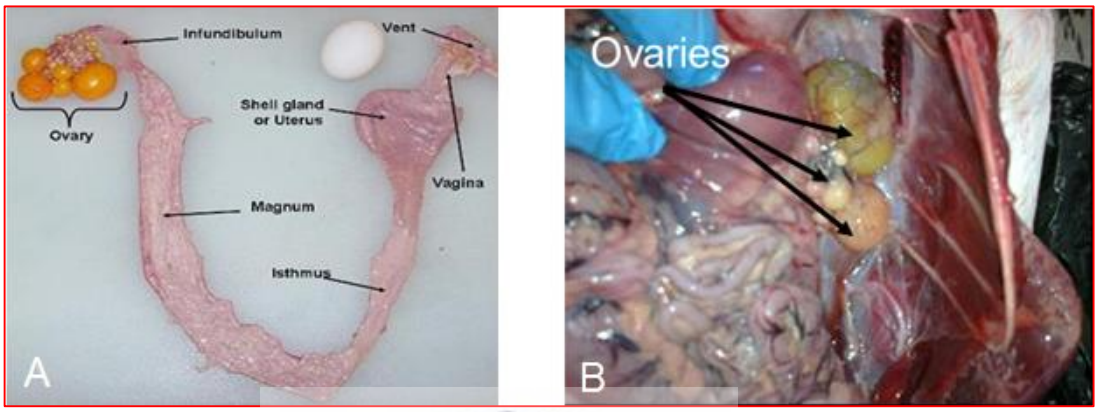

Figure 3.4: A) Parts of the reproductive tract of a female chicken, (Jacob *et al*., 2001) **B**) Photograph of the dissected female reproductive tract of the African penguin indicating the location of the ovaries.

3.3.3. Histology

After fixation in Bouin's solution, 3 – 4 mm, representative samples of testes and ovaries were placed in properly labeled cassettes (n=6 for males and n=22 for females). These tissue samples were dehydrated through a sequence, ethanol, cleared in xylene and infiltrated in wax, using an automated Leica TP 1020 tissue processor (Leica Biosystems, Germany) in an 18 hour cycle as shown in Table 3.1.

Table 3.1: Eighteen hour fixing and embedding cycle for processing of tissues (Buesa, 2010)

Steps	Solution	Time (hour)
1	70% ethanol	2
2	80% ethanol	2
3	90% ethanol	2
4	100% ethanol 1	2
5	100% ethanol 2	2
6	Xylene 1	2
7	Xylene 2	2
8	Wax bath 1	2
9	Wax bath 2	2

After the 18 hour cycle has been completed, the tissue samples were embedded in paraffin wax and cut into sections using a microtome (ERMA Disposable Microtome Blades PATHO CUTTER, Erma INC Japan) and section thickness was set at 4 µm. Sections were then placed on a staining rack and put in a hot air oven at 60 ºC for approximately 30 min to further fix and melt the wax, then stain with haematoxylin and eosin as shown in Table 3.2. Haematoxylin and eosin (H&E) stain is a common stain used for light microscopy to give contrast and to highlight a particular feature of interest of the tissue samples.

Table 3.2: Procedure for haemotoxylin and eosin staining (Buesa, 2010).

Steps	Solution	Time (minutes)
1	Xylene (x2)	5 min each
2	100% ethanol (x2)	5 min each
3	90 and 80% ethanol	5 min each
4	Haematoxylin	7 min
5	Rinse in tap water	1 min
6	Scott's tap water	2 min
7	1% acid alcohol	2 min
8	Eosin	2min
9	Rinse in tap water	1 min
10	80, 90 and 100% ethanol	2 min each
11	Xylene (x2)	2 min each
12	Mount for observation	

On the histological examination of the gonads, where appropriate, the diameter of testis and ovaries was measured using the Sperm Class Analyser (SCA) 6.1 CASA system (Microptic S.L., Barcelona, Spain). The slides were viewed using a Basler A312fc digital

camera (Microptic S.L., Barcelona, Spain), mounted onto a Nikon Eclipse 50i microscope (IMP Solutions, Cape Town) and a 10x and 40X positive phase contrast objective. In total, cross-sections from 12 ovaries and 14 testes as well as randomly selected seminiferous tubules in the testes were measured. The diameter of the seminiferous tubules was measured across the minor and major axes and mean diameter was obtained. The follicles in the ovaries were counted. At the stage of sexual maturity in birds, many follicles enlarge and they are over 100 visible (Johnson *et al*., 2014), to the naked eye varying in size from about 0.5mm to 20.5mm of diameter.

3.3.4. Testes and ovary ultrastructure

African penguins (*Spheniscus demersus*) which were euthanized or accidentally deceased were dissected and reproductive organs were collected. Both the right and left testis of males and the ovary of females were removed and fixed in 10% phosphate buffered formalin for light microscopy but for TEM we fixed in glutaraldehyde. Reproductive tissues (n=6, for male and females) of African penguins were examined and used for ultrastructure descriptions and histology.

Toluidine blue (TB) stains tissues based on the principle of metachromasia. The dye reacts with the tissues to produce a colour different from that of the original dye and from the rest of the tissue. Tissue were prepared on pre-cleaned defatted slides and then air dried for 30–60 min. Dried smears were fixed with freshly made 96% ethanol: acetone (1:1) at 4°C for 30 min–12 h and air dried. Hydrolysis was performed with 0.1 mol/l HCl at 4°C for 5 min followed by three changes of distilled water, 2 min each. TB (0.05% in 50% McIlvain's citrate phosphate buffer at pH3.5, Gurr-BDH Chemicals Ltd, Poole, UK) was applied for 5 min. Slides were rinsed briefly in distilled water, lightly blotted with filter paper, dehydrated in tertiary butanol at 37°C (2×3 min) and xylene at room temperature (2×3 min) and mounted with DPX. The stained tissues were analysed

with Nikon Eclipse 50i microscope bright field optics, using a blue filter and 10x, 20x and 40x magnification. The 10x, 20x were used to measure and see the whole section of the tissue organ and different stages of cells present.

Tissue samples were prepared for transmission electron microscopy (TEM) by cutting a small part of testis and ovary with visible follicles. The tissue samples were then transferred to 2.5% phosphate buffered glutaraldehyde followed by post-fixation in 1% osmium tetroxide in the same buffer. These fixed samples were subsequently processed for TEM including contrasting with lead citrate and uranyl acetate using standard procedures (Maree *et al*., 2010). A Reichert ultramicrotome (SMM Instruments, Johannesburg, South Africa) with a diamond knife (Agar Scientific, Randburg, South Africa) was used to make silver to gold sections. Thin sections on copper grids were examined using a Jeol JEM 1011 transmission electron microscope at 80 kV (Advanced Laboratory Solutions, Johannesburg, South Africa).

3.4. Hormone profiling

3.4.1. Study animals

This part of the study was also conducted at SANCCOB rehabilitation centre in Cape Town (Table View) by using individual birds that are incapable to return to the wild and are kept in the centre's home pens and assigned with different numbers. African penguins (*Spheniscus demersus*) were housed outdoors at SANCCOB, exposed to sunlight and to fluctuations in air temperature and wind, roofed with a transparent net to eliminate mosquitoes who pose a malaria threat in African penguins. Faecal and blood samples were collected from twelve (six females and six males, *n=4* were breeding pairs, n=2 juveniles and n=2 non-breeding) captive African penguins. All breeding pairs used in this study had reached the adult moulting stage by the start of

sample collection.

The annual cycle in hormone levels of African penguin was studied by using faecal samples (non-invasive method) and blood samples (invasive method). The non-invasive method was used because the technique allows for the subject to be investigated without introducing variables, such as stress due to capturing and handling, which may influence the results. However, plasma and faecal hormonal levels are different. The blood plasma samples represent a 'snapshot' of circulating hormone concentrations at a specific point in time, whereas faecal samples represent an accumulation of metabolized and excreted hormone over a species-specific time period, signifying the intestinal passage time from duodenum to rectum (Ninnes *et al*., 2010).

3.4.2. Sample collection and processing

A total of 235 faecal samples were collected between June 2014 and November 2015, a period covering one complete breeding cycle of the African penguin. Samples were collected once per week prior to and after the egg-laying period. Sample collection involved observing the penguins from a distance until a bird was observed defecating. Faecal samples were stored at -20 ºC within five minutes of collection until further analysis. Where possible, previously reported ELISA analysis techniques were modified to allow the exact protocol to be used for African penguins Ganswindt *et al.* (2002).

Frozen faecal samples were placed in a VIRTIS Bench Top "2K" series lyophilizer (freeze-drier) (Lasec, South Africa) for 24 hr. Once freeze-dried, samples were grounded (pulverized) and sifted using a mesh to remove remaining fibrous material. Dry faecal powder (0.050-0.055 g) was placed in a test tube with 1.5 mL of 90% methanol and vortexed at high speed in a multitude vortexer (VX-2500) (Henry Troemner LLC, USA) for 30 min. Samples were then centrifuged (Heraeus Magafuge

1.0 R) (Separations Scientific, South Africa) at 3000 rpm for 20 min, and the supernatant was stored at -20 ºC in a new tube until the hormone assays were performed.

Blood sampling was conducted between March 2014 and August 2015. The blood collection was restricted to samples taken on a monthly basis in order to reduce stress levels in African penguins. Blood was collected in the morning before feeding, time was kept constant. A total of 110 blood samples were collected (1 ml) from the penguin foot using 18 gauge needles and heparinized micro-hematocrit capillary tubes. All blood samples were collected within 30 min after a bird was caught. The blood samples were centrifuged (micro-hematocrit centrifuge, hawksley) at 200 rpm for 5 min within an hour of collection and the plasma was stored at -80 ºC until the hormone assays were performed.

3.4.3. Hormone analysis

Faecal extracts were assessed for levels of epiandrosterone (Ep) for males and progestogens (Pg) and estrogens (Es) for females using enzyme immunoassays (EIA). Assay procedures followed protocols published by Ganswindt *et al.* (2002). Each hormone was assayed in a single run to avoid inter-assay variation. For determination of hormone concentrations, 50 µl aliquots of assay buffer, standards, quality controls and diluted sample extracts were pipetted in duplicate into the microtiter plate wells. Hereafter, 50 µl biotin-labelled steroid was added to each well, as well as 50 µl antibody, except for the blank, where 50 µl assay buffer was added instead. The loaded plate was covered with cling wrap and the contents mixed gently before it was incubated overnight at 4 °C. Following incubation, the plates were washed with PBS four times and 150 µl streptavidin-peroxidase was added to each well. Following second incubation at room temperature for 45 minutes, plates were washed with PBS again before 150 µl substrate solution was added, and plates were further incubated under the same conditions until

maximal optical density was 1.0. The reaction was terminated by adding 50 ml of 4N H_2SO_4 and the absorbance was measured at 450 nm using BioTek EL800 (Analytical & Diagnostic Products CC) microplate reader. Sensitivities of the assay were 0.24 ng/g for Ep, 10 ng/g for the Pg and 12 ng/g for Es. Intra- and inter-assay coefficient of variation, determined by repeated measurements of high and low value quality controls, ranged between 9.8-13.3% for T, 5.6-15.2% for P and 7.3-15.3% for E.

Estradiol ELISA, dihydrotestosterone ELISA and testosterone ELISA were purchased from BIOCOM-Biotech (DRG Instrument GmbH, Germany). A 1:20 dilution of blood plasma in washing buffer (10% PBS, 0.1% Tween20 in dH_2O) was prepared for the estradiol (E) and testosterone (T) enzyme-linked immunosorbent assays (ELISA). A 1:40 dilution of blood plasma in washing buffer was prepared for the dihydrotestosterone (DHT) ELISA.

The plasma concentration of E, DHT and T were assayed by double-antibody sandwich ELISA. All samples were measured in duplicate using Multiskan EX (Thermo Electron Corporation, cat. No. 1507300) and the ELISA assay as provided by the manufacturing company. The concentration of each hormone was corrected for dilution and expressed as ng/ml plasma.

3.5. Semen analysis

3.5.1. Study animals

This part of the study was conducted at the Two Oceans Aquarium in Cape Town, South Africa, using penguins housed in a captive colony showing breeding success. The light regime in their indoor enclosure was manipulated throughout the year to mimic natural seasonal changes. Penguins were given a standardized optimal fish diet that was supplemented with vitamins and minerals (Crawford *et al*., 2011).

Captive-born male penguins used during this study had reached the adult moulting stage and were suspected to be fertile (aged four at start of study). Two African penguins were habituated for the semen collection procedure from an early age. The males used were never pair-bonded with females prior to the study and were the only available breeding-age birds to collect semen samples from as part of an ongoing study on penguin reproductive biology.

3.5.2. Semen collection

Semen was collected on five occasions during the 2014–2016 breeding seasons by using an unrestrained, cooperative method as described for the Magellanic penguin (O'Brien *et al*., 1999). The annual breeding season is divided into two parts, with the long breading season lasting from June to November and the short breeding season lasting two months, namely January and February. At the beginning of the breeding season, males displayed breeding behavior, including vocalizations, flipper spreading, head shaking and body uplifting. The birds voluntarily followed their keepers to a quiet area of the indoor enclosure where the males willingly mounted the keepers' legs whilst continuing to exhibit breeding behavior. Ejaculates were collected once a week, on a petri dish held in position close to the averted cloaca. After ejaculation, neat semen was deposited into a collection vial and kept at 35 ºC for transport purposes until semen analysis commenced in less than 30 min after collection.

A total number of 51 ejaculates (44 from Penguin 1 and 7 from Penguin 2) were collected and standard semen parameters were analysed to assess the quantity and quality of these ejaculates. Thirty five sample were collected during the long breeding season and sixteen samples were collected during the short breeding samples. The macroscopic analysis included assessment of semen colour and volume, while microscopic analysis involved measurement of sperm concentration from the neat

sample, total number of sperm in the ejaculate and percentage total motility (see 3.5.3).

3.5.3. Sperm motility and concentration

Microscopic analysis was performed using the Sperm Class Analyser (SCA) 6.1 CASA system (Microptic S.L., Barcelona, Spain) (Fig 3.5). The analysis of sperm motility and concentration involved the pipetting of 3 µl and 2 µl semen into a 20 micron four and eight chamber Leja slide (Leja Products B.V., Nieuw Vennep, The Netherlands) respectively, pre-warmed to 40 ºC (body temperature of penguins). The slides were viewed using a Basler A312fc digital camera (Microptic S.L., Barcelona, Spain), mounted onto a Nikon Eclipse 50i microscope (IMP Solutions, Cape Town) fitted with a heated stage (40 ºC) and a 10x positive phase contrast objective. However, pseudo-negative phase contrast microscopy was employed using the positive phase contrast lens at phase contrast condenser settings 2 or 3.

The following motility and kinematic parameters were analysed using CASA: percentages total sperm motility, rapid, medium and slow swimming sperm as well as curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), straightness (STR), linearity (LIN), wobble (WOB), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF). Sperm motility tracks were captured at 50 frames per second, with the optics set at Ph+ (Pseudo-negative phase as explained above). Between three and five fields were captured for each sample until a total number of at least 200 motile spermatozoa were analysed per sample. The percentage rapid spermatozoa was determined using default SCA settings for birds and VCL cut-off values of 20<50>80 µm/s to identify slow, medium and rapid swimming spermatozoa. The various sperm kinematic parameters and their derivatives are described in Table 3.3 and Fig 3.6 is a diagrammatic presentation of some of these parameters.

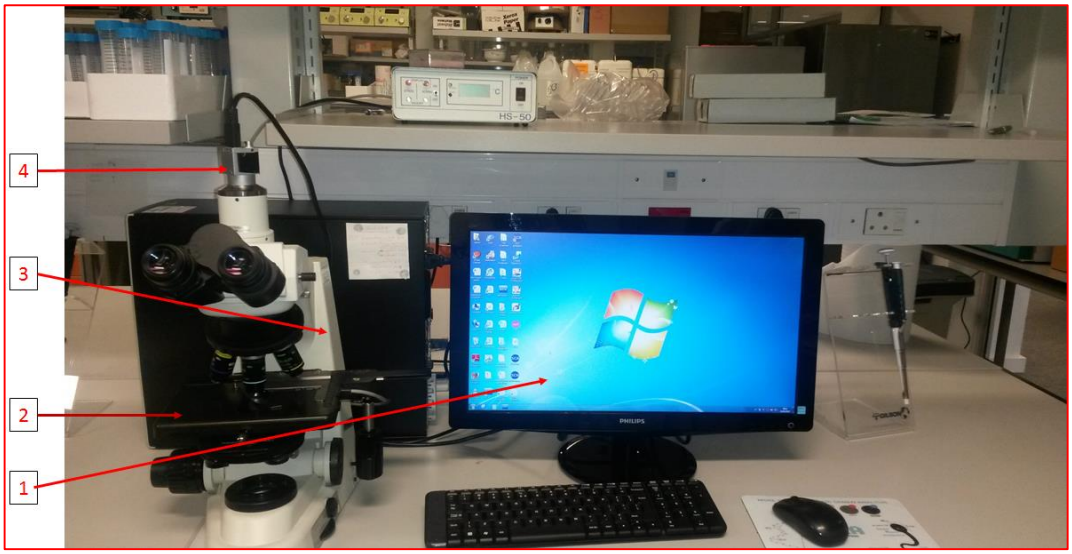

Figure 3.5: Equipment used for assessment of semen parameters as well as CASA analysis of sperm concentration, motility, vitality, and morphometry. 1) PC with SCA® software for CASA analysis, 2) Heated stage, 3) Nikon Eclipse 50i microscope, 4) Basler ACE 1300 digital camera.

Table 3.3: Definitions of eight sperm kinematic parameters determined by SCA® system.

Parameter	Description	Measurement
VCL (μm/s)	Curvilinear velocity	Time-averaged velocity of a sperm head along its actual curvilinear path.
VSL (μm/s)	Straight-line velocity	Time-averaged velocity of a sperm head along the straight line between its first detected position and its last.
VAP (μm/s)	Average-path velocity	Time-averaged velocity of a sperm head along its average path.
LIN (%)	Linearity	Linearity of the curvilinear path = 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 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.

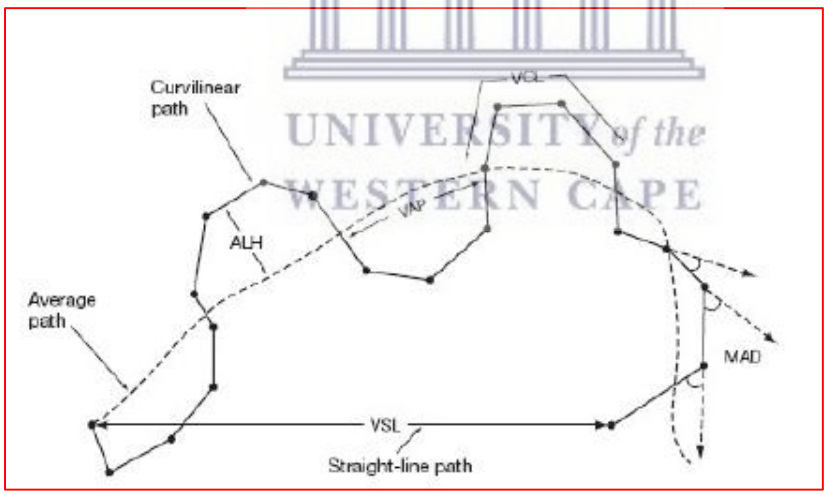

Figure 3.6: Diagrammatic presentation of kinematic parameters measured by CASA. ALH = amplitude of lateral head displacement, VAP = average path velocity, VCL = curvilinear velocity, VSL = straight-line velocity, MAD = mean angular displacement (not determined in this study). (WHO, 2010)

3.5.4. Swim-up technique

The swim-up technique involved the selection of motile spermatozoa by their ability to swim up into a culture medium (Jameel, 2008). The technique used in this study is a direct swim-up sperm preparation whereby spermatozoa leave semen and swim into the medium. The spermatozoa that are found or collected furthest away from the semen have the greatest probability of being motile and morphological normal. In the cervix of the female reproductive tract potentially fertile spermatozoa are separated from immotile spermatozoa. For ART purposes the development of sperm separation techniques are needed to distinguish potential fertile spermatozoa to non-motile sperm, in this study a swim-up method was used.

After a semen sample was collected, a volume of 3 µl was carefully pipetted underneath 300 µl of pre-warmed Ham's F10 (Sigma Aldrich, SA) medium which was placed in 1.5 ml Eppendorf tube (a 1:100 ratio). The preparation was kept at 37 ºC for the duration of the experiment. In order to make sure a motile sperm population was selected for evaluation, a sample of 3 µl was taken in the middle of Ham's F10 fraction to measure sperm parameters after 15, 30, 45 and 60 min of incubation. The comparison for time was done in two males for two breeding seasons.

3.5.5. Flush technique

This technique was first introduced Van der Horst *et al.,* (2010) and involves the exposure of semen to chemicals or media using Leja slides while being kept incubated on a pre-warmed microscope stage. The advantage of the flush technique is that it mimics the natural situation of semen release into the female reproductive tract compared to simply mixing semen with a cell culture medium, as is usually done in an experimental setup. This technique comprises of the flushing of semen from the entrance of the Leja chamber towards the furthest end of the chamber by the specific

medium tested. Fundamentally, no mixing occurs at the beginning of the displacement of semen and medium tested. The spermatozoa from the semen will swim in all directions and subsequently spermatozoa will come in contact with the medium that is introduced. The sperm motility analysis was only performed when spermatozoa in the particular section of the slide chamber were visually clear, which was established on the basis that no semen debris or any seminal particles were present except for spermatozoa. In the flush technique, spermatozoa are allowed to move towards the medium through slow progression from semen to the environment outside seminal plasma.

Three different media (Ham's F10 medium, 8 mM caffeine or 2 mM procaine hydrochloride) were used to induce hyperactivation. The selection of the chemical concentration was based on results of preliminary studies in other species, as nothing has been reported in other avian species. An aliquot of 0.25 µl semen was injected into a pre-warmed four chamber Leja slide followed by flushing of the semen into the slide by using either 2.5 µl Ham's F10 medium, 8 mM caffeine or 2 mM procaine hydrochloride, both prepared in Ham's F10 medium. Thereafter, sperm motility was analysed after 15, 30, 45 and 60 min while keeping the slide warm on the microscopic stage.

3.5.6. Semen cryopreservation

In order to determine the effect of cryoprotectant, semen samples for freezing were diluted with Triladyl diluent (Embryo Plus, SA) at a ratio of 1:16, because of high concentration of African penguin semen. Research by Rekha *et a*l., 2016 showed that semen preserved in Triladyl had better motility, viability, and functional integrity compered to other extenders. The straws (0.2- 2µM, Embryo Plus, SA) were filled at 37 ºC and sealed (PVC sealing powder, Embryo Plus, SA). The straws were cooled in a

fridge until the target temperature of 5 ºC was reached and held for 30 min. Straws were frozen in a closed styrofoam box, suspended in a liquid nitrogen vapour (-40 ºC) for 11min before being submerged under liquid nitrogen at -196 ºC for two hours. Post freezing the straws were thawed in a beaker containing 35 ml of water which was maintained in a temperature of 37 ºC. The straws were wiped dry and samples decanted into an eppendorf. The sperm motility was assessed immediately after Triladyl was added, then again after 30 min (before freezing) and immediately after thawing each sample. The sperm analysis was performed as described in section 3.5.3 including measurement of the percentages total sperm motility, rapid, medium and slow swimming sperm as well as eight kinematic parameters.

3.5.7. Sperm vitality

The vitality of sperm was assessed using nigrosin–eosin staining. The underlying principle of this method is that spermatozoa with an intact plasma membrane remain unstained and are assumed to be functional, while non-viable spermatozoa whose plasma membrane is disintegrating will absorb the stain. Using bright field microscopy, the viable sperm remain pearly white, while eosin will stain non-viable sperm a pink to magenta colour. The nigrosin serves as a background to enhance differentiation between the non-viable and viable sperm. Eosin-nigrosin (E/N) smears were prepared by adding 5 µl raw/neat semen to 50 µl of pre-warmed E/N stain at 37 ºC. Thereafter, 5 µl of the mixture was pipetted onto a pre-warmed StarFrost slide, a smear was made and allowed to air dry overnight. Once the stained smear was air dry, a cover slip was mounted using DPX mounting medium. To examine sperm vitality, microscopy analysis was performed using the Sperm Class Analyser (SCA) 6.1 CASA system (Microptic S.L., Barcelona, Spain) at 10x and 40x objective of a Nikon Eclipse 50i microscope no analyse. The use of no analyse function in SCA is not for automatic analysis but it is used rather for manual analyses. A total of 100 spermatozoa were assessed per

sample.

3.5.8. Sperm morphology and morphometry

SpermBlue (Microptic S.L., Barcelona, Spain) was used as morphology stain according to the technique described by van der Horst and Maree (2009). Semen was fixed in 2.5% phosphate buffered glutaraldehyde and smears were made using 10 µl semen, where after slides were air-dried for one hour. Subsequently, smears were stained for 12 min with SpermBlue. After staining, excess stain was drained and washed off by gently dipping slides into distilled water for 3 seconds. Slides were air dried at an angle of 60–80° and mounted with a cover slip using DPX mounting medium.

The stained spermatozoa were analysed with Nikon Eclipse 50i microscope bright field optics, using a blue filter and 40x and 60x objectives as well as a 100x oil immersion objective. The morphology parameters assessed included the acrosome, head, mid-piece and tail) for African penguin spermatozoa. The 40x and the 60x objectives were used for measuring the total length of spermatozoa, while the 100x oil immersion were used to measure individual sperm components.

Additionally, Normarski differential interference contrast microscopy (NDICM) was used to describe and evaluate sperm morphology. Preparations for NDICM involved pipetting 5 µl fixed semen (2.5% phosphate buffered glutaraldehyde) on a clean slide and covering it with a cover slip.

Measurement of morphometric parameters involved semi-automated image analysis using the analySIS® FIVE soft imaging system (Wirsam, Cape Town, South Africa). Microscopic analysis was performed using a Zeiss Photomicroscope III (Zeiss, Cape Town, South Africa) equipped with bright field optics, including a 40x objective (sperm

principal piece measurements) and a 100x oil immersion objective (all other sperm component measurements). Micrographs were taken using an Olympus Astra 20 digital camera (Wirsam, Cape Town, South Africa) fitted on the microscope.

3.5.9. Sperm ultrastructure

Semen samples were prepared for transmission electron microscopy (TEM) by adding 5 µl semen to 100 µl 2.5% phosphate buffered glutaraldehyde followed by post-fixation in 1% osmium tetroxide in the same buffer. These fixed samples were subsequently processed for TEM including contrasting with lead citrate and uranyl acetate using standard procedures (Maree *et al*., 2010). A Reichert ultramicrotome (SMM Instruments, Johannesburg, South Africa) with a diamond knife (Agar Scientific, Randburg, South Africa) was used to make silver to gold sections. Thin sections on copper grids were examined using a Jeol JEM 1011 transmission electron microscope at 80 kV (Advanced Laboratory Solutions, Johannesburg, South Africa).

3.6. Statistical analysis

Every sample collected was considered as an individual sample during statistical analysis. For semen analysis multiple test were performed in the same individuals in different semen ejaculates. Medcalc version 17.2 Medcalc Software, Mariakerke, Belgium, was used for basic statistical analyses. Tests were executed for normality of distribution and most data sets represented normal distributions. Kolmogorov-Smirnoff test for equality of variances was applied and when $P>0.05$, one way analysis of variance (ANOVA) analysis was performed for parametric data distributions. In subsections of data that appeared to have non-parametric ($P<0.05$) data distributions, the Mann-Whitney test for independent samples was used. The significance between the means, $P<0.05$ was designated as significantly different. Data is represented as the mean ± SD in the tables and $P < 0.05$ was considered significant. For each hormone

(and hormone metabolites), the relationship between faecal and plasma values was examined across the sexes using linear regression (r=-0.8833). This correlation was also examined separately at each of the seasons (breeding vs non-breeding) stages. Effect trend, rather than statistical significance, was emphasized in interpreting results. The data is represented as the mean ± standard deviation in the tables.

Chapter 4:

Results

4.1. Macro-anatomy of the male and female reproductive structures in African penguin

This section is intended to describe the structure and basic anatomical features of the male and female reproductive tract of African penguin and is not an in depth anatomical study. The information presented is based on observations from accidentally deceased birds and relates to what is reflected in the literature on ratites (Valdez *et al*., 2014).

4.1.1. Male reproductive macro-anatomy

The reproduction system of the male African penguin is similar to that of other birds but different to that of the mammals. The testes are bean- or egg-shaped and surrounded by a well-developed fibrous capsule that includes connective tissue and contractile fibers composed of actin and desmin intermediate filaments (Fig 4.1). The testes are situated in the abdominal cavity, ventral to both the vertebral column and the kidneys. The bean-shaped left testis and the egg-shaped right testis are directed towards the midline and the caudal vena cava lies in the narrow gap between their dorsomedial surfaces. Differences in testicular size and weight were noticeable according to age. The testes in juveniles are very small, with an average length and width of 7.31 ± 0.61 mm and 2.43 ± 0.97 mm respectively. Furthermore, juvenile testes have no clear visible blood vessels macroscopically, are cream in colour and cylindrical in shape (Fig 4.1A). The testes of adult penguins are creamy-white or yellow in colour (Fig 4.1B and C).

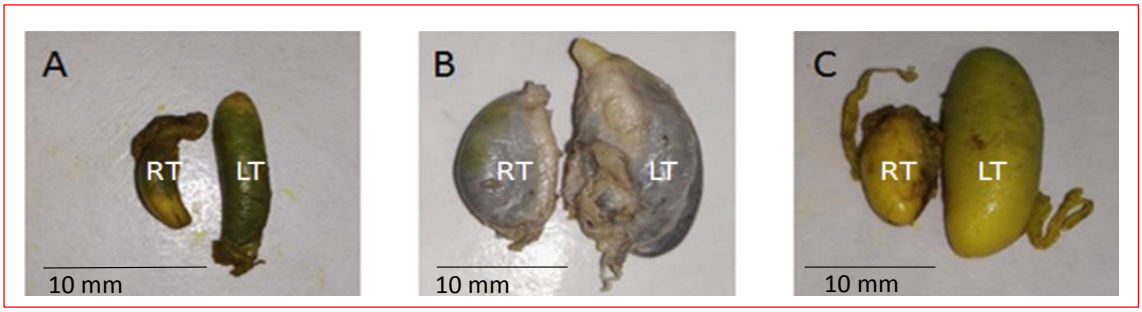

Figure 4.1: The excised testes of A) juvenile, B and C) sexually mature African penguins (ventral view). The difference in size and shape between the right (RT) and the left (LT) testes. The large LT is bean-shaped and the RT in B and C is round to oval in shape. The epididymis is not clearly distinct on either testis

The mean and range for left and right adult testis dimensions are presented in Table 4.1. The testes are asymmetrical in adults, with the right testis on average tending to be smaller in length, width and volume respectively (16.80 ± 4.37 mm; 7.93 ± 2.63 mm; 0.75 mL) compared to the left testis (25.39 ± 5.85 mm; 11.48 ± 4.09 mm; 2.46 mL). No significant differences (P>0.05) were observed, however, observed between right and left testis dimensions, probably due to large range in values for each parameter. For instance, the right testis length varied from 8.21 mm to 25.13 mm while the left testis varied between 14.10 mm and 39.09 mm in length. The mean weight for right and left adult penguin testis were 1.15 ± 0.75 g and 2.71 ± 1.46 g (n = 25) respectively. The range of weight for right and left adult penguin testis were 0.10 – 4.00 g and 0.39 – 7.41 g (n = 35) respectively. It was observed that during the breeding season, the adult testis greatly enlarges, however, there was no significant difference (P>0.05) observed between the two seasons. Table 4.2 and 4.3 present the mean and range for left and right adult testis dimensions during the two seasons.

Table 4.1: Summary of mean testis dimensions for all African penguins (mean ± SD). (n = 36***).***

	Right testis		Left testis	
	Mean ± SD	Range	Mean ± SD	Range
Length (mm)	16.80 ± 4.37	8.21 – 25.13	25.39 ± 5.85	14.10 – 39.09
Width (mm)	7.93 ± 2.63	2.95 – 15.25	11.48 ± 4.09	5.11 – 23.10
Volume (mL)	0.75 ± 0.62	0.05 – 3.45	2.46 ± 2.23	0.22 – 10.26
Weight (g)	1.15 ± 0.75	0.10 – 4.00	2.71 ± 1.46	0.39 – 7.41

SD= Standard deviation

Table 4.2: Summary of mean testis dimensions for adult breeding African penguins (mean ± SD). (n = 26)

	Breeding			
	Right testis		Left testis	
	Mean ± SD	Range	Mean ± SD	Range
Length (mm)	18.11 ± 4.07	8.21 – 25.13	28.10 ± 4.63	22.68 – 39.09
Width (mm)	9.10 ± 2.03	5.58 – 15.25	13.24 ± 3.66	7.43 – 23.10
Weight (g)	1.41 ± 0.77	0.43 – 4.00	3.27 ± 1.36	1.11 – 7.41

SD= Standard deviation

Table 4.3: Summary of mean testis dimensions for adult non-breeding African penguins (mean ± SD). (n = 9)

	Non-Breeding			
	Right testis		Left testis	
	Mean ± SD	Range	Mean ± SD	Range
Length (mm)	15.51 ± 8.48	9.98 – 18.82	21.14 ± 3.33	14.40 – 26.32
Width (mm)	6.12 ± 1.82	2.95 – 9.03	8.64 ± 1.53	5.11 – 9.94
Weight (g)	0.57 ± 0.23	0.10 – 0.91	1.34 ± 0.50	0.39 – 2.22

SD= Standard deviation

The avian epididymis is a poorly developed structure embedded within connective tissue and it is attached to the testis towards the dorsal body wall. The epididymis is closely adjacent to both the testis and the kidney. The penguin epididymis is a small spindle-shaped structure that is closely attached to the medial border of the testis. It is a short duct when compared to the elongated epididymis present in mammals. In this study the epididymis measurements were not taken due to difficult in trying to separate the epididymis from the testis.

4.1.2. The female reproductive macro-anatomy

The female African penguin reproductive structures include only a left ovary, an oviduct, a uterus and a vagina. The ovary is attached to the bodies of the lumbar vertebrae by the mesovarium and located dorsomedial to the spleen. The ovary of a juvenile female is flattened and it resembles a pad of fat tissue (Fig 4.2A). In many respects, the ovary of juveniles appears comparable in size to that of an adult penguin. There were no externally visible follicles in juvenile ovaries (Fig 4.2A), which makes it difficult to differentiate it from the testis of a juvenile male. In adult females, the ventral surface of

the ovary has a grape-like cluster of small but noticeable follicles that are easy to identify (Fig 4.2C and D). Although lots of variation was noted in the ovarian shape and size among the penguins observed in this study, the adult ovary has a mean length of 25.72 ± 5.37 mm and a mean width of 9.02 ± 3.87 mm (n = 37). After the breeding season, the ovary of an adult penguin goes into a resting phase where it decreases in length and width (Fig 4.2B); such an ovary is then described as being mature but out of breeding season. The mean weight of an adult ovary was 1.32 ± 1.07 g (n = 28).

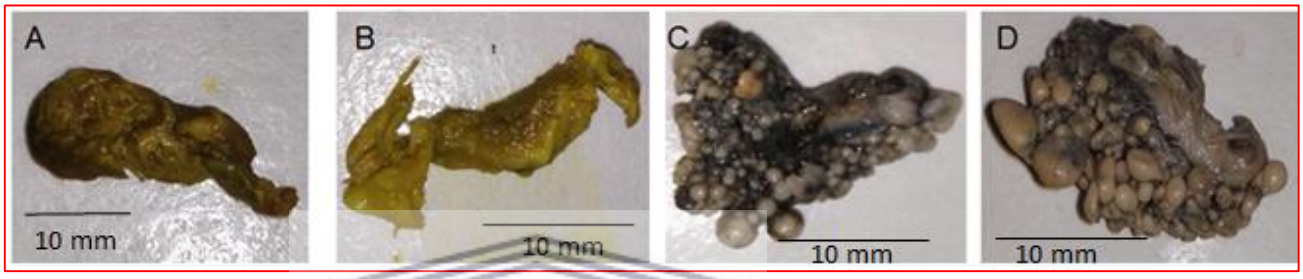

Figure 4.2: The macroscopic view of ovaries of A) juvenile (n=6), B) adult non-breeding penguin and C and D n=10) adult breeding (n=13) penguin, with follicles in the ovaries appearing as a bunch of grapes.

Macroscopic observation of the ovaries revealed changes in size and colour during follicle development, where small to large follicles can be observed in each period of the season (breeding or non-breeding season). Follicles ranged from white small follicles with a diameter of <0.01 mm to mature, yellow large follicles which had a maximum diameter of 22 mm (Fig 4.2C and D). The mean follicle diameter was 2.10 ± 1.58 mm. The number of follicles at each stage of development suggests ovarian activity and can be used to classify a female penguin as breeding or non-breeding. The well-developed, yolk-filled follicles can be described as mature and it represents a reproductive active penguin. The number of follicles for reproductively breeding penguins ranged from 30 to 160 per ovary (Fig 4.3), with most ovaries having between 55 to 80 follicles.

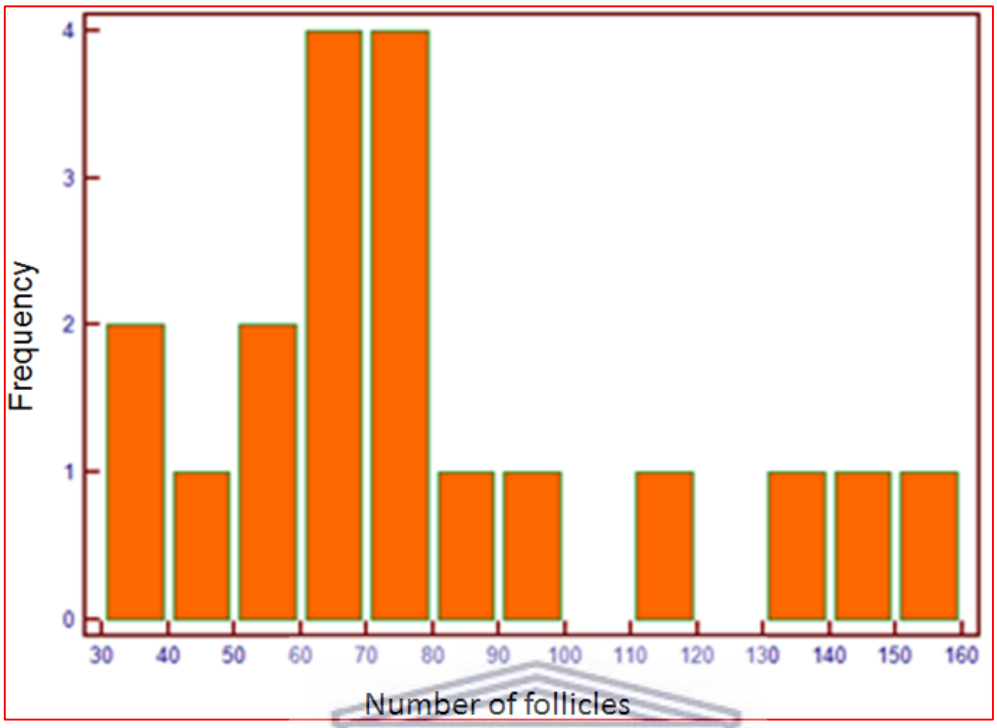

Figure 4.3: Number of follicles per ovary in breeding penguins (n = 19).

The fully developed left oviduct of the African penguin could be clearly seen with its sub-divisions. The oviduct is divided into segments in succession from infundibulum, magnum, isthmus, and connected to the expanded uterus and vagina. In juveniles, the oviduct is underdeveloped and can be distinguished as a small, straight tube stretching from the ovary towards the cloaca without clear connections to the uterus and eventually the vagina.

4.2. Histology of the testis and ovary

4.2.1. Histology of the testis

The testis of the African penguin is covered by a dense irregular connective tissue capsule, namely the thin, tightly adherent tunica albuginea. Since this capsule is very

thin, unlike in mammals, and it does not give off septa to divide the testis into separate lobules, the capsule is consequently soft. Generally in birds, the testicular capsule is composed of three main tissue layers: an outer, thin tunica serosa, a thick tunica albuginea and the innermost, very thin tunica vasculosa. Smooth muscle cells in the testicular capsule are responsible for the spontaneous contractions of the capsule. The additional histological characteristics of the testes depend on the age and sexual activity/breeding cycle, with maximal development during the breeding season (Fig 4.4 – 4.6 and Fig 4.8).

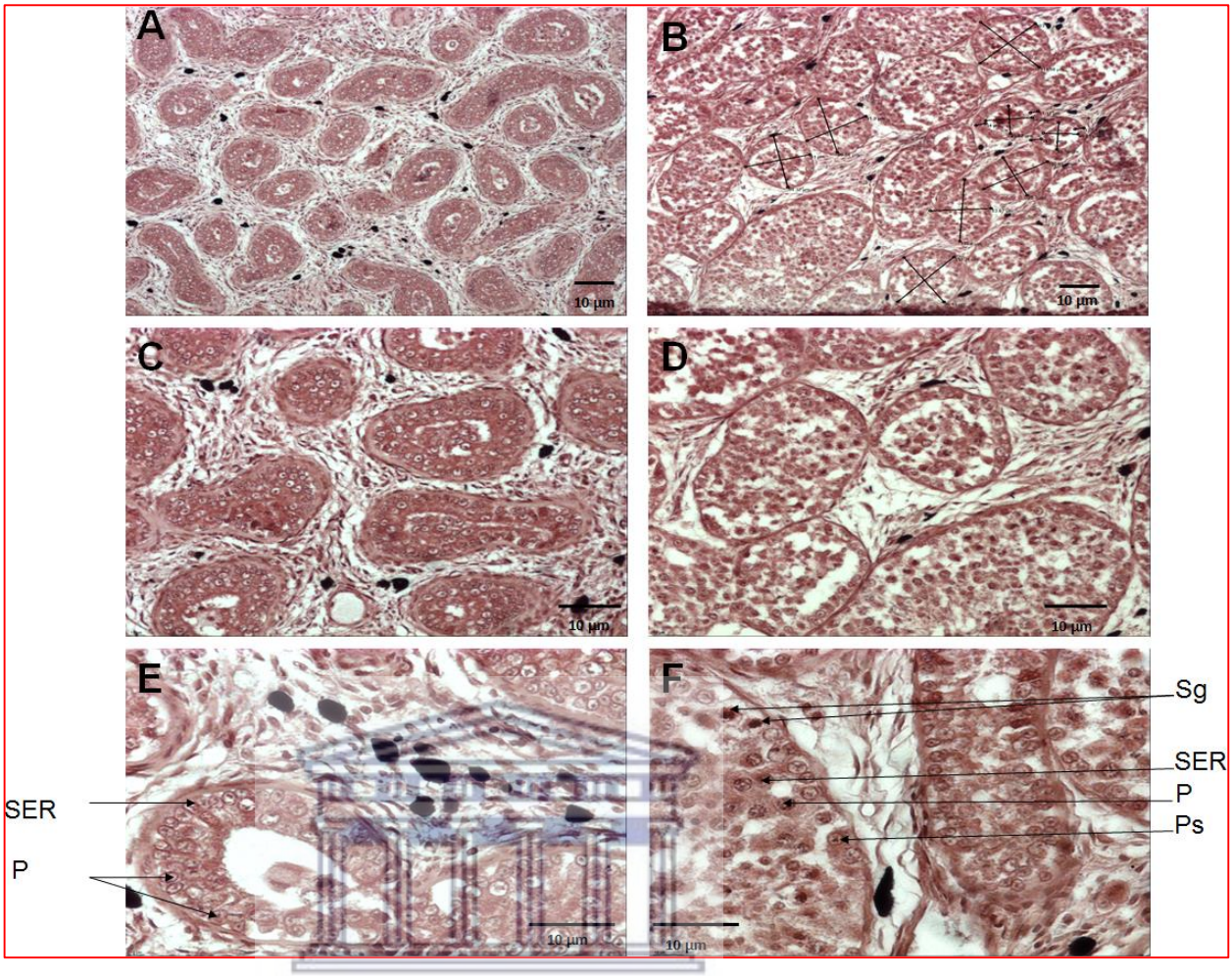

Figure 4.4: Micrographs of the juvenile African penguin seminiferous tubules showing complex branching of these tubules. A – B) Micrographs taken at X100 initial magnification displaying different shapes of the seminiferous tubules, C – D) X200 initial magnification, noting that the lumen is not clearly visible open and E – F) X400 initial magnification showing Sertoli cells (SER), spermatogonia (Sg), primary spermatocytes (P),secondary spermatocyte (Ps).

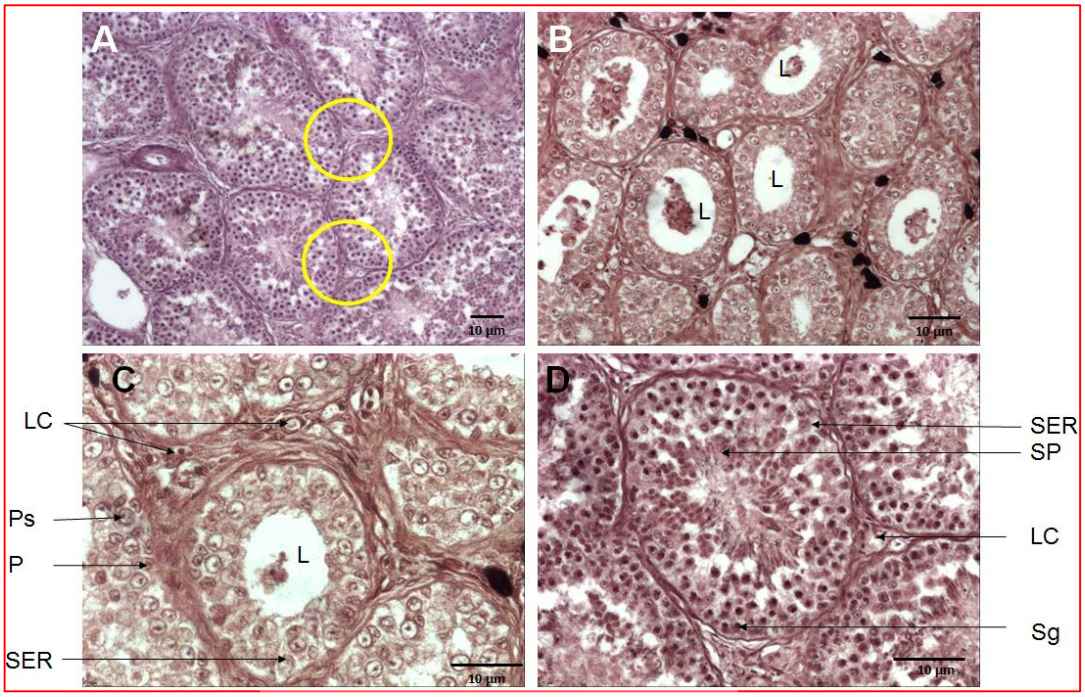

Figure 4.5. Micrographs of non-breeding African penguin testis demonstrating the closely packed seminiferous tubules separated by thin strips of boundary tissue. A) Note the interstices between groups of three seminiferous tubules (yellow circled). B) Lumen of seminiferous tubule (L). C – D) Higher magnification (400X) showing lumen (L), Sertoli cells (SER), spermatogonia (Sg), primary spermatocytes (P), secondary spermatocyte (Ps), spermatids (SP) and Leydig cells (LC).

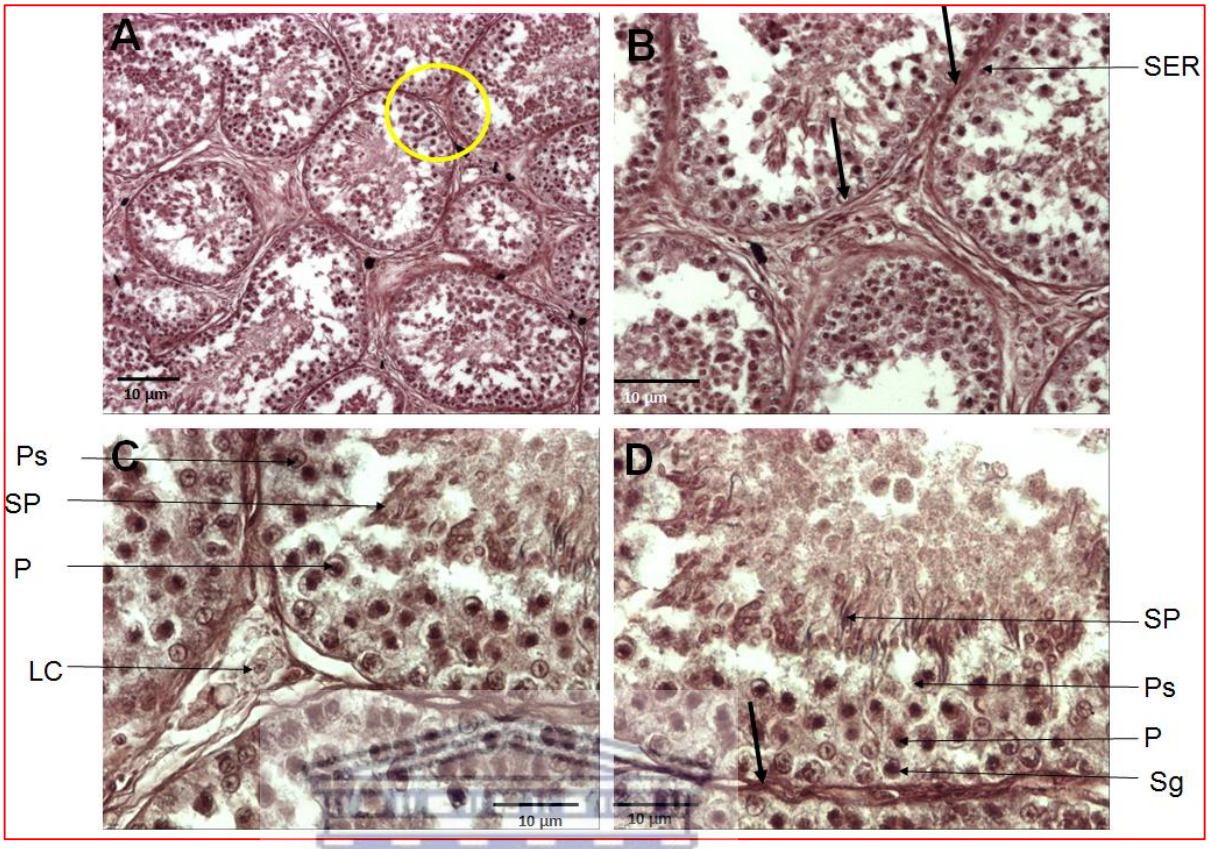

Figure 4.6: H & E photomicrographs of the seminiferous tubules of adult breeding African penguins. A) Note the interstices between groups of three seminiferous tubules (yellow circled). B) Closely packed seminiferous tubules separated by thin strips of boundary tissue (arrows) and Sertoli cells (SER). C-D) The organization of the seminiferous epithelium at higher magnification X40 view of the seminiferous tubules displaying various germ cells in the epithelium, spermartogonia (Sg), primary spermatocytes (P), secondary spermatocyte (Ps), spermatids (SP) and Leydig cells (LC).

The seminiferous tubules are bound together by the interstitial tissue which consists of blood vessels, lipids and Leydig cells (LC) containing lipid droplets (Fig 4.5 and 4.6). The abundant lipid content of the LC observed are believed to be responsible for the secretion of testosterone (Fig 4.9). The LC are polymorphic cells with a spherical, polyhedral shape and have large nuclei with distinct nucleoli (Fig 4.5B). They were

located in the angular space among the ST (Fig 4.5, 4.6 and Fig 4.9). Blood vessels are closely associated with the LC and provide a route for testosterone transport.

A basement membrane separates the thin mesothelium cells of the tunica serosa from the connective tissue of the tunica albuginea. The seminiferous tubules (ST) are bound by a constant number of clearly defined layers, namely a myoid layer, basal lamina and collagen fibers (Fig 4.9B). The basal lamina is attached to the seminiferous epithelium. A layer of flattened cells, the myoid cells, are adjacent to the basal lamina. The myoid cells contain many extremely fine cytoplasmic filaments. These cells are believed to be responsible for the rhythmic contraction of the ST and also the propulsive movement of sperm from the lumen to the excurrent ducts.

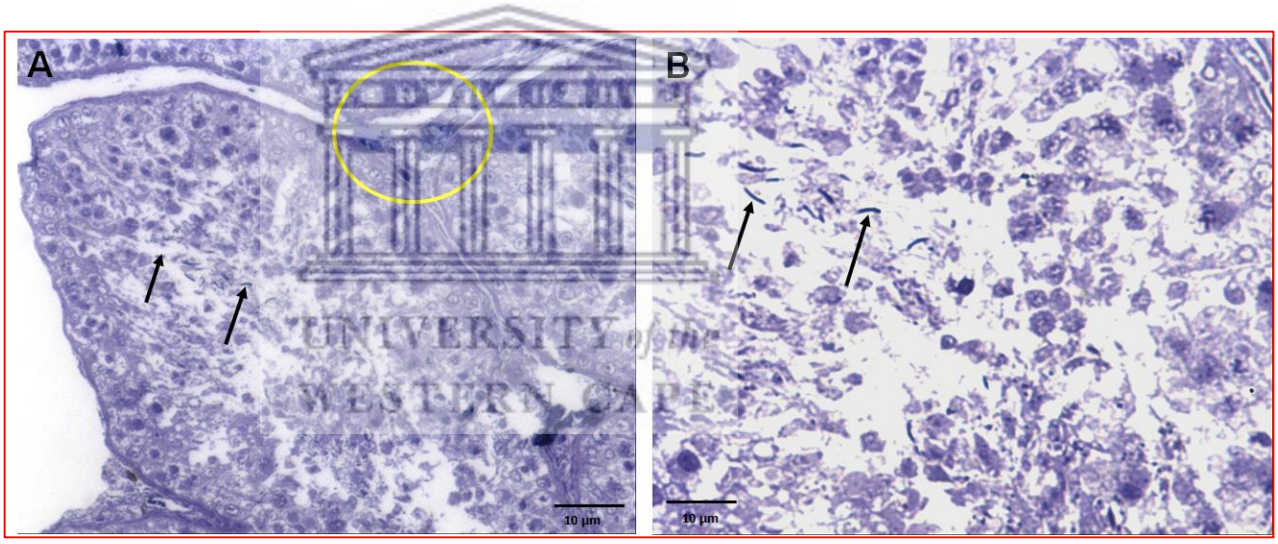

Figure 4.7: A section of the seminiferous tubules of an adult breeding African penguin, stained with toluidine blue. A) The interstitial space (yellow circle) between the different tubes of the seminiferous tubules and B) different phases of the spermatids (arrows).

The ST form the greater part of the testicular parenchyma and they are tightly packed. The ST of birds, contrary to those of mammals, form a highly complex anastomotic,

non-blind ending network of tubules (Aire, 2000) (Fig 4.4 – 4.7). The ST open directly into the rete testis as in other avian species. The ST had a round to oval shape due to the complex coiling of the tubules at different levels. The mean diameter of ST adult males was 133.05 ± 64.12 µm (Table 4.4), containing various types of spermatogenic and other somatic/sustentacular cells (Fig 4.4 - 4.7, 4.9 (A & D) and 4.10). In this study the ST diameter ranged from 48.27 µm to 303.42 µm and the frequency is displayed in Fig 4.8. and Table 4.5. The most frequent ST diameter between 60.00 µm and 140 µm. The smallest diameter (48.27 µm) was observed in a non-breeding penguin. The lumen and epithelium thickness means were 62.34 ± 32.58 µm and 33.99 ± 23.21 µm, respectively. In an adult penguin, the testes have a sizeable amount of fluid content in the lumen of the ST.

Table 4.4: Measurements of morphological parameters (mean ± SD) for seminiferous tubules (n=145) in adult African penguins (n=6).

	Mean ± SD	Range
Total diameter (µm)	133.05 ± 64.12	48.27 – 303.42
Lumen diameter (µm)	62.34 ± 32.58	23.01 – 151.54
Epithelium thickness (µm)	33.99 ± 23.21	11.52 – 94.48

SD= Standard deviation

Table 4.5: Measurements of morphological parameters (mean ± SD) for seminiferous tubules between breeding versus non-breeding (African penguins (n=6).

	Breeding		Non-Breeding	
	Mean ± SD	Range	Mean ± SD	Range
Total diameter (µm)	184.62 ± 52.54	93.3 – 303.42	74.72 ±14.86	48.27 -111.36
Lumen diameter (µm)	96.03 ± 36.07	42.32 – 151.54	43.67 ± 13.20	23.01- 88.44
Epithelium thickness (µm)	57.61± 18.91	16.93 – 94.48	21.23 ± 12.47	14.09 – 90.05

SD= Standard deviation

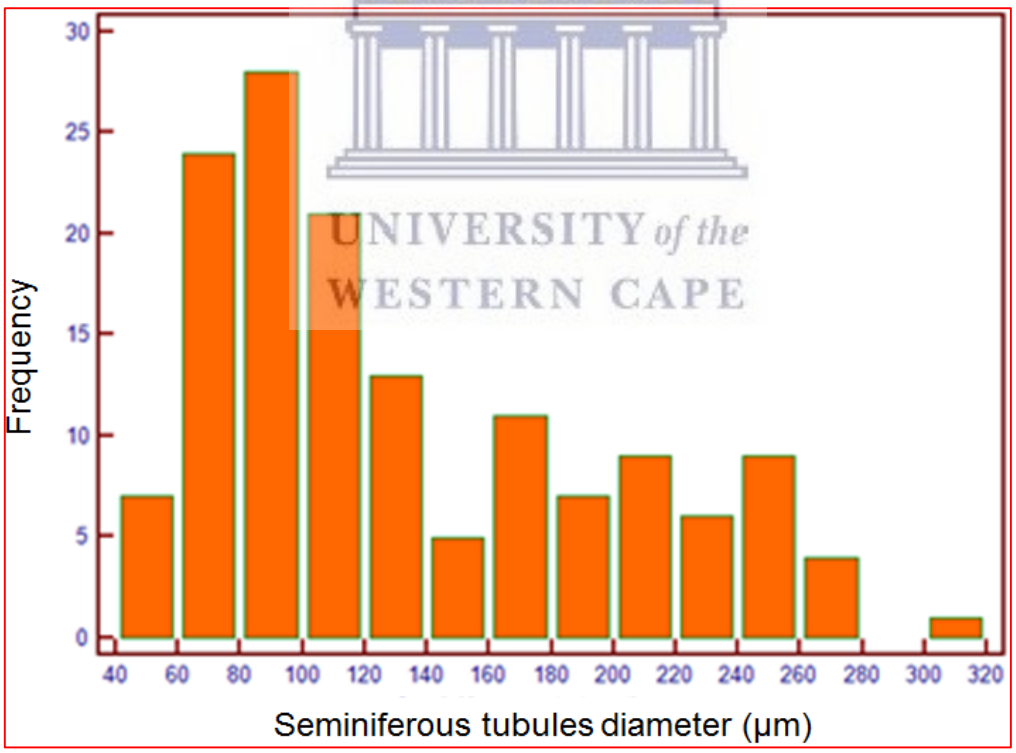

Figure 4.8: Frequency distribution of seminiferous tubule diameter in African male penguins (n=6).

The various cell stages typical of spermatogenesis could be identified in the seminiferous epithelium during the breeding and non-breeding seasons for the adult African penguin, making use of both light and transmission electron microscopy. The stages are arranged in well-defined concentric layers within the epithelium. These cells include, from the periphery to the lumen of the tubules, spermatogonia, primary and secondary spermatocytes and elongated immature and mature spermatids (Fig 4.5, 4.6, 4.9 and 4.10). Spermatogonia are typically small, round cells that lie adjacent to the basement membrane and are characterized by having large, round nuclei which arose from the primordial germ cells. They are located at the periphery of the tubule and are frequently in direct contact with the basal lamina. Spermatogonia have a lightly stained nucleus lying eccentrically in the cytoplasm. On the basis of nuclear appearance, two types of primary spermatocytes have been described (Fig 4.10). Generally, the primary spermatocytes are observed to be the largest germinal cells in the testis of African penguin. They differ from secondary spermatocytes in relation to their nuclei, with primary spermatocytes having large, rounded nuclei. In addition to the size difference, secondary spermatocytes may be identified by their spherical nucleus containing granular chromatin (Fig 4.10B). The secondary spermatocytes were considerably smaller and only slightly larger than the spermatogonia. The annual cyclic changes that occur in the testis of an African penguin are illustrated in Fig 4.9 and Fig 4.10.

Within the epithelium, non-dividing, tall columnar Sertoli cells (SER) also occurred and were considerably fewer in numbers compared to spermartogonia. The SER extend from the basal lamina of the seminiferous epithelium to the lumen and were identified by their oval-shaped nuclei with a well-developed nucleolus (Fig 4.10A and B). In non-breeding birds, the Sertoli cells were randomly distributed and their nuclei were smaller than spermtogonial nuclei. The cytoplasm of the SER was not easily recognized due to the presence of many germinal cells, but it has been reported by Lake (1956) that they

form an extensive complex around and between the germinal cells, making contact with them. The SER are the supporting cells for the developing spermatozoa that become attached to their surface after meiosis.

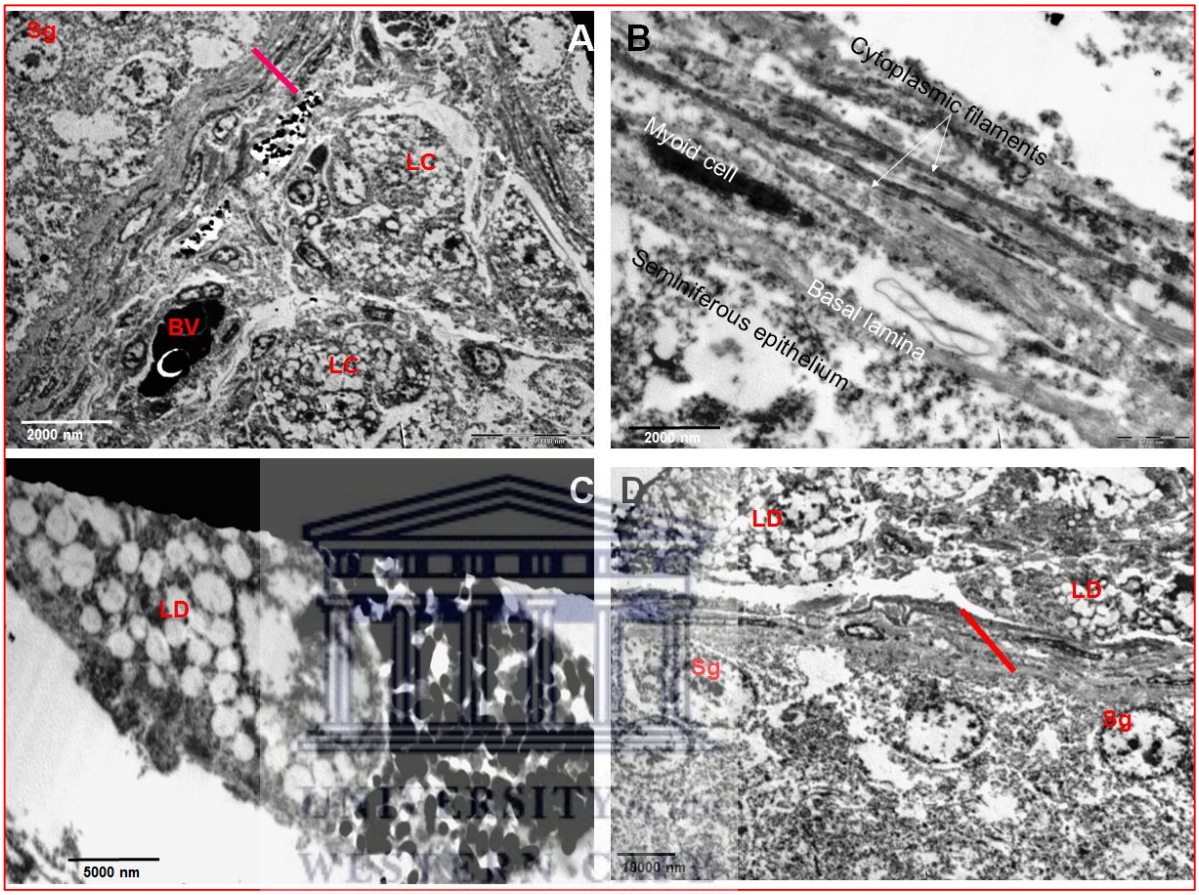

Figure 4.9: TEM micrographs of the seminiferous epithelium of breeding African penguins. A). Section of interstitial tissue including blood vessels (BV) and Leydig cells (LC). The basement membrane (red bar) separates the tunica serosa from the connective tissue of the tunica albuginea. The BV and LC are located within the interstitial and are close in contact with each other. B) Electron micrograph of the African penguin tunica propria. The basal lamina with deep collagen fibres rests on amorphous material adjacent to myo-fibroblast cells of the boundary tissue. The myoid cells are characterized by densely packed filaments. The cytoplasmic filaments of the myoid cells and basal lamina surround the seminiferous tubules of the African penguin. C) Leydig cells with lipid droplets (LD). D) Section of the seminiferous epithelium showing

spermatogonia (Sg) next to the basal lamina and presence of lipid droplets (LD) in the interstitial space.

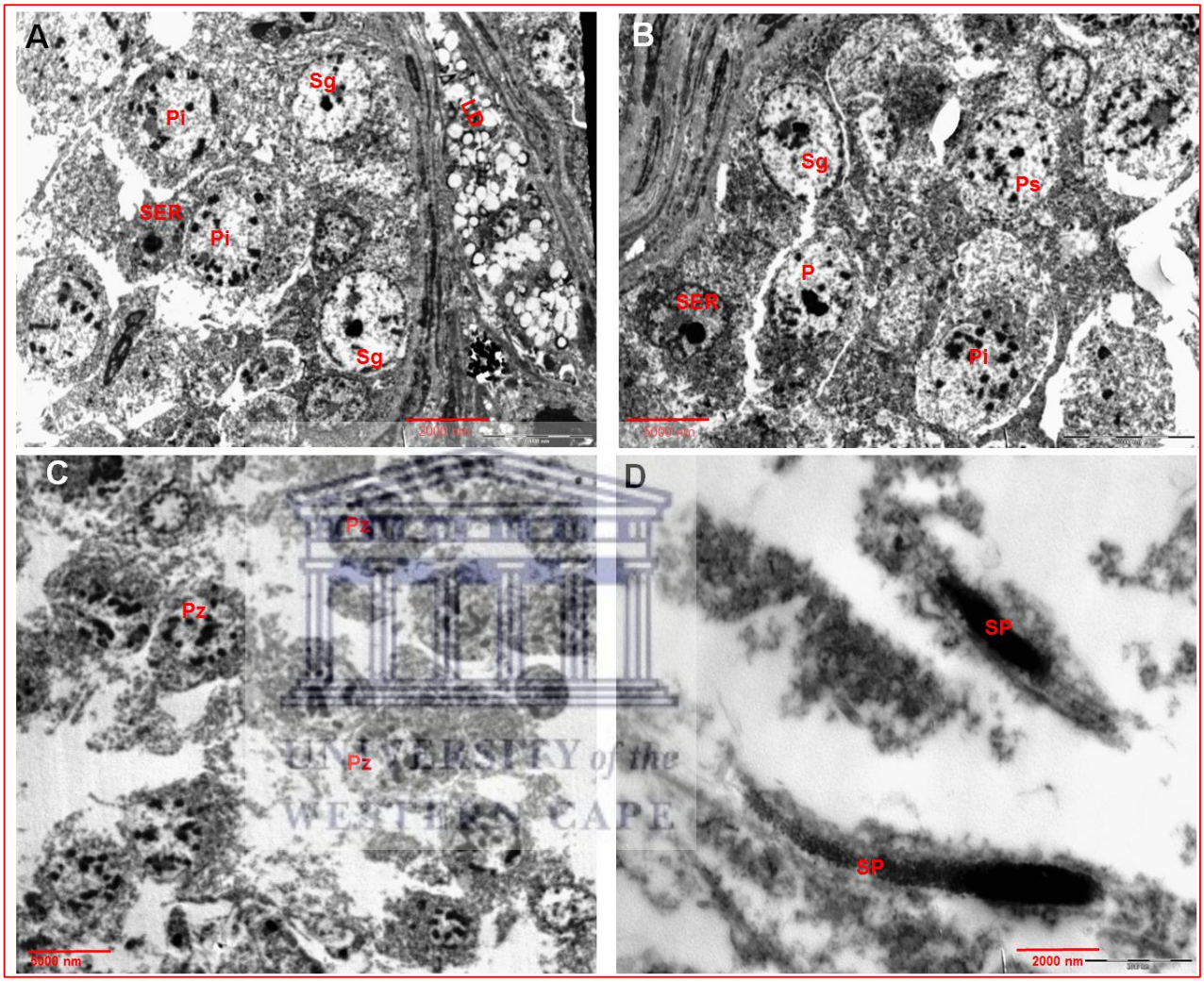

Figure 4.10: Electron micrographs of the African penguin portraying the cellular components of the seminiferous tubules. (A – B) Organization of the seminiferous epithelium, including Sertoli cells (SER), spermatogonia (Sg), primary spermatocytes (P), secondary spermatocytes (Ps), different stages of spermatids (SP) and lipid droplets (LD). In the leptotene (Pi) stage, the chromosome are evident as thin and delicate filaments. C) As the cells enter the pachytene stage (Pz), the chromosomes become shorter and thicker and each one split into two chromatids. D) Different stages of spermatids (SP) in the lumen of the ST

4.2.2. Histology of the ovary

Adult African penguin ovaries revealed uneven surface topography, with prominences separated by grooves. The outside of the ovary is covered by a thin continuous mesothelium composed of a single layer of cuboidal epithelium. The tunica albuginea is a layer of collagenous connective tissue on the outside that is covered by the thin mesothelium. Internally, the ovary basically consists of an outer cortex (zona parenchymatosa) surrounding a vascular medulla (zona vasculosa). The outer cortex contains ova while the medulla is composed of primarily connective tissue. Attached within the loose connective tissue of the medulla are nerves and blood vessels. In the cortex of the ovary, only small follicles were present in juveniles and non-breeding penguins, while in breeding females there were numerous follicles showing different stages of development, ranging from primordial follicles to mature follicles (Fig 4.11 - 4.13). Inside an ovisac, the mature follicles occupy the deeper area of the cortex, while the primordial and primary follicles are situated peripherally (Fig 4.12 and Fig 4.13). The follicles with the largest diameter were only observed in breeding penguins. These results demonstrate that an adult African penguin female has follicles in both the breeding and non-breeding seasons.

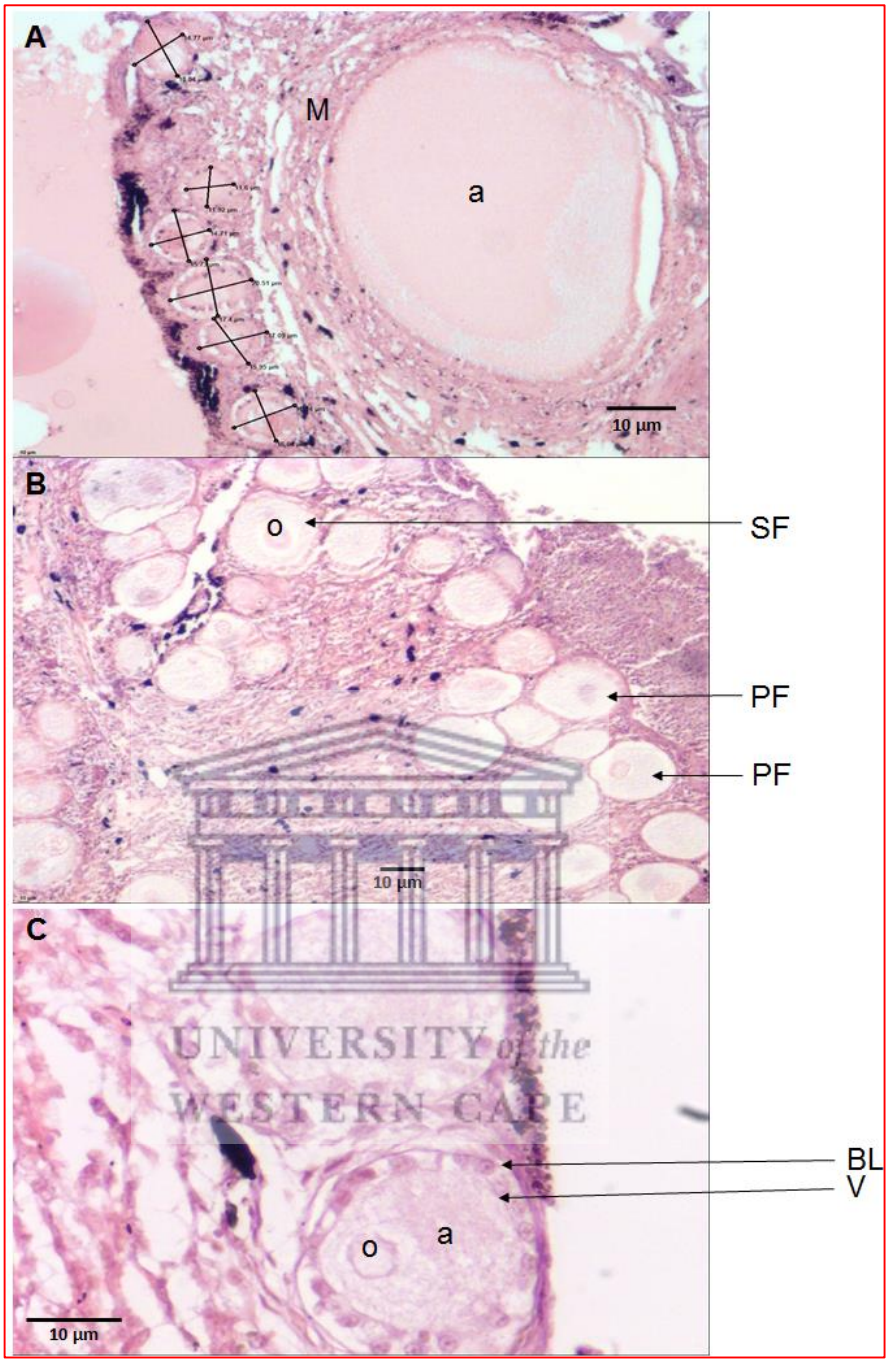

Figure 4.11: Micrographs of juvenile female African penguin ovaries (n=6). (A) Ovary showing different follicle sizes and how the follicles were measured using SCA measurement tools (see Materials and Methods). B) Primary follicles (PF), secondary follicles (SF) in the cortical stroma of the ovary, C) Growth of follicles in size which contain an enlarged oocyte (o), containing a nucleus, and (a) antrum, surrounded by flattened vitelline membrane (V) and basal lamina (BL)

Table 4.6: Summary of mean follicle diameter (mean ± SD) of different classes of follicles for all African penguins primarily based on size (n=22).

	Mean ± SD (µm)	Range (µm)
Primary follicles	11.21 ± 1.50	7.12 – 13.45
Secondary follicles	16.25 ± 1.99	13.06 – 20.75
Tertiary follicles	25.17 ± 5.73	18.95 – 42.46

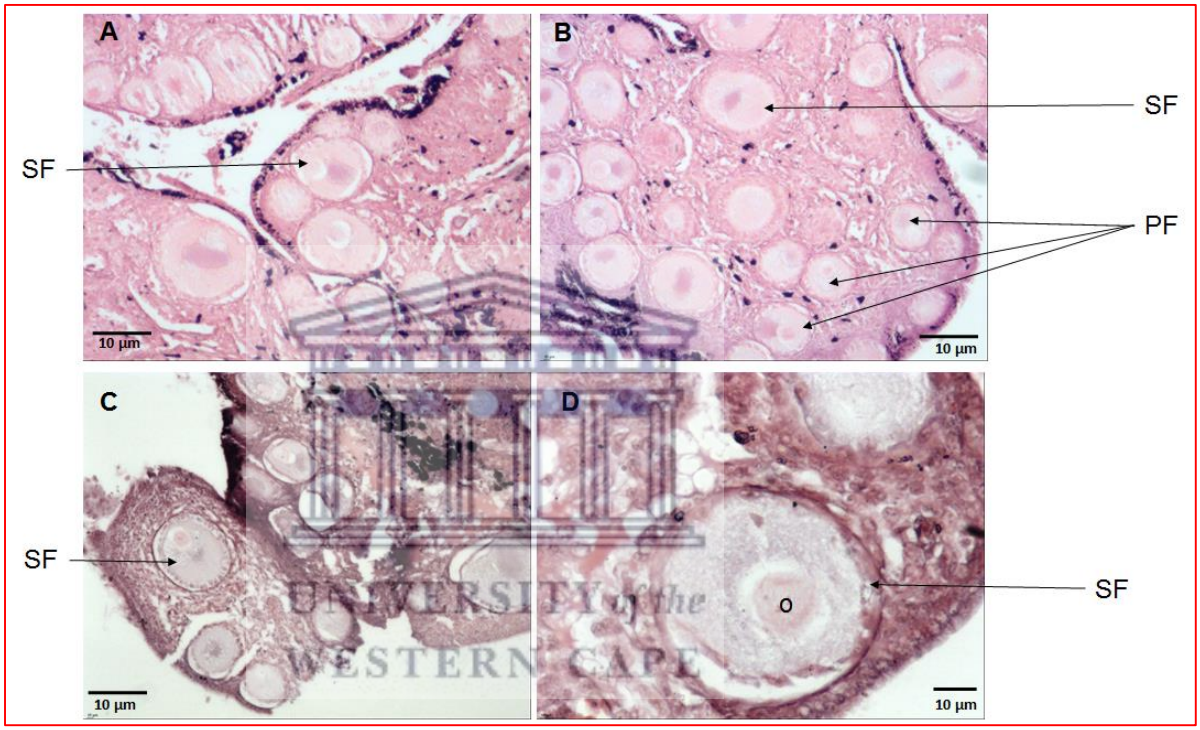

Figure 4.12: Histological view of follicular configuration in ovaries of non-breeding penguin females (n=8). A - C) Primary follicles (PF) and secondary follicles (SF) in the cortical stroma of the ovary. D) Grown follicle in size which contain an enlarged oocyte (o), a nucleus, surrounded by a tinny vitelline membrane.

Table 4.6 show the different classes of follicles primarily based on their size. The mean and range of follicle diameter for juvenile and adult (breeding and non-breeding)

penguins are displayed in Table 4.7. Most of follicles ranged between 10.00 – 20.00 µm in diameter (Fig 4.15), and the mean diameter was 16.55 ± 5.50 µm. The primordial follicles are small (diameter 5.00 – 11.81 µm) compared to the mature (vitelline) follicle (diameter 13.5 – 42.46 µm). Abundant follicles were found in adult non-breeding and breeding penguins.

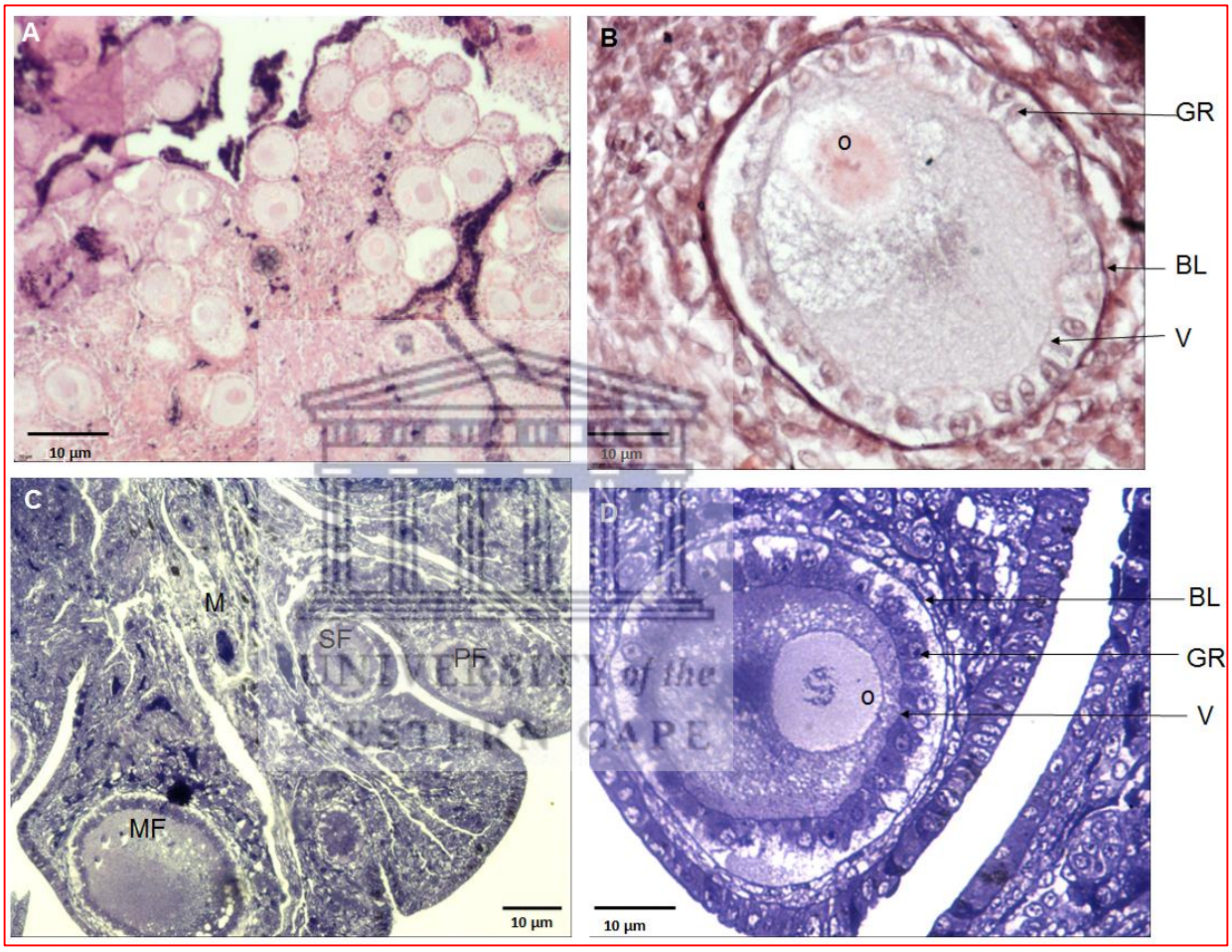

Figure 4.13: Histological view of follicles of breeding African penguins (n=11) stained with H & E and toluidine blue. H & E (A and B) and toluidine blue (C and D). A) Primary follicles (PF) and secondary follicles (SF) in the cortex captured at 100X magnification. C) Primary follicle (PF), secondary follicle (SF) and mature follicle (MF) in the cortex and blood vessels in the medulla (M). B and D) Enlarged follicle and oocyte (o), containing nucleus, surrounded by a thin vitelline membrane (V), proliferated granulosa cells (GR) and basal lamina (BL).

Primordial follicles containing primary oocytes remain embedded within the stromal tissue in the cortex and lack an epithelium cell layer (Fig 4.13 A). The demarcation between these follicles were not clear, we use size to differentiate them (Table 4.6). The granulosa and theca layers are separated by an acellular basement membrane, namely the basal lamina. Granulosa cells of the follicles are cuboidal and situated in between the basal lamina and the vitelline membrane (Fig 4.13 B and D). The vitelline membrane is an acellular fibrous layer homologous to the mammalian zona pellucida. The layers of theca interna and external layers surround the basal lamina. Further follicular growth lead to antrum type follicles in which granulosa cells have proliferated and the fully grown oocyte which is surrounded by a vitelline membrane and has follicular fluid in the antrum.

There appears to be a larger number of individual primordial follicles, consisting of a larger oocyte enclosed in a single layer of columnar cells, in the ovarian cortex of juveniles compared to adult African penguins (Fig 4.11 and Fig 4.12). The oocyte in juveniles is large, is surrounded by granulosa cells and has a large vesicular nucleus with finely dispersed chromatin and large nucleoli. The primordial follicles grow to become primary follicles, which include a change from flattened to cuboidal epithelial cells, but no antrum formation is evident. The presence of the vitelline membrane is unconfirmed in juveniles compared to the clear visible membrane in adults. The oocyte (Fig 4.13C) has a large nucleus with one or more large nucleoli. The growth of the primary follicle was shown by the accumulation of follicular fluid, enlarge, and come together to form a fluid filled cavity called the follicular antrum (Fig 4.14). The fully grown oocyte was developed and covered by the antrum and was encompassed by a fully developed vitelline membrane (Fig 4.14).

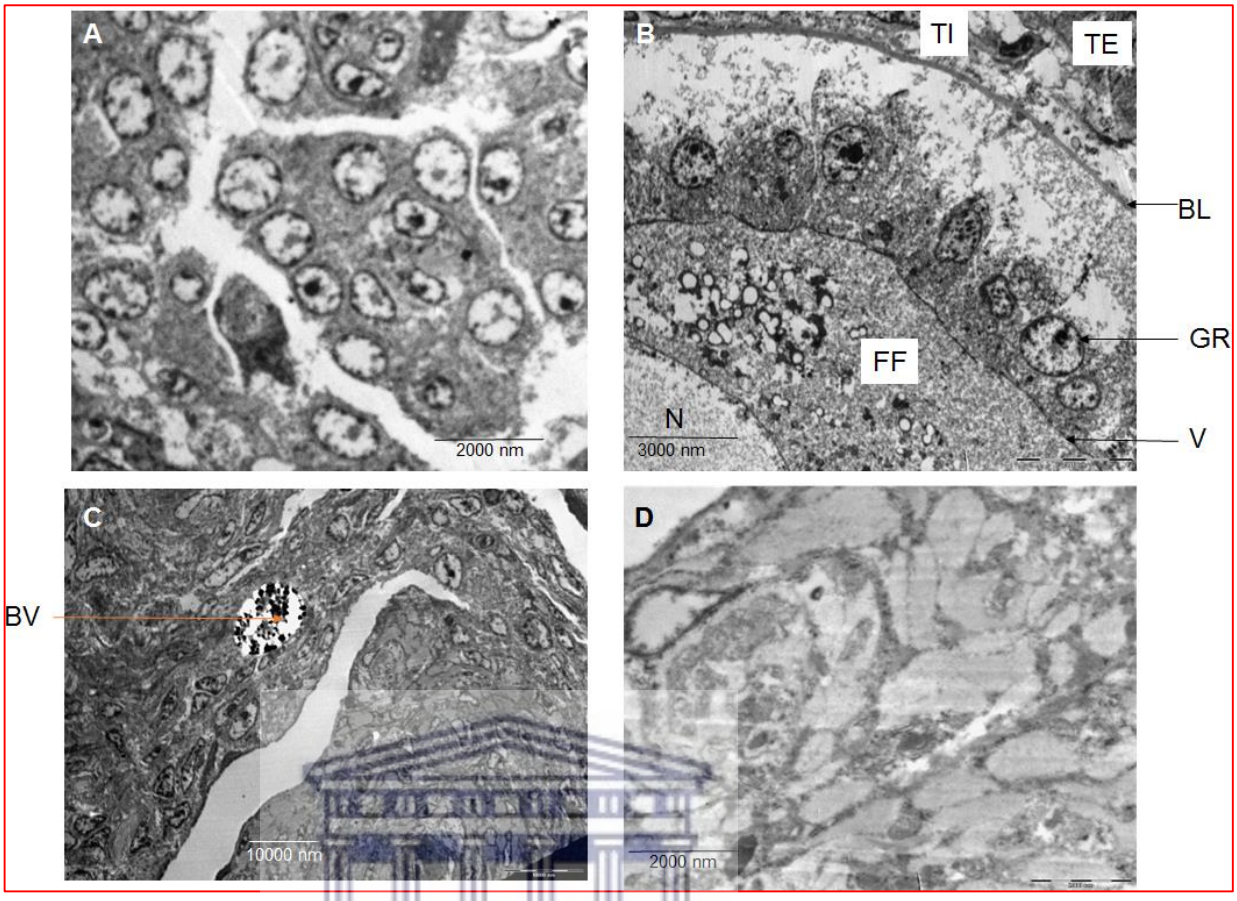

Figure 4.14: Micrographs of breeding adult penguin ovaries (n=6). A) Cortical stroma of the ovary contains a number of connective tissue cells and fibers. B) On the outside of the granulosa cells (GR) is a basal lamina (BL) on which the theca interna (TI) and theca externa (TE) rest. The TI and TE remain separated to the GR cells by the BL, which is not penetrated by blood vessels, leaving the GR cells avascular, however the TI is vascular. Follicular fluid (FF) present in the antrum surrounding. C) Luteal cells are subsequently formed after ovulation as the blood vessels (BV) of the TI and GR grow into the shrunken antrum and form the corpus luteum. D) A corpus luteum being transformed into an early corpus albicans.

The ovulated follicle is transformed into a new highly vascular, glandular structure, the corpus luteum (Fig 4.16). The corpus luteum is characterised by the follicular theca cells which luteinize into small luteal cells (Fig 4.16). The luteal cells enlarged and became filled with lipid droplets which subsequently form estrogen and progesterone.

Table 4.7: Summary of mean follicle diameter for juvenile and adult (non-breeding and breeding) African penguins (mean ± SD)

	n-value	Mean ± SD (µm)	Range (µm)
Juvenile	6	15.97 ± 4.41	7.12 – 24.85
Adult non-breeding	10	15.36 ± 5.33	9.12 – 31.42
Adult breeding	6	18.60 ± 5.15	11.81 – 42.46
All	22	16.55 ± 5.50	7.12 - 42.46

SD= Standard deviation

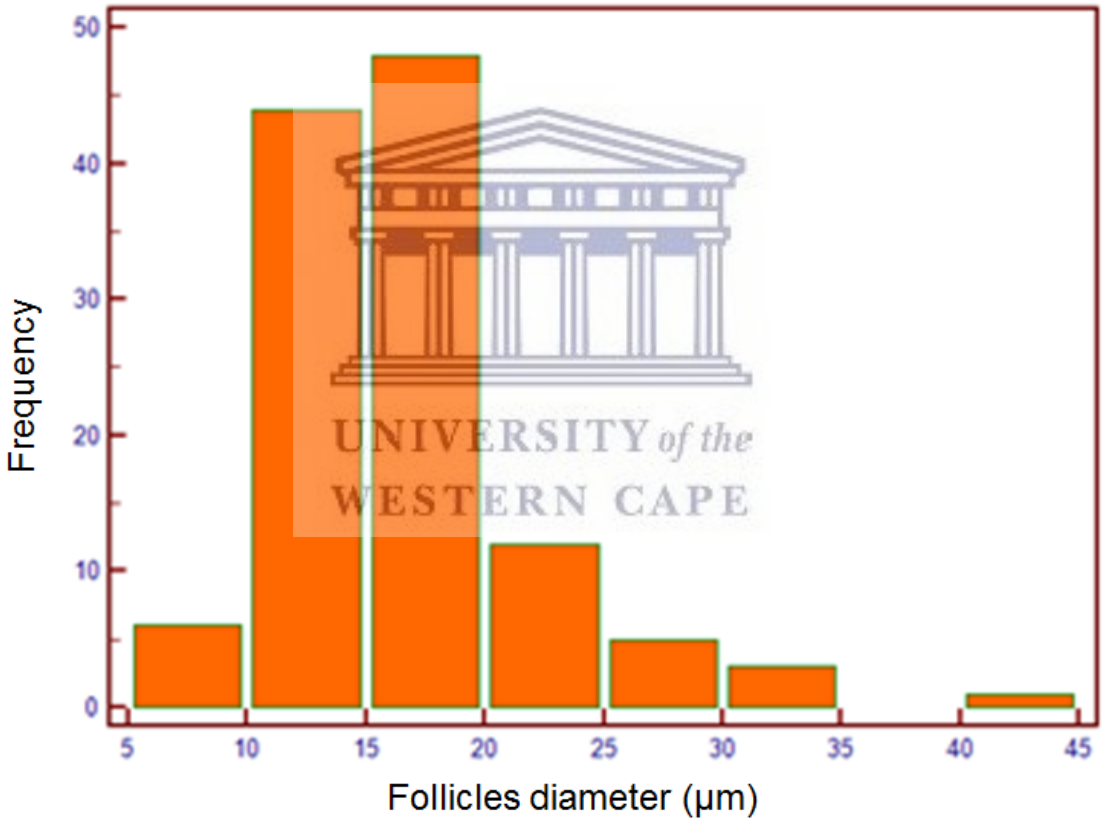

Figure 4.15: Frequency distribution of follicle diameter in all African penguins (n=19).

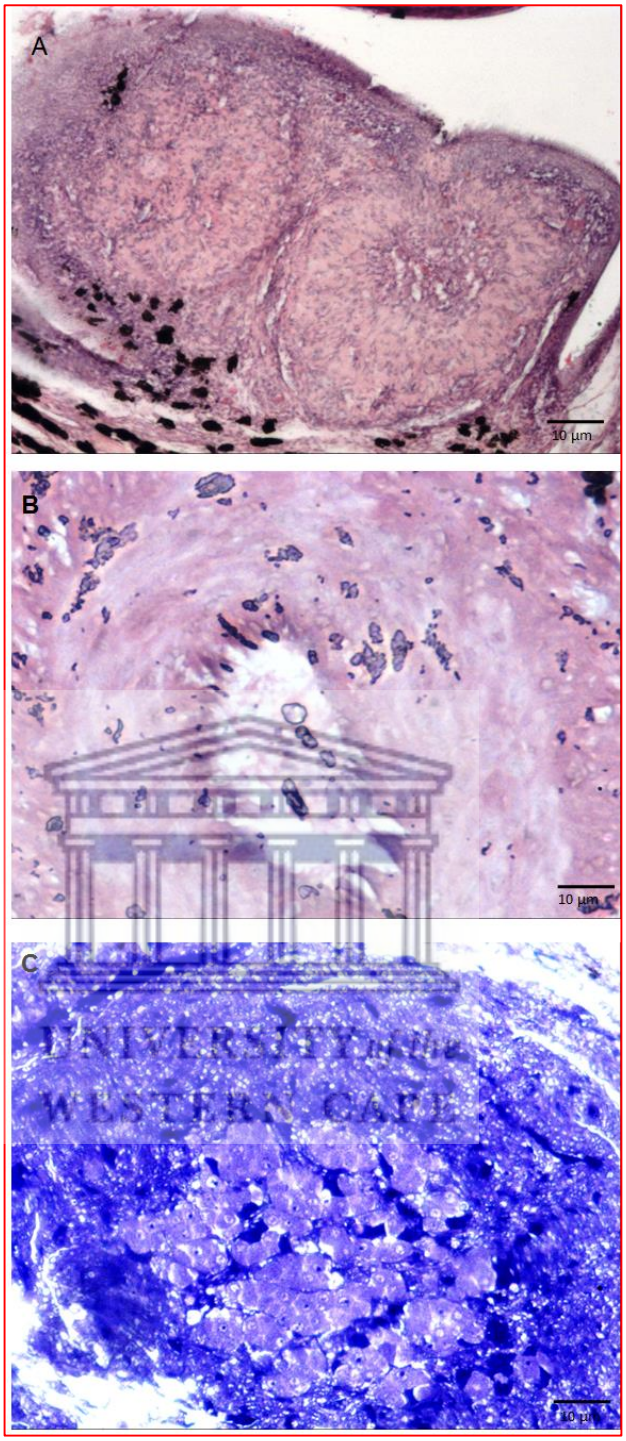

Figure 4.16: Micrographs showing formation of corpus luteum (CL), after rupture of follicle. A - B) H & E staining showing early and late stages of CL, C) Toluidine blue staining showing the luteal cells in the centre.

4.3. Plasma and faecal hormone levels

4.3.1. Hormonal profiles of males and females during different seasons

In this study we observed male and female penguin breeding activity and collected blood and faecal samples before and after the egg-laying (breeding) period. Therefore, the results will be presented by comparing the breeding season and non-breeding season. The individual plasma concentrations for both sexes indicating the difference between breeding and non-breeding seasons for three reproductive hormones are presented in Table 4.8, 4.9 and 4.10. One limitation of the hormonal assays in this study was the availability of penguins and thus the number of samples collected was often too small for more detailed statistical analysis.

4.3.1.1. Seasonal changes in plasma and faecal hormones levels in males

A clear difference between breeding and non-breeding seasons was observed in blood plasma levels of testosterone, with the lowest values observed during the non-breeding seasons ($P>0.05$). Blood plasma testosterone levels during both breeding seasons ranged from 545.33 pg/mL to 4384.98 pg/mL, while during the non-breeding seasons these levels ranged from 1.78 pg/mL to 7.95 pg/mL (Table 4.8). Although differences in plasma testosterone levels were observed among individual penguins, all the individuals showed low values during the non-breeding seasons. Interestingly, during both breeding seasons, male 4 (M4) had the highest levels of testosterone in blood plasma compared to the other three males included in the study. When comparing faecal androgen metabolite (fAM) concentrations, there was no significant ($P>0.05$) difference observed in epiandrosterone levels between breeding and non-breeding seasons. In faecal samples, penguin M3 showed a higher level of testosterone in July compared to the other three males, while M4 showed higher levels compared to the other males in November (Fig. 4.17). Except for the aforementioned two highest faecal

testosterone levels observed in the two males, the level of testosterone was relatively similar for most males in both breeding seasons ranging between 1.8 pg/ml to 7525.7 pg/ml.

Table 4.8: Individual plasma testosterone concentrations (pg/ml) for penguins (n=8), indicating the difference between breeding and non-breeding seasons.

Penguin sex	Month						Median
	BR	NBR	NBR	BR	BR	NBR	
	Jan	Apr	May	June	July	Oct	
M1	1875.9	2.4	6.7	704.9	745.0	5.7	355.8
M2	946.8	1.8	1.8	1428.1	628.8	5.2	327.2
M3	763.9	1.8	6.3	1152.9	545.3	4.3	275.8
M4	4384.9	7.9	7.3	1054.7	3142.6	-	531.3
F1	842.3	2.4	6.7	566.9	594.4	4.4	286.8
F2	3366.8	9.2	4.5	890.2	795.1	5.8	402.1
F3	2955.1	3.1	3.8	448.1	1849.1	-	448.1
F4	7525.7	3.1	3.9	408.1	1489.3	-	448.1

BR = Breeding, NBR= Non-breeding, M=Male, F=Female

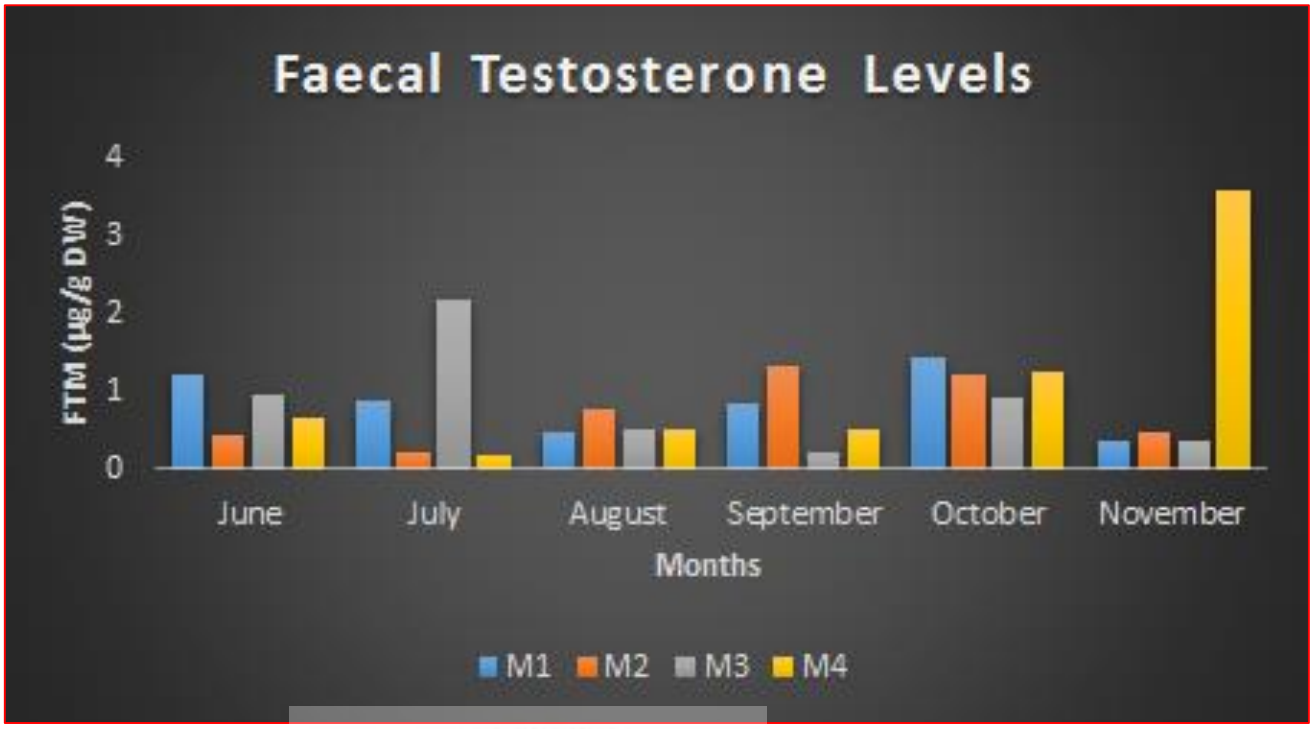

Figure 4.17: Changes in faecal testosterone levels in adult African penguin males, showing difference between breeding and non-breeding seasons.

FTM= Faecal testosterone metabolites, M= Male

The plasma levels for dihydrotestosterone (DHT) displayed no significant difference ($P>0.05$) between breeding and non-breeding seasons. However, much lower levels of DHT were observed during the month of July (long breeding season) when compared to other months (Table 4.9). Two males (M1 and M4), presented more than fivefold concentration of DHT in January compared to other males in the study, while males showed similar levels of DHT during May.

Table 4.9: Individual plasma dihydrotestosterone concentrations (pg/ml) for penguins (n=8), indicating the difference between breeding and non-breeding seasons.

Penguin sex	Month						Median
	BR	NBR	NBR	BR	BR	NBR	
	Jan	Apr	May	June	July	Oct	
M1	7050.5	488.4	2357.6	394.3	15.5	2056.0	1272.2
M2	418.5	749.4	2272.6	1177.1	7.1	364.1	583.9
M3	275.3	491.9	3312.7	1889.2	7.7	387.6	439.7
M4	5199.3	1213.6	3934.8	1350.1	44.5	0.00	1350.1
F1	842.3	364.6	3927.8	8406.4	22.7	938.5	890.4
F2	3366.8	842.2	2146.9	685.6	10.5	1802.2	1322.2
F3	2955.1	385.3	1294.0	524.4	36.8	-	524.4
F4	7525.7	2596.2	2272.6	2647.8	21.8	-	2647.8

BR = Breeding, NBR= Non-breeding, M=Male, F=Female

There were no seasonal differences observed in plasma estradiol levels observed between the two seasons compared in this study, although May and October showed lower values than the other months. The highest level of estradiol was displayed by M1 during the short breeding season. During April, all males displayed a much higher level of estradiol compared with other non-breeding months, which was also comparable to the estradiol levels observed in the two breeding seasons (Table 4.10.).

Table 4.10: Individual plasma estradiol concentrations (pg/ml) for penguins (n=8), indicating the difference between breeding and non-breeding seasons.

Penguin sex	Month					Median
	BR	NBR	NBR	BR	NBR	
	Jan	Apr	May	July	Oct	
M1	26231.6	15624.1	226.4	4436.4[a]	676.1[b]	4436.4
M2	7778.3	7167.9	150.7	6619.2[a]	401.9[b]	6619.2
M3	185.8	7703.9	148.1	6966.4[a]	320.1[b]	320.1
M4	1553.3	16356.4	236.3	14167.7		7860.5
F1	1060.5	14924.4	204.3	5198.[9a]	424.5[b]	1060.5
F2	6291.4	18873.4	285.7	8741.9[a]	670.3[b]	6291.4
F3	1282.6	16667.5	4874.8	14049.6	-	9462.2
F4	5422.7	7294.9	136.8	26.3	-	2785.2

BR = Breeding, NBR= Non-breeding, M=Male, F=Female

[a,b,] Different letters in the same row denote a significant difference (P<0.05)

4.3.1.2. Seasonal changes in plasma and faecal hormones levels in females

Females presented similar results to that observed in males, displaying a difference in circulating testosterone levels between breeding and non-breeding seasons (P≥0.05). Blood plasma testosterone levels for females ranged from 408.1 pg/mL to 7525.7 pg/mL for the breeding seasons and these levels were 31 times higher than the range of 2.4

pg/mL to 6.69 pg/mL during the non-breeding season. The range of plasma testosterone levels in females was similar to that observed in males. The highest blood plasma testosterone level was displayed by F4 during the short breeding season (Table 4.8).

There was no significant difference (P>0.05) in the plasma levels for DHT in females when breeding and non-breeding seasons were compared. The levels of DHT were similar through the seasons (Table 4.9).

A significant difference (P<0.05) was observed in plasma estradiol levels when April (non -breeding period) was compared to May and October (breeding season). All four females had an estradiol peak during the month of April (Table 4.10). A significant difference (P<0.05) was observed in faecal estrogen levels during the long breeding season when comparing the pre-egg laying period (July) and the incubation period (November). The estrogen levels were higher before egg laying commenced except in for one of the birds (Fig 4.18).

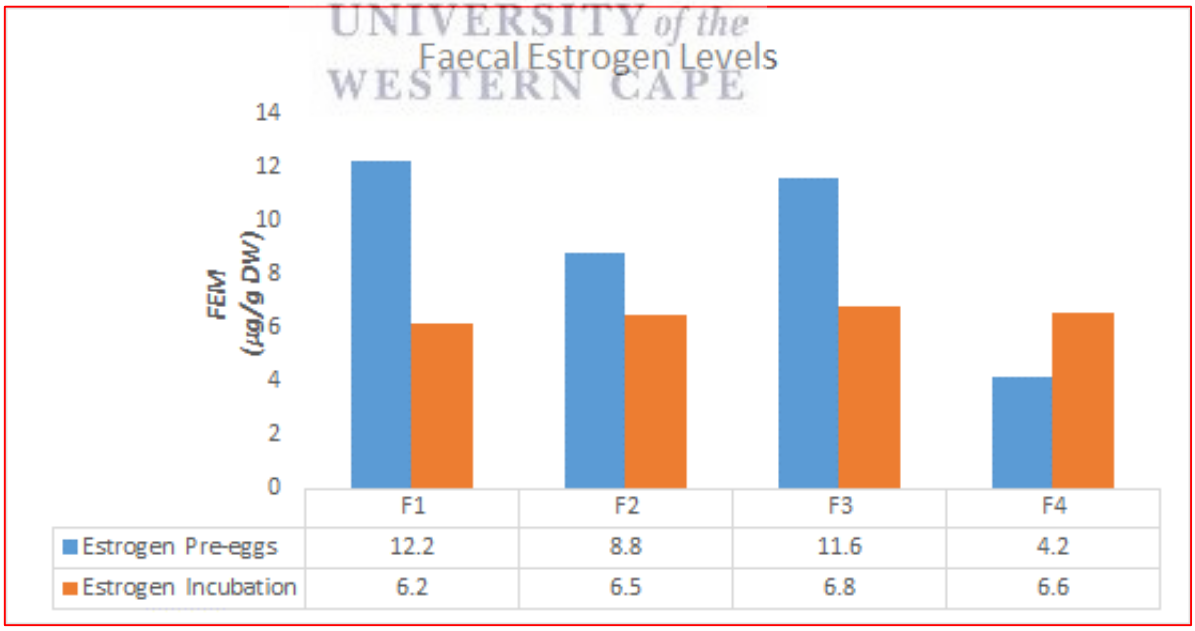

Figure 4.18: Relationship between egg production and changes in faecal estrogen hormones in

females during the long breeding season. FEM= Faecal estrogen metabolites, F= Female

The mean faecal progestogen levels showed no significant difference (P>0.05) between the pre-egg laying period and the incubation period during the long breeding season. However, faecal progestogens levels seemed to be slightly higher (P>0.05) before egg laying compared to the incubation period (Fig 4.19).

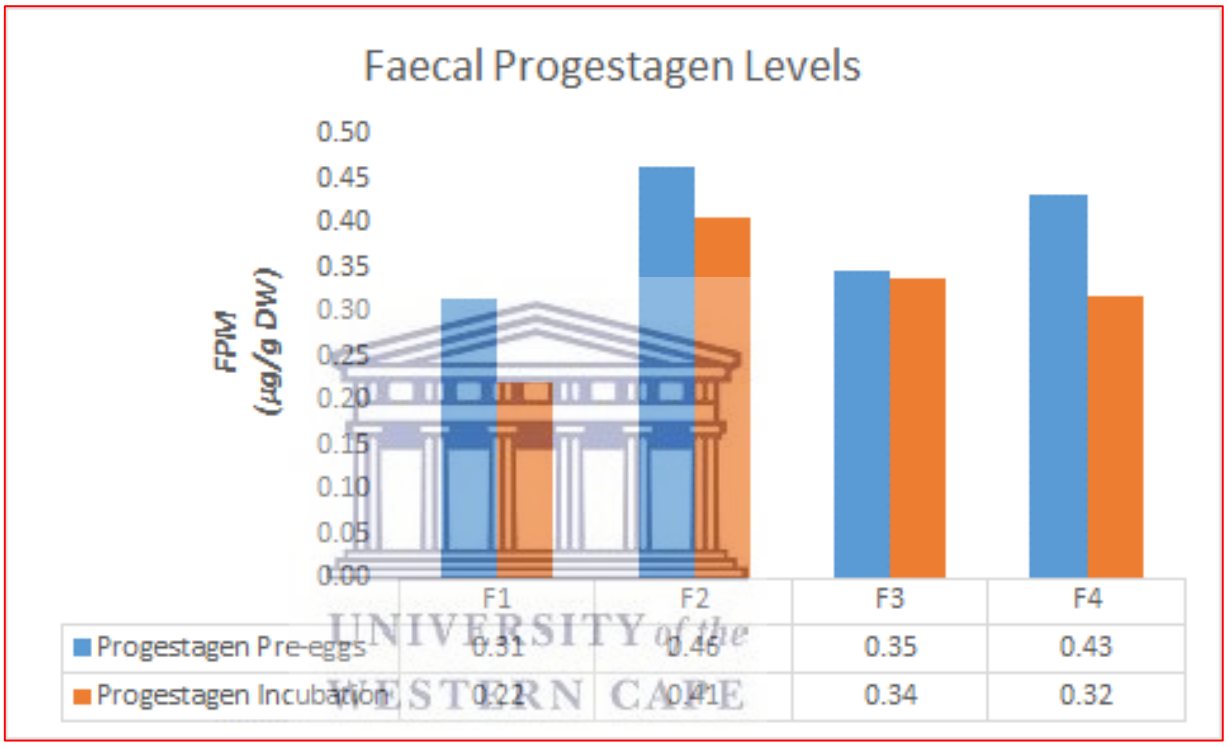

Figure 4.19: Relationship between egg production and changes in faecal progestogen hormones in females during the long breeding season. FPM= Faecal progestagen metabolites, F= Female

4.3.2. Comparison of blood plasma hormones levels between males and females

4.3.2.1. Testosterone in males and females

The blood plasma level of testosterone in females compared to males, mean values in females displayed generally high values when the two seasons were compared.

Hormonal levels varied during the seasons with a similar pattern between the females and males (Fig 4.20). All females also displaying low values during the non-breeding seasons

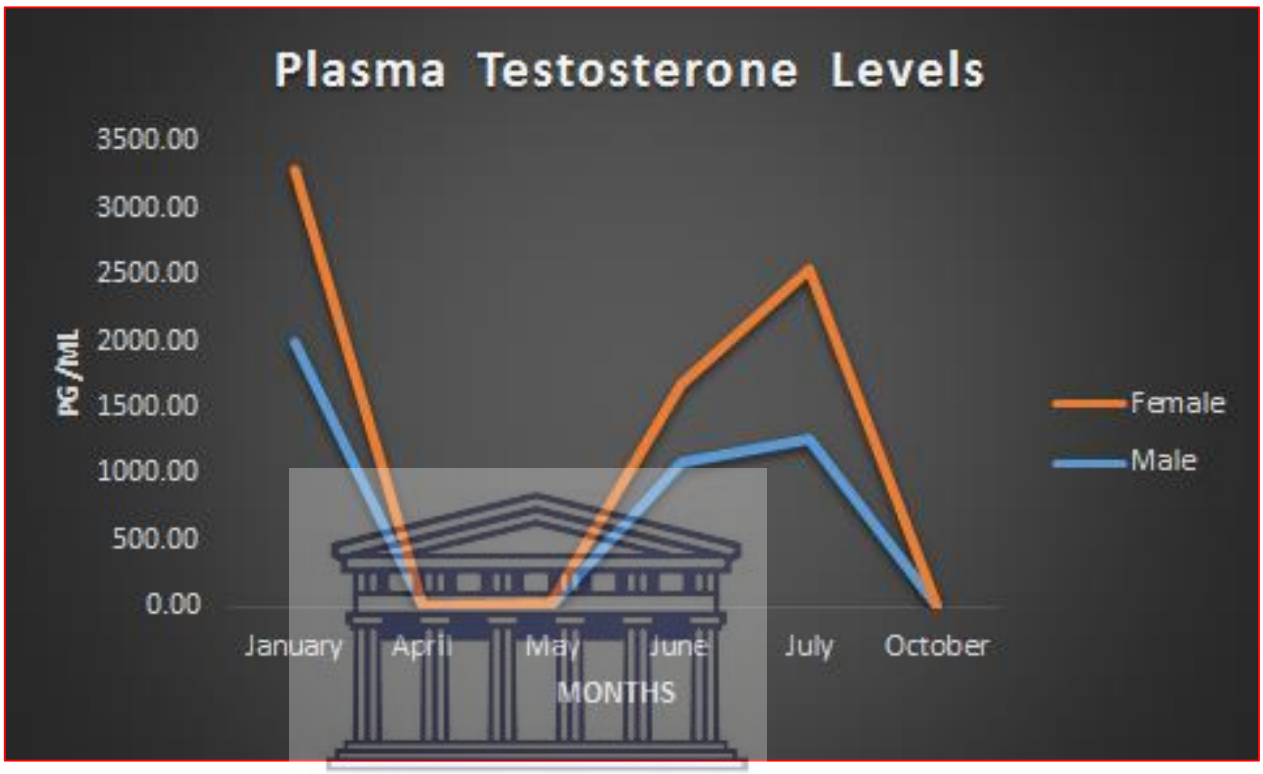

Figure 4.20: Changes in plasma testosterone level of African penguin adult males compared with females (n=8).

4.3.2.2. DHT in males and females

The blood plasma level in DHT for both sexes remain low in both seasons investigated. The females had higher mean values compere to males but no significant difference observed. The only peak observe was in June in females. Females showed to secrete more DHT than males (Fig 4.21).

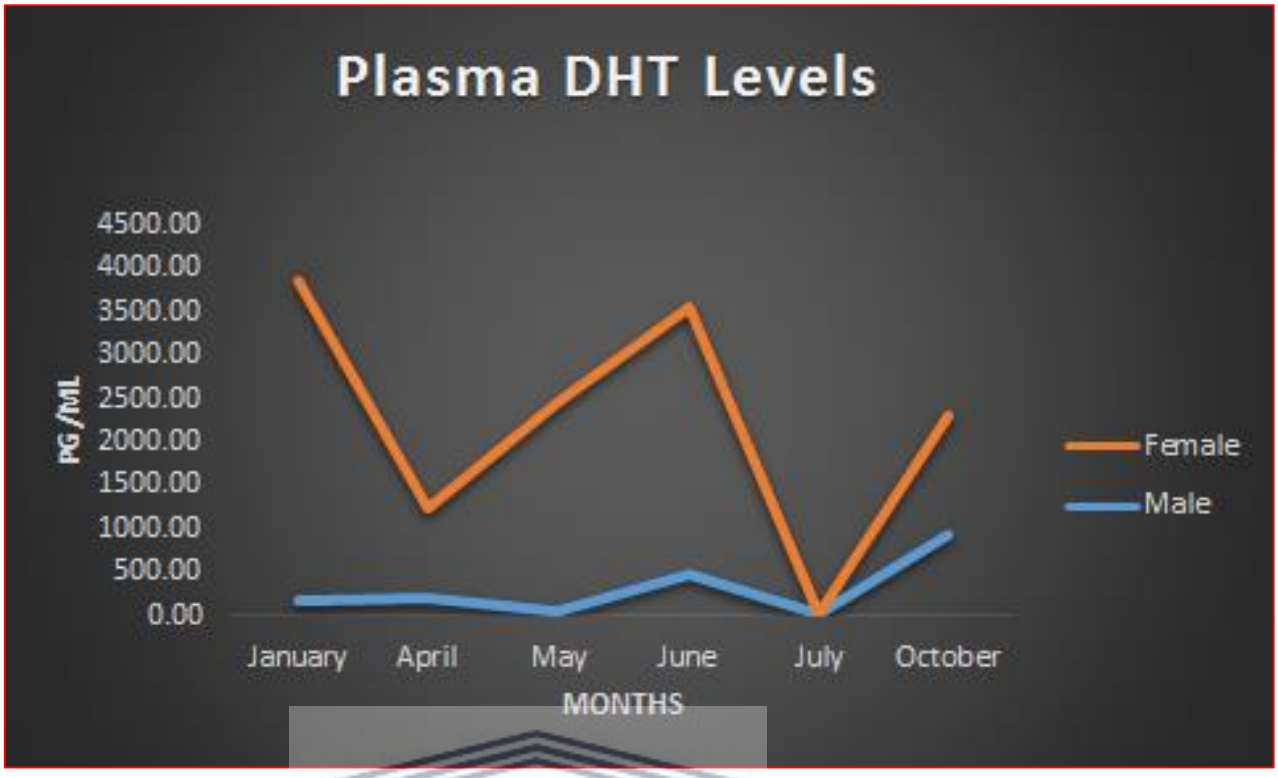

Figure 4.21: Changes in plasma level in DHT of African penguin adult males compared with females (n=8).

4.3.2.3. Estradiol in males and females

The estradiol levels between the two sexes followed a similar pattern with variations among the months in this study. On average females tend to have a higher estradiol level than males in April and July, but similarly low mean levels was recorded in the month of May and October for both sexes (Fig 4.22).

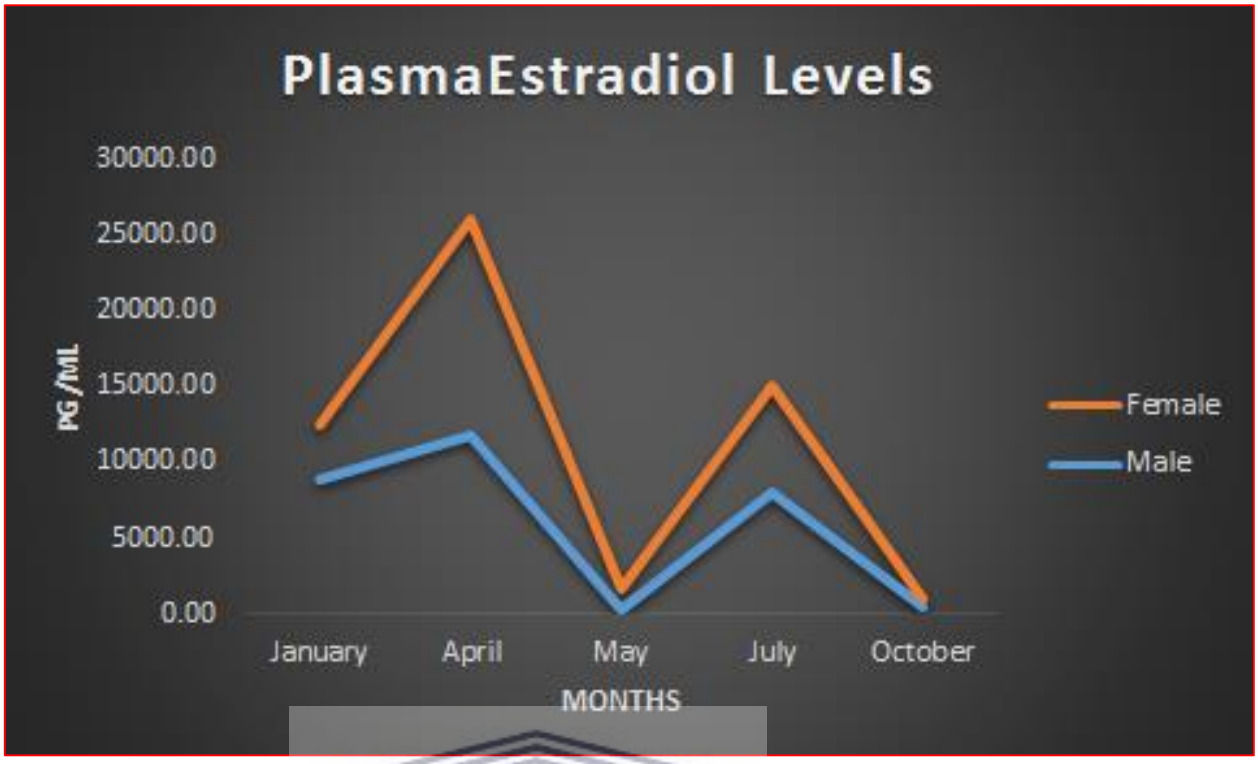

Figure 4.22: Changes in plasma level in estradiol of African penguin adult males compared with females (n=8).

4.4. Semen and sperm analyses

4.4.1. Standard semen parameters

The mean and range for standard semen parameters (colour, volume, total motility (TM), sperm concentration and total number of sperm) are displayed in Table 4.11. Generally the colour of African penguin semen was whitish to grey and the volume range between 0.01 to 0.10 ml. The other semen parameters, percentage total motility (TM) mean was above 72% and sperm concentration averaged 3244.7 ± 192.4 $x10^6$. No differentiation could be made in the colour of semen between the two penguins. For three semen parameters, namely volume, sperm concentration and number of sperm in the ejaculate, the mean values were significantly ($P<0.05$) higher in Penguin 1 as compared to Penguin 2. However, no differences were observed in the TM between the

two penguins. Although the ranges for semen volume, TM, sperm concentration and number of sperm in the ejaculate overlapped between the two African penguins used in this study, much larger ranges were observed in Penguin 1.

Table 4.11: Summary of standard semen parameters (mean ± SD) assessed for African penguin (n=55).

	Penguin 1		Penguin 2		All	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Number of ejaculates	44		7		51	
Colour	Whitish – grey		Whitish – grey		Whitish – grey	
Volume (ml)	0.05 ± 0.04[a]	0.01-0.10	0.02 ± 0.01[b]	0.01-0.03	0.05 ± 0.03	0.01-0.10
Total motility (%)	71.7 ± 7.8	40.1-80.4	73.5 ± 8.4	62.3-87.1	72.1 ± 6.9	40.1-87.1
Sperm concentration (x10^6/ml)	3494.5 ± 2047.6[a]	802.6 - 7808.8	2099.4 ± 741.1[b]	1527.5 - 3569.8	3274.7 ± 192.4	802.6 - 7808.8
Number of sperm in ejaculate (x10^6)	86.94 ± 154.52[a]	3.42 - 368.08	45.76 ± 16.16[b]	15.27 - 60.06	186.94 ± 154.52	3.42 - 740.18

[a,b,] Different letters in the same row denote a significant difference (P<0.05).

SD = standard deviation

4.4.2. Effect of different techniques used to evaluate sperm motility and sperm kinematic parameters over time

The mean perTM, swimming speed classes and sperm kinematics of the swim-up and flush techniques using Ham's F10 are displayed in Table 4.12. When comparing the swim-up and flush techniques (Fig 4.23), the percentage TM and rapid swimming sperm were significantly higher ($P<0.05$) in swim-up compared to flush technique at all-time points. On the contrary, the percentage slow swimming sperm was significantly lower ($P<0.05$) in swim-up than that of the flush technique. Furthermore, sperm velocity kinematics (VCL, VSL and VAP) were significantly higher in swim-up compared to flush technique at most time points. Other significant differences were found in LIN, STR and WOB after 30 min of incubation, with swim-up still resulting higher values than flush technique. ALH and BCF, were also significantly higher in swim-up preparation for some of the time points. Thus, overall the swim-up technique resulted in significantly higher sperm motility and kinematic parameters values than the flush technique. In both techniques percentage TM was above 70%, while 40% of these sperm could be classified as rapid swimming sperm in the swim-up technique. Interestingly, the percentage rapid swimming sperm in the swim-up was always more than double the percentage found for the flush technique. The range for straight swimming sperm was more than 68% in both techniques.

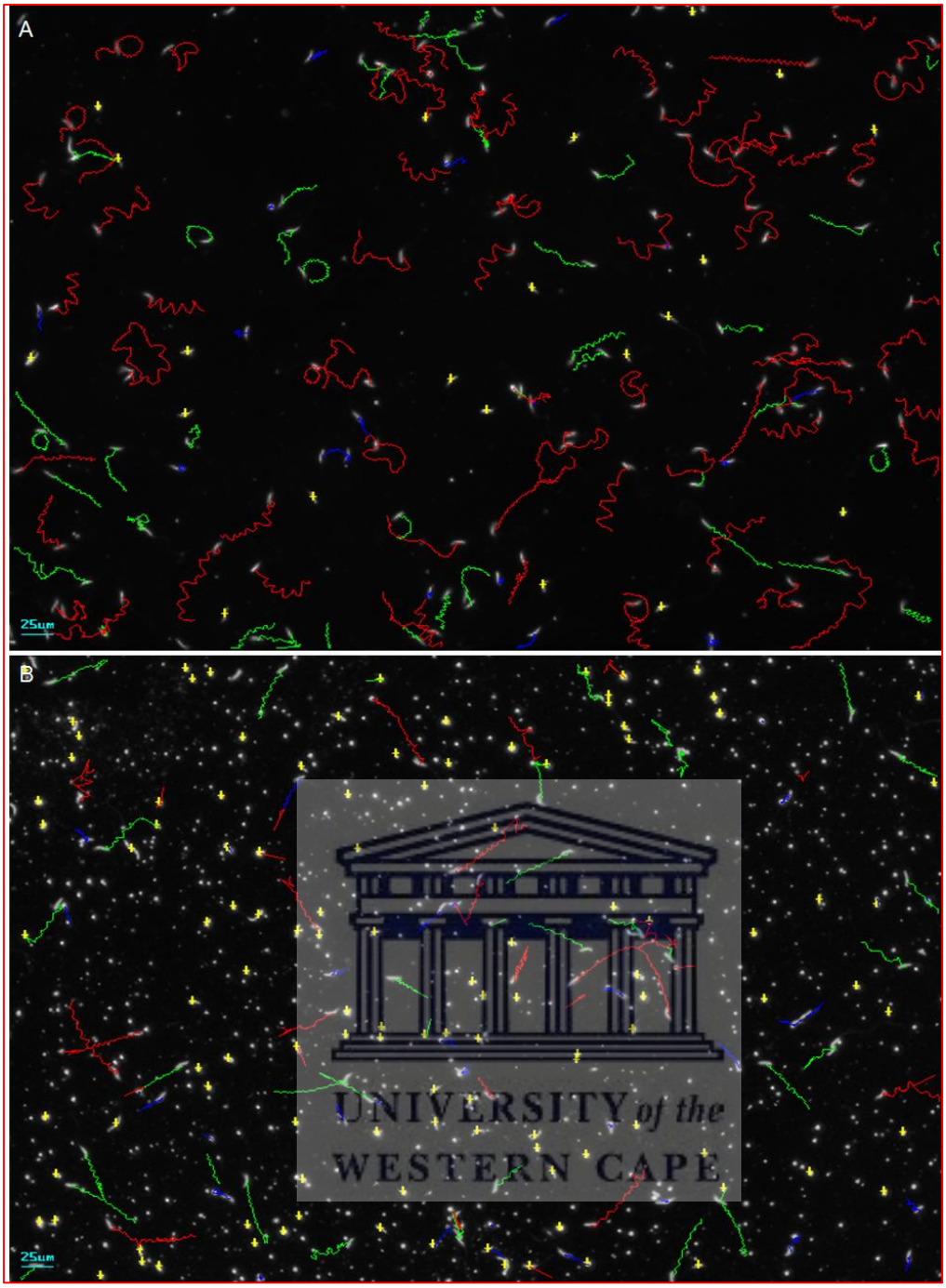

Figure 4.23: Sperm motility tracks of African penguins captured by SCA system at 50 frames per second, illustrating the difference in kinematics parameters. A) swim-up in Ham's F10 and B) Ham's F10 flush technique. Red tracks = rapid progressive swimming spermatozoa, green tracks = rapid swimming spermatozoa, blue tracks = medium progressive swimming spermatozoa, yellow crosses = static spermatozoa

Table 4.12: Total sperm motility, swimming speed class and sperm kinematics parameter measurements (mean ± SD) for African penguin as a result of swim-up and flush techniques over a 60 minute incubation period (n = 51).

Swim up	0 Min		15 Min		30 Min		45 min		60 Min	
	Swim-up	Flush	Swim-up	Flush	Swim-up	Flush	Swim-up	Flush	Swim-up	Flush
TM	91.3 ± 3.6[a]	77.9 ± 9.8[b]	92.1 ± 5.1[a]	78.2 ± 15.3[b]	94.7 ± 4.4[a]	80.0 ± 17.2[b]	93.3 ± 5.0[a]	70.2 ± 20.8[b]	91.0 ± 7.2[a]	61.8 ± 22.9[b]
Rapid	40.9 ± 24.2[a]	15.2 ± 10.2[b]	40.6 ± 19.7[a]	20.6 ± 16.5[b]	51.2 ± 16.7[a]	17.0 ± 14.5[b]	55.4 ± 21.9[a]	14.9 ± 13.4[b]	48.6 ± 26.1[a]	11.8 ± 10.9[b]
Med	34.6 ± 12.5	32.8 ± 9.4	34.8 ± 8.6	32.5 ± 12.3	30.9 ± 8.8	34.0 ± 11.3	26.4 ± 10.6	27.8 ± 10.4	28.9 ± 11.3	22.6 ± 12.8
Slow	16.4 ± 10.5[a]	29.9 ± 9.9[b]	18.1 ± 14.6	25.1 ± 8.2	12.5 ± 7.7[a]	28.9 ± 11.2[b]	11.5 ± 8.0[a]	27.6 ± 8.3[b]	13.5 ± 9.8[a]	27.4 ± 11.6[b]
VCL	79.7 ± 13.6[a]	64.4 ± 6.9[b]	79.6 ± 11.7[a]	66.3 ± 9.7[b]	85.2 ± 11.1[a]	64.1 ± 9.6[b]	87.1 ± 13.8[a]	61.9 ± 7.3[b]	83.2 ± 15.5[a]	58.5 ± 10.5[b]
VSL	45.3 ± 8.7[a]	34.7 ± 6.3[b]	44.5 ± 10.1[a]	34.9 ± 8.5[b]	49.7 ± 10.0[a]	31.6 ± 8.7[b]	49.8 ± 9.5[a]	29.2 ± 6.1[b]	47.3 ± 10.4[a]	28.1 ± 8.4[b]
VAP	58.9 ± 10.4[a]	46.4 ± 6.2[b]	58.6 ± 9.9[a]	47.3 ± 8.6[b]	63.8 ± 9.6[a]	44.8 ± 9.5[b]	93.3 ± 5.0[a]	70.2 ± 20.8[b]	61.9 ± 12.0[a]	39.9 ± 9.3[b]
LIN	56.9 ± 7.3	53.3 ± 5.9	55.5 ± 7.3	51.7 ± 7.0	58.1 ± 7.9[a]	48.1 ± 8.7[b]	58.1 ± 7.9[a]	48.2 ± 8.7[b]	56.7 ± 7.2[a]	47.1 ± 9.4[b]

STR	76.3 ± 6.2	73.3 ± 5.4	74.7 ± 6.9	71.9 ± 7.1	77.0 ± 7.5[a]	68.3 ± 7.7[b]	77.1 ± 5.6[a]	69.5 ± 7.9[b]	75.7 ± 6.4[a]	68.5 ± 8.3[b]
WOB	74.0 ± 5.1	71.9 ± 5.1	73.6 ± 4.2	71.1 ± 5.2	49.7 ± 10.0[a]	31.6 ± 8.7[b]	77.6 ± 4.3[a]	66.5 ± 6.7[b]	74.4 ± 4.9[a]	67.8 ± 8.2[b]
ALH	2.2 ± 0.2	2.0 ± 0.1	2.3 ± 0.2[a]	2.0 ± 0.2[b]	2.3 ± 0.2[a]	2.1 ± 0.2[b]	2.4 ± 0.2[a]	2.1 ± 0.2[b]	2.3 ± 0.3	2.0 ± 0.3
BCF	18.9 ± 4.3	16.8 ± 3.8	17.6 ± 4.6	16.2 ± 2.0	18.4 ± 4.9	16.3 ± 4.0	18.7 ± 4.3[a]	14.9 ± 3.8[b]	18.1 ± 4.6[a]	14.8 ± 2.5[b]

TM = Total motility (%), Rapid = Rapid swimming sperm (%), Medium = Medium swimming sperm ((%)), Slow = Slow swimming sperm ((%)), VCL= Curvilinear velocity (µm/s), VSL= Straight-line velocity (µm/s), VAP = Average path velocity (µm/s), ALH = Amplitude of lateral head displacement (µm), LIN = Linearity (%), STR = Straightness (%), WOB = Wobble (%), BCF = Beat cross frequency (Hz).

[a,b] Different superscript letters in the same row for each time point denote a significant difference between the two techniques applied ($P<0.05$).

Hams= Hams F12, Caff= Caffeine, Proc= Procaine.

SD = standard deviation

4.4.3. Effect of three different media on sperm motility and sperm kinematic parameters over time

Table 4.13 displays the results of sperm motility and kinematic parameters when three different media, namely Ham's-F10, caffeine (8 mM), and procaine (2 mM), were used to flush the semen into the Leja slide (Fig 4.24). The only significant differences observed were in the percentages TM and medium swimming sperm, displaying higher mean values ($P<0.05$) in procaine when compared to caffeine and Ham's F10 at 0 minutes (Table 4.13). There were no significant difference observed ($P>0.05$) between the two African penguins in any of the swimming sperm classes, percentage totally motility and average sperm kinematic parameters captured at 50 frames/second. At all--time points an overlap of values was observed when these three media were used to flush the semen, procaine in (TM, VCL, VSL, VAP and WOB).

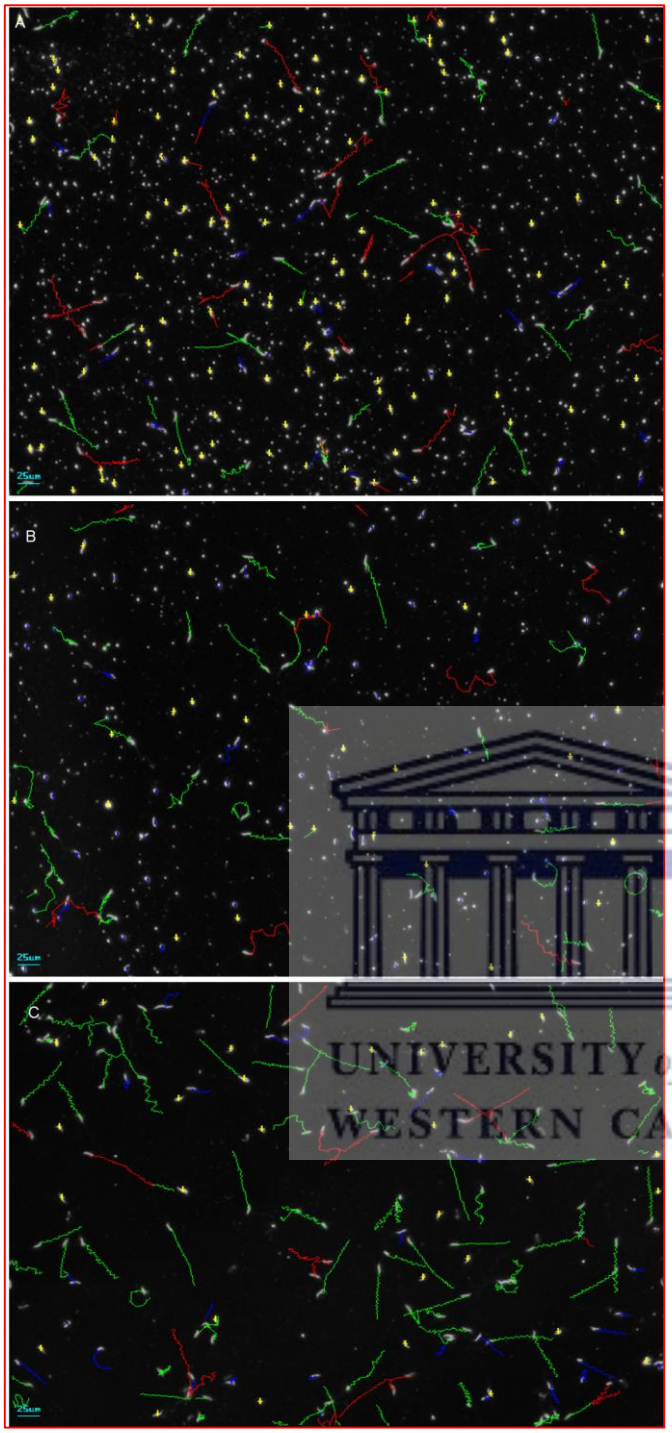

Figure 4.24: Sperm motility tracks of African penguin spermatozoa captured by the SCA system to illustrate the effect of three different media. A) Ham's F10 flush B) caffeine flush and C) procaine flush. Red tracks = rapid progressive swimming spermatozoa, green tracks = rapid swimming spermatozoa, blue tracks = medium progressive swimming spermatozoa, yellow crosses = static spermatozoa.

Table 4.13: Sperm motility and sperm kinematics parameters measured (mean ± SD) for African penguin after exposure to three different media, captured at different time points using the flush technique (n=40)

	0 Min			15 Min			30 Min			45 Min			60 Min		
	Hams	Caff	Proc	Hams	Caff	Proc	Hams	Caff	Proc	Hams	Caff	Proc	Hams	Caff	Proc
TM	77.9 ± 9.8	72.4 ± 21.0[a]	86.9 ± 8.1[b]	78.2 ± 15.3	73.8 ± 18.5	81.1 ± 11.12	79.9 ± 17.2	66.9 ± 29.1	72.4 ± 15.8	70.2 ± 20.8	62.1 ± 26.6	64.4 ± 19.4	61.8 ± 22.9	61.7 ± 25.9	67.7 ± 18.5
Rapid	15.2 ± 10.1	24.2 ± 15.4	27.8 ± 13.6	20.6 ± 16.5	23.1 ± 15.7	16.6 ± 13.2	16.9 ± 14.5	16.1 ± 8.6	16.9 ± 18.1	14.9 ± 13.4	16.3 ± 14.5	10.9 ± 17.2	11.8 ± 10.9	20.1 ± 21.9	11.4 ± 11.9
Med	32.8 ± 9.3	27.2 ± 12.0[a]	39.9 ± 10.7[b]	32.5 ± 12.2	31.5 ± 15.2	38.8 ± 10.7	34.0 ± 11.4	31.3 ± 15.9	32.2 ± 11.3	27.8 ± 10.4	28.0 ± 13.2	28.4 ± 15.6	22.6 ± 12.8	21.7 ± 11.6	31.5 ± 10.8
Slow	29.9 ± 9.9	21.0 ± 8.9	19.3 ± 14.6	25.1 ± 8.2	19.2 ± 7.5	25.8 ± 13.3	28.9 ± 11.2	19.7 ± 9.1	24.4 ± 12.2	27.5 ± 8.3	22.3 ± 12.3	25.1 ± 12.6	27.5 ± 11.6	19.7 ± 8.5	24.8 ± 9.4
VCL	64.4 ± 6.6	69.2 ± 9.9	72.5 ± 9.7	66.3 ± 9.9	68.4 ± 9.0	66.5 ± 9.1	64.1 ± 13.0	66.9 ± 5.6	66.1 ± 9.2	61.9 ± 7.3	63.9 ± 8.5	59.4 ± 11.9	58.5 ± 10.5	64.5 ± 12.7	60.5 ± 7.3
VSL	34.7 ± 6.3	35.9 ± 8.2	38.6 ± 8.6	34.8 ± 8.5	34.1 ± 7.1	35.4 ± 7.1	31.6 ± 8.7	31.8 ± 8.9	31.6 ± 8.2	29.2 ± 6.1	31.5 ± 7.6	30.4 ± 7.6	28.1 ± 8.4	34.0 ± 10.7	30.2 ± 6.0
VAP	46.4 ± 6.2	49.4 ± 8.7	52.3 ± 7.9	47.3 ± 8.6	49.1 ± 7.6	47.6 ± 7.3	44.8 ± 9.5	46.6 ± 7.3	44.8 ± 7.6	41.4 ± 6.4	44.2 ± 7.6	41.8 ± 8.3	39.9 ± 9.3	46.4 ± 11.9	41.9 ± 5.8
LIN	53.3 ± 5.9	50.7 ± 7.0	52.8 ± 6.9	51.7 ± 7.0	49.0 ± 5.7	52.6 ± 7.5	48.2 ± 8.7	46.9 ± 12.2	47.7 ± 10.0	46.9 ± 7.7	48.6 ± 8.4	50.5 6.5	47.2 9.4	51.4 7.8	49.8 ± 7.1

STR	73.3 ± 5.4	70.1 ± 6.6	72.2 ± 6.2	71.9 ± 7.1	67.5 ± 5.9	72.4 ± 6.8	68.3 ± 7.7	65.2 ± 12.6	68.2 ± 10.0	69.5 ± 7.9	69.5 ± 6.8	70.0 ± 6.4	68.6 ± 8.3	70.5 ± 6.1	71.2 ± 5.9
WOB	71.9 ± 5.1	70.8 ± 4.4	75.3 ± 3.8	71.1 ± 5.2	71.2 ± 3.9	70.9 ± 4.8	69.2 ± 7.7	69.3 ± 9.3	67.8 ± 5.9	66.5 ± 6.7	68.7 ± 6.4	70.1 ± 5.1	67.8 ± 8.2	71.1 ± 6.2	69.4 ± 5.34
ALH	2.0 ± 0.1	2.1 ± 0.2	2.1 ± 0.2	2.0 ± 0.1	2.2 ± 0.2	2.1 ± 0.3	2.1 ± 0.2	2.2 ± 0.3	2.2 ± 0.3	2.1 ± 0.3	2.1 ± 0.3	1.9 ± 0.3	2.0 ± 0.3	2.0 ± 0.3	2.1 ± 0.2
BCF	16.9 ± 3.6	16.2 ± 3.9	16.9 ± 4.4	16.2 ± 3.9	15.7 ± 3.9	16.9 ± 4.2	16.3 ± 4.0	15.1 ± 4.2	15.5 ± 4.4	14.9 ± 3.8	15.5 ± 3.8	15.7 ± 3.3	14.8 ± 2.5	15.4 ± 3.1	14.9 ± 3.6

TM = Total motility (%), Rapid = Rapid swimming sperm (%), Medium = Medium swimming sperm ((%)), Slow = Slow swimming sperm ((%)), VCL= Curvilinear velocity (µm/s), VSL= Straight-line velocity (µm/s), VAP = Average path velocity (µm/s), ALH = Amplitude of lateral head displacement (µm), LIN = Linearity (%), STR = Straightness (%), WOB = Wobble (%), BCF = Beat cross frequency (Hz).

[a,b, c] Different superscript letters in the same row for each time point denote a significant difference between the two techniques applied ($P<0.05$).

SD = standard deviation

4.4.4. Effect of time on sperm motility and sperm kinematic parameters

The total motility, swimming speed classes and sperm kinematics parameters evaluated at five time points using the swim-up technique are illustrated in Table 4.14. None of the parameters measured showed a significant difference ($P<0.05$) among the time points. Assessing the effect of time on only rapid swimming sperm kinematics also revealed no significant difference ($P>0.05$) among the different time points. As expected, rapid swimming spermatozoa had higher mean values for all sperm kinematics parameters except WOB when compared to the mean kinematics of all motile spermatozoa ($P<0.05$).

4.4.5. Sperm motility and sperm kinematics parameters of two African penguins

Table 4.15 displays individual results for the two African penguins, comparing their sperm motility characteristics over time during the long breeding season. These two penguin displayed similar results in TM, the percent rapid swimming spermatozoa together with three sperm velocity parameters (VCL, VSL and VAP). The overall results showed that Penguin 2 had higher mean values than Penguin 1 for only a few motility parameters. The only significant difference observed at 0 minutes was in WOB ($P<0.05$), showing higher mean value in Penguin 2 than that of Penguin 1. Between the two penguins there were few differences during the time points, but a trend was observed. When 30 min time point was compared with 0 min and 15 min, most swimming speed classes and sperm kinematics parameters were higher or equal ($P\leq0.05$).

Table 4.14: Sperm motility and sperm kinematic parameter measurements (mean ± SD) captured

	0 Min	15 Min	30 Min	45 Min	60 Min
TM	91.3 ± 6.6	92.1 ± 5.1	94.7 ± 4.4	93.3 ± 5.0	91.0 ± 7.2
Rapid	40.4 ± 24.2	40.6 ± 19.7	51.2 ± 16.7	55.4 ± 21.9	48.6 ± 26.1
Med	34.6 ± 12.5	34.8 ± 8.6	30.9 ± 8.8	26.4 ± 10.6	28.9 ± 11.3
Slow	16.37 ± 10.5	18.1 ± 14.6	12.5 ± 7.7	11.5 ± 8.0	13.5 ± 9.8
VCL	76.7 ± 13.6[a]	79.6 ± 11.7[a]	85.2 ± 11.1[a]	87.1 ± 13.8[a]	83.3 ± 15.5[a]
Rapid VCL	105.7 ± 4.0[b]	105.2 ± 4.0[b]	107.1 ± 5.7[b]	107.9 ± 4.3[b]	106.5 ± 4.5[b]
VSL	45.3 ± 8.6[a]	44.5 ± 10.1[a]	49.7 ± 10.0[a]	49.90 ± 9.5[a]	47.3 ± 10.4[a]
Rapid VSL	62.2 ± 7.8[b]	60.1 ± 6.6[b]	62.8 ± 8.4[b]	62.6 ± 6.2[b]	60.6 ± 9.8[b]
VAP	58.9 ± 10.4[a]	58.6 ± 9.9[a]	63.8 ± 9.6[a]	64.2 ± 11.7[a]	61.9 ± 12.4[a]
Rapid VAP	77.1 ± 4.2[b]	76.0 ± 6.1[b]	79.1 ± 5.9[b]	78.6 ± 5.1[b]	77.6 ± 7.7[b]
LIN	56.9 ± 7.3[a]	55.5 ± 7.3[a]	58.1 ± 5.7[a]	56.9 ± 5.7[a]	56.7 ± 7.2[a]
Rapid LIN	79.9 ± 7.8[b]	77.7 ± 4.4[b]	78.4 ± 6.7[b]	78.9 ± 5.4[b]	76.5 ± 7.5[b]
STR	76.3 ± 6.2[a]	74.8 ± 4.2[a]	77.0 ± 7.5[a]	77.1 ± 5.6[a]	75.7 ± 6.4[a]
Rapid STR	58.9 ± 7.4[b]	57.3 ± 5.5[b]	58.8 ± 7.8[b]	58.4 ± 6.0[b]	57.0 ± 8.6[b]
WOB	74.0 ± 5.1	73.6 ± 4.2	74.9 ± 6.7	73.7 ± 4.3	74.4 ± 6.4
Rapid WOB	73.1 ± 3.1	72.4 ± 4.2	74.0 ± 4.0	73.3 ± 3.7	74.0 ± 3.7
ALH	2.2 ± 0.2[a]	2.3 ± 0.2[a]	2.3 ± 0.2[a]	2.3 ± 0.2[a]	2.3 ± 0.3[a]
Rapid ALH	2.6 ± 0.2[b]	2.7 ± 0.2[b]	2.7 ± 0.2[b]	2.7 ± 0.2[b]	2.7 ± 0.2[b]
BCF	18.9 ± 4.3[a]	17.6 ± 4.6[a]	18.4 ± 4.9[a]	18.7 ± 4.3[a]	18.1 ± 4.8[a]
Rapid BCF	23.3 ± 3.1[b]	22.6 ± 2.6[b]	23.3 ± 3.2[b]	24.4 ± 2.1[b]	23.3 ± 2.3[b]

at 50 frames/second for African penguin to indicate the effect of time using the swim-up technique (n = 51).

TM = Total motility (%), Rapid = Rapid swimming sperm (%), Medium = Medium swimming sperm ((%)), Slow = Slow swimming sperm ((%)), VCL= Curvilinear velocity (µm/s), VSL= Straight-line velocity (µm/s), VAP = Average path velocity (µm/s), ALH = Amplitude of lateral head

displacement (μm), LIN = Linearity (%), STR = Straightness (%), WOB = Wobble (%), BCF = Beat cross frequency (Hz).

[a,b,] Different superscript letters in the same row for each time point denote a significant difference between the two techniques applied (P<0.05).

SD = standard deviation.

Table 4.15: Sperm motility and sperm kinematic parameter measurements (mean ± SD) captured at 50 frames/second to compare samples from two individuals African penguins at different time points using the swim-up technique (n = 51).

Swim up	0 Min		15 Min		30 Min		45 min		60 Min	
	P1	P2	P1	P2	P1	P2	P1	P2	P1	P2
TM	88.4±7.9	94.3±3.6	89.3±4.7[a]	95.9±2.6[b]	92.5±4.9	97.4±1.4	92.5±3.9	94.5±6.5	91.8±4.7	89.9±10.7
Rapid	42.1±22.1	38.7±28.7	35.13±17.6	48.25±21.7	49.5±12.1	53.3±22.4	50.5±21.2	62.1±23.3	49.6±19.3	46.9±37.2
Med	31.9±11.9	37.2±13.8	35.1±6.7	34.2±11.7	31.7±3.7	29.9±13.2	28.4±10.1	23.7±11.6	30.6±11.0	26.1±12.4
Slow	14.4±7.9	18.4±13.3	21.5±17.6	13.4±8.5	11.1±6.3	14.3±9.5	13.5±8.2	8.7±7.7	11.5±5.7	16.8±14.5
VCL	75.7±12.2	83.6±14.9	73.7±8.8[a]	87.7±10.8[b]	80.1±6.3	91.4±13.1	80.4±11.2[a]	96.4±12.2[b]	80.3±10.5	88.0±21.9
VSL	40.9±6.8	49.6±8.6	39.8±9.3[a]	51.1±7.5[b]	47.2±5.6	52.8±13.9	45.2±6.6[a]	56.2±9.7[b]	43.9±7.1	52.6±13.3
VAP	53.3±9.3	64.4±8.9	52.9±7.7[a]	66.5±7.1[b]	59.5±3.9	69.0±12.3	57.8±9.1[a]	73.1±9.1[b]	58.1±8.5	68.0±15.9
LIN	54.3±5.4	59.7±8.5	53.4±8.2	58.4±5.3	59.3±8.2	56.6±7.9	56.5±6.1	57.7±5.6	55.2±8.9	59.1±1.7
STR	77.1±5.6	75.5±7.4	74.3±8.3	75.4±5.0	79.4±7.5	74.2±7.1	78.5±4.9	75.1±6.3	75.9±8.2	75.3±2.4

WOB	70.3±3.0[a]	77.7±4.0[b]	71.7±3.9	76.4±2.9	74.5±4.0	75.3±3.5	71.8±4.7	76.1±1.9	72.4±5.3	77.6±1.0
ALH	2.2±0.2	2.1±0.3	2.2±0.1	2.3±0.2	2.3±0.2	2.4±0.2	2.3±0.2	2.4±0.2	2.3±0.2	2.2±0.3
BCF	14.6±0.9	23.7±1.9	14.2±1.8	22.5±1.5	14.5±1.3[a]	22.9±2.9[b]	15.3±0.4[a]	23.5±1.5[b]	14.6±0.9	23.7±1.8

TM = Total motility (%), Rapid = Rapid swimming sperm (%), Medium = Medium swimming sperm ((%)), Slow = Slow swimming sperm ((%)), VCL= Curvilinear velocity (µm/s), VSL= Straight-line velocity (µm/s), VAP = Average path velocity (µm/s), ALH = Amplitude of lateral head displacement (µm), LIN = Linearity (%), STR = Straightness (%), WOB = Wobble (%), BCF = Beat cross frequency (Hz).

[a,b,] Different superscript letters in the same row for each time point denote a significant difference between the two techniques applied (P<0.05).
SD = standard deviation

4.4.6. Sperm motility and sperm kinematics parameters comparing long and short breeding seasons

In the data presented in Table 4.16 most spermatozoa were motile, having an average speed (VCL) above 75 µm/s at all-time points. The mean values observed for (VCL, VAP and WOB) in the short breeding seasons presented with higher mean values compared to that of the long breeding seasons. However, a statistically significance difference ($P<0.05$) was only observed at 15 min and 60 min for VCL, VAP, BCF and medium swimming sperm. In contrast the short breeding displayed a significantly lower mean values for LIN and STR at 30 min and 45 min respectively. During the two breeding seasons results displayed that in all the sperm kinematics parameters and swimming sperm classes were not exclusively independent of each other.

Table 4.16: Sperm motility and kinematic parameters measurements (mean ± SD) captured at 50 frames/second for African penguin comparing short and long breeding seasons (n = 12).

Swim up	0 Min		15 Min		30 Min		45 min		60 Min	
	Short	Long	Short	Long	Short	Long	Short	Long	Short	Long
TM	84.8 ± 18.7	88.4 ± 7.9	92.2 ± 6.6	89.3 ± 4.7	91.4 ± 6.78	92.5 ± 4.9	87.5 ± 13.9	92.5 ± 3.9	94.6 ± 3.5	91.8 ± 4.7
Rapid	28.2 ± 18.9	42.1 ± 22.1	43.4 ± 23.3	35.1 ± 17.6	40.8 ± 20.9	49.5 ± 12.1	39.6 ± 27.4	50.5 ±21.3	74.8 ± 3.4[a]	49.6 ± 19.3[b]
Med	41.6 ± 31.9	31.9 ± 11.9	36.1 ± 12.3	35.1 ± 6.7	37.4 ± 9.8	31.7 ± 3.7	33.2 ± 11.1	28.4 ±10.1	15.5 ± 1.1[a]	30.6 ± 11.0[b]
Slow	14.9 ± 10.3	14.4 ± 7.7	12.7 ± 10.4	21.5 ± 17.6	13.3 ± 9.4	11.1 ± 6.3	14.8 ± 13.0	13.5 ± 8.2	4.2 ± 1.9	11.5 ± 5.7
VCL	77.2 ± 7.7	75.7 ± 12.2	86.6 ± 8.2[a]	73.8 ± 8.9[b]	83.7 ± 8.1	80.1 ± 6.3	82.9 ± 14.1	80.4 ±11.2	97.7 ± 2.0[a]	80.3 ± 10.5[b]
VSL	40.8 ± 8.4	40.9 ± 6.8	43.2 ± 4.3	39.7 ± 9.3	41.1 ± 6.1	47.2 ± 5.6	40.5 ± 8.0	45.3 ± 6.6	45.9 ± 9.4	43.9 ± 7.0
VAP	58.2 ± 7.7	53.32± 9.3	63.1 ± 6.2[a]	52.9 ± 7.7[b]	60.5 ± 6.1	59.5 ± 3.9	60.9 ± 10.9	57.8 ± 9.1	70.9 ± 5.1[a]	58.1 ± 8.5[b]
LIN	52.6 ± 11.2	54.3 ± 5.4	49.8 ± 4.7	53.4 ± 8.2	49.3 ± 7.4[a]	59.3 ± 8.3[b]	48.9 ± 7.2	56.5 ± 6.1	46.9 ± 8.7	55.2 ± 8.9
STR	68.5 ± 9.7	77.1 ± 5.6	67.5 ± 4.6	74.3 ± 8.1	66.7 ± 8.1	79.4 ± 7.5	65.8 ± 7.8[a]	78.5 ± 4.9[b]	64.3 ± 8.4	75.9 ± 8.2
WOB	75.3 ± 6.5	70.3 ± 3.0	72.9 ± 3.3	71.7 ± 3.9	73.1 ± 2.2	74.5 ± 4.0	73.6 ± 2.2	71.8 ± 4.7	72.5 ± 4.1	72.4 ± 5.3

ALH	2.3 ± 0.2	2.2 ± 0.2	2.32 ± 0.23	2.2 ± 0.13	2.3 ± 0.2	2.3 ± 0.2	2.3 ± 0.2	2.3 ± 0.2	2.5 ± 0.2	2.3 ± 0.3
BCF	18.0 ± 6.2	15.0 ± 1.2	18.7 ± 3.8[a]	14.2 ± 1.8[b]	18.3 ± 4.3	14.5 ± 1.2	18.2 ± 4.7	15.3 ± 0.4	14.7 ± 1.5	14.6 ± 0.9

TM = Total motility (%), Rapid = Rapid swimming sperm (%), Medium = Medium swimming sperm ((%)), Slow = Slow swimming sperm ((%)), VCL= Curvilinear velocity (µm/s), VSL= Straight-line velocity (µm/s), VAP = Average path velocity (µm/s), ALH = Amplitude of lateral head displacement (µm), LIN = Linearity (%), STR = Straightness (%), WOB = Wobble (%), BCF = Beat cross frequency (Hz).

[a,b,] Different superscript letters in the same row for each time point denote a significant difference between the two techniques applied ($P<0.05$).

SD = standard deviation

4.4.7. Effect of cryopreservation on sperm motility and sperm kinematic parameters

As the chemical environment of cryopreservation diluents can have a profound effect on sperm quality, we examined the effect of diluent and the freezing process on the sperm motility in African penguins (Fig 4.25). No significant difference was observed between the semen treated with Triladyl and the thawed samples, although the semen parameters (TM, Rapid, Med and Slow), tend to have higher mean values before freezing. After the addition of Triladyl and thawing, TM decreased by half for the long breeding season and by 3 fold for the short breeding season. No significant difference ($P>0.05$) was observed between the two seasons, however VCL, VSL and VAP were significant higher before freezing. Overall in Table 4.17 there was no significant difference displayed in all the sperm kinematics parameters ($P>0.05$) of semen before freezing and after thawing for two African penguins. Generally in Table 4.18 none of the parameters measured showed a significant difference ($P<0.05$) among the time points.

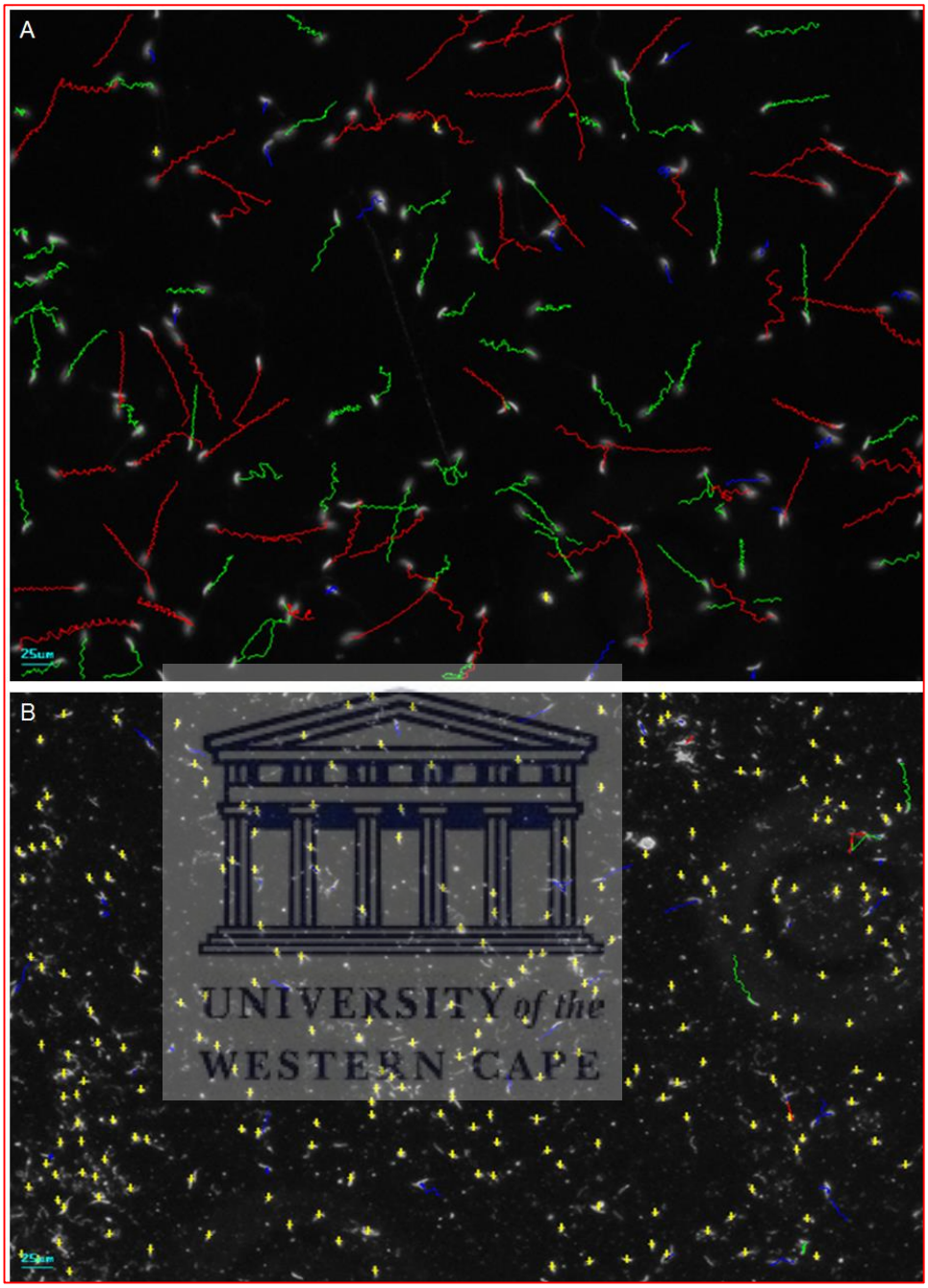

Figure 4.25: Sperm motility tracks of African penguin spermatozoa captured by the SCA system at 50 frames per second displaying swimming patterns exposed to medias. A) Ham's F10 media and B) triladyl extender. Red tracks = rapid progressive swimming spermatozoa, green tracks = rapid swimming spermatozoa, blue tracks = medium progressive swimming spermatozoa, yellow crosses = static spermatozoa.

Table 4.17: Sperm motility and sperm kinematics parameter measurements (mean ± SD) of semen before freezing and after thawing for two African penguins to indicate the effect of Triladyl over time (n = 10).

	Penguin 1			Penguin 2		
	Before freezing		After thawing	Before freezing		After thawing
	0 Min	30 Min	2 HRs	0 Min	30 Min	2 HRs
TM	60.0 ± 37.9	53.6 ± 33.9	27.12 ± 10.6	61.9 ± 8.0[a]	48.7 ± 18.5[b]	16.8 ± 5.8[c]
Rapid	14.2 ± 23.6	7.0 ± 10.9	1.2 ± 1.8	6.2 ± 4.3[a]	1.8 ± 2.2[b]	1.1 ± 0.9[c]
Med	19.9 ± 18.9	21.0 ± 20.0	5.6 ± 4.8	18.3 ± 7.3	11.8 ± 13.5	3.3 ± 2.9
Slow	25.8 ± 15.5	35.6 ± 25.3	20.3 ± 6.0	37.4 ± 7.8[a]	35.0 ± 8.8[b]	12.4 ± 2.7[c]
VCL	51.5 ± 23.7	46.6 ± 13.5	40.3 ± 5.8	55.5 ± 8.7	46.1 ± 9.7	46.5 ± 16.1
VSL	20.2 ± 10.3	16.9 ± 7.9	16.8 ± 6.4	23.4 ± 8.8	20.5 ± 7.9	15.9 ± 8.2
VAP	33.8 ± 16.6	29.7 ± 11.1	24.8 ± 6.5	37.8 ± 10.4	31.9 ± 9.9	27.3 ± 12.2
LIN	38.4 ± 11.7	35.1 ± 8.9	40.3 ± 11.1	41.9 ± 14.8	43.2 ± 10.1	33.7 ± 13.9
STR	58.4 ± 10.2	55.3 ± 8.8	65.2 ± 8.6	59.8 ± 10.8	61.6 ± 6.6	54.0 ± 12.7
WOB	64.5 ± 9.0	62.6 ± 7.4	60.96 ± 9.06	67.7 ± 12.6	68.3 ± 9.3	57.8 ± 13.1
ALH	1.90 ± 1.1	1.8 ± 1.0	1.6 ± 0.9	2.4 ± 0.5	1.9 ± 0.3	1.8 ± 0.7
BCF	7.4 ± 4.6	6.6 ± 4.2	7.3 ± 4.5	9.2 ± 2.9	9.6 ± 2.8	6.2 ± 4.7

TM = Total motility (%), Rapid = Rapid swimming sperm (%), Medium = Medium swimming sperm ((%)), Slow = Slow swimming sperm ((%)), VCL= Curvilinear velocity (µm/s), VSL= Straight-line velocity (µm/s), VAP = Average path velocity (µm/s), ALH = Amplitude of lateral head displacement (µm), LIN = Linearity (%), STR = Straightness (%), WOB = Wobble (%), BCF = Beat cross frequency (Hz).

SD = standard deviation

Table 4.18: Sperm motility and sperm kinematic parameter measurements (mean ± SD) of semen before freezing (0 min and 30 min) and after thawing (2 hrs.) for two African penguins comparing short and long breeding seasons (n = 10)

	0 Min		30 Min		2 HRS	
	Long	Short	Long	Short	Long	Short
TM	60.0 ± 37.9	53.6 ± 33.9	27.12± 10.6	61.9 ± 8.0	48.7 ± 18.5	16.8 ± 5.8
Rapid	14.2 ± 23.6	7.0 ± 10.9	1.2 ± 1.8	6.2 ± 4.3	1.8 ± 2.2	1.1 ± 0.9
Med	19.9 ± 18.9	21.0 ± 20.0	5.6 ± 4.8	18.3 ± 7.3	11.8 ± 13.5	3.3 ± 2.9
Slow	25.8 ± 15.5	35.6 ± 25.3	20.3 ± 6.0	37.4 ± 7.8	35.0 ± 8.8	12.4 ± 2.7
VCL	51.5 ± 23.7	46.6 ± 13.5	40.3 ± 5.8	55.5 ± 8.7	46.1 ± 9.7	46.5 ± 16.1
VSL	20.2 ± 10.3	16.9 ± 7.9	16.8 ± 6.4	23.4 ± 8.8	20.5 ± 7.9	15.9 ± 8.2
VAP	33.8 ± 16.6	29.7 ± 11.1	24.8 ± 6.5	37.8 ± 10.4	31.9 ± 9.9	27.3 ± 12.2
LIN	38.4 ± 11.7	35.1 ± 8.9	40.3 ± 11.1	41.9 ± 14.8	43.2 ± 10.1	33.7 ± 13.9
STR	58.4 ± 10.2	55.3 ± 8.8	65.2 ± 8.6	59.8 ± 10.8	61.6 ± 6.6	54.0 ± 12.7
WOB	64.5 ± 9.0	62.6 ± 7.4	60.96 ± 9.06	67.7 ± 12.6	68.3 ± 9.3	57.8 ± 13.1
ALH	1.90 ± 1.1	1.8 ± 1.0	1.6 ± 0.9	2.4 ± 0.5	1.9 ± 0.3	1.8 ± 0.7
BCF	7.4 ± 4.6	6.6 ± 4.2	7.3 ± 4.5	9.2 ± 2.9	9.6 ± 2.8	6.2 ± 4.7

TM = Total motility (%), Rapid = Rapid swimming sperm (%), Medium = Medium swimming sperm ((%)), Slow = Slow swimming sperm ((%)), VCL= Curvilinear velocity (µm/s), VSL= Straight-line velocity (µm/s), VAP = Average path velocity (µm/s), ALH = Amplitude of lateral head displacement (µm), LIN = Linearity (%), STR = Straightness (%), WOB = Wobble (%), BCF = Beat cross frequency (Hz).

4.5. Assessment of sperm vitality

A comparison of sperm vitality between the two individual African penguins as well as between the two breeding seasons (for Penguin 1 only) are presented in Table 4.19. No significant difference in viability was observed when comparing Penguin 1 with Penguin 2 or between the long and short breeding seasons (P>0.05). In Penguin 1 the average mean vitality was 87.82 ± 4.38% whereas Penguin 2 displayed an average of 78.88 ± 13.23%. An example of spermatozoa stained with eosin/nigrosine for vitality assessment is displayed in Fig 4.26. The head of a live spermatozoon is unstained (white), whereas a dead spermatozoon's head is stained pink (Fig 4.26).

Table 4.19: Summary of sperm vitality assessment of African penguin spermatozoa (mean ± SD) for different males and in different breeding seasons.

	P1	P2	All	P1 Short	P1 Long
Live %	87.8 ± 4.3	78.8 ± 13.2	82.4 ± 10.1	87.6 ± 4.0	88.4 ± 4.7

P1 = Penguin one, P2 = Penguin Two.

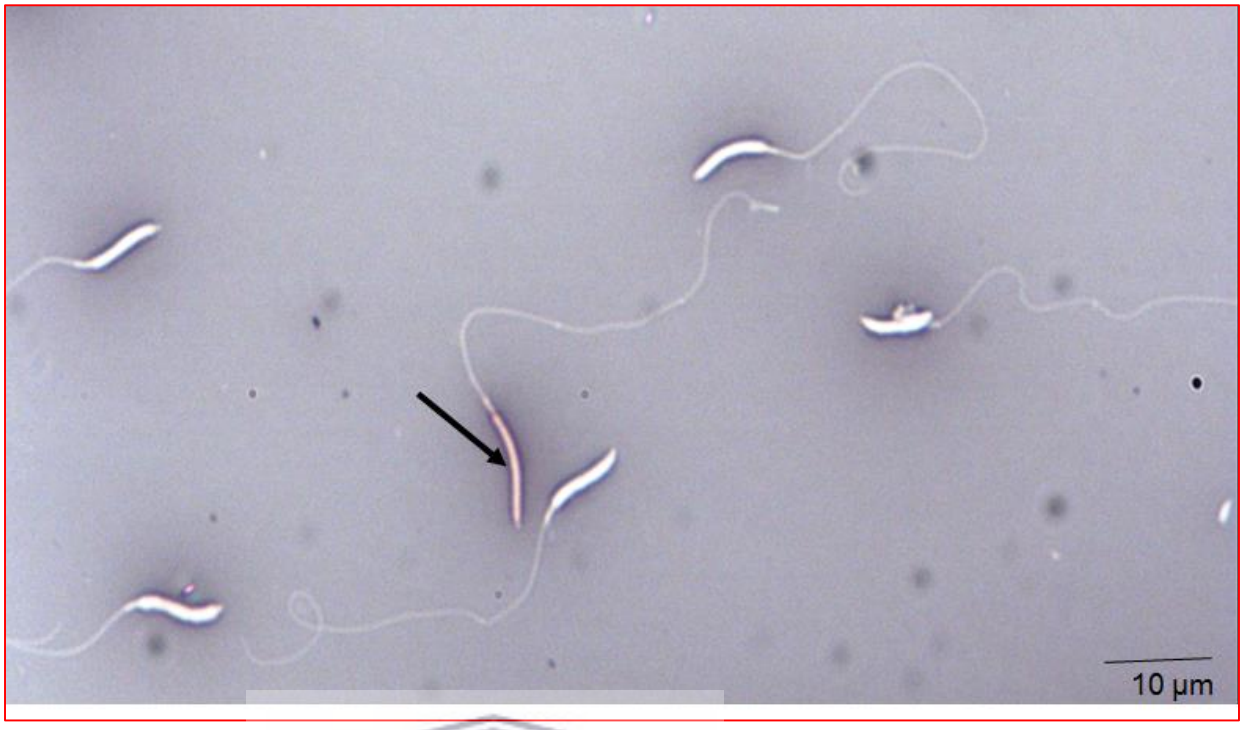

Figure 4.26: Micrograph of African penguin spermatozoa after staining with eosin and the background stain nigrosin. All spermatozoa display an intact plasma membrane (live spermatozoon, no stain uptake) with the exception of one spermatozoon (arrow, dead spermatozoon).

4.6. Sperm morphology

The spermatozoa of African penguin are filiform, flagellate cells which can classically be divided into three parts, namely the head, the mid-piece and the tail (Fig 4.27). When viewed by light microscopy, SpermBlue stained all sperm components differentially and accordingly resulted in clear boundaries between acrosome, head, mid-piece, principal piece and end piece which allowed for accurate measurements, as displayed in Fig 4.27. The fairly straight head is elongated anteriorly and contains a clearly defined, small acrosome (Fig4.27 A and C). Three different head shapes, namely spoon (Fig 4.27A), curved (Fig 4.27B) and linear (Fig 4.27C) heads were observed. When using

phase contrast microscopy, the various components of the spermatozoon could also clearly be distinguished from each other (Fig 4.28). The tail is composed of three distinct segments, namely the mid-piece, principal piece and end-piece. The short mid-piece and elongated tail are evident in both Fig 4.27 and Fig 4.28. The base of the head is continuous with the mid-piece, the first segment of the tail. The mid-piece revealed no specific features on light microscopy and phase contrast microscopy. After the distal end of the mid-piece, the tail continue as the long principal piece. The principal and the end piece are thinner in diameter than the mid-piece (Fig 4.28). NDICM revealed a small round acrosome covering the filiform-shaped head as well as a clear mid-piece containing several small mitochondria (Fig 4.29). In the 51 semen samples studied, the spermatozoa displayed similar morphologic features (head, mid-piece and tail) in both short and long breeding seasons.

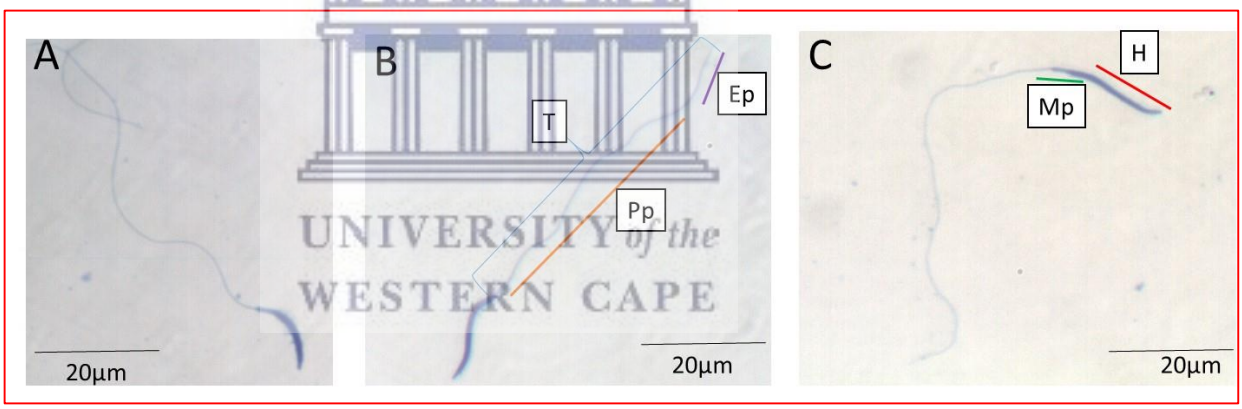

Figure 4.27: Sperm morphology of African penguins. (A, B and C) stained with SpermBlue and analysed with brigthfield optics. The sperm head (H), mid-piece (Mp), tail (T), principal piece (Pp) and end-piece (Ep) could be distinguished and measured.

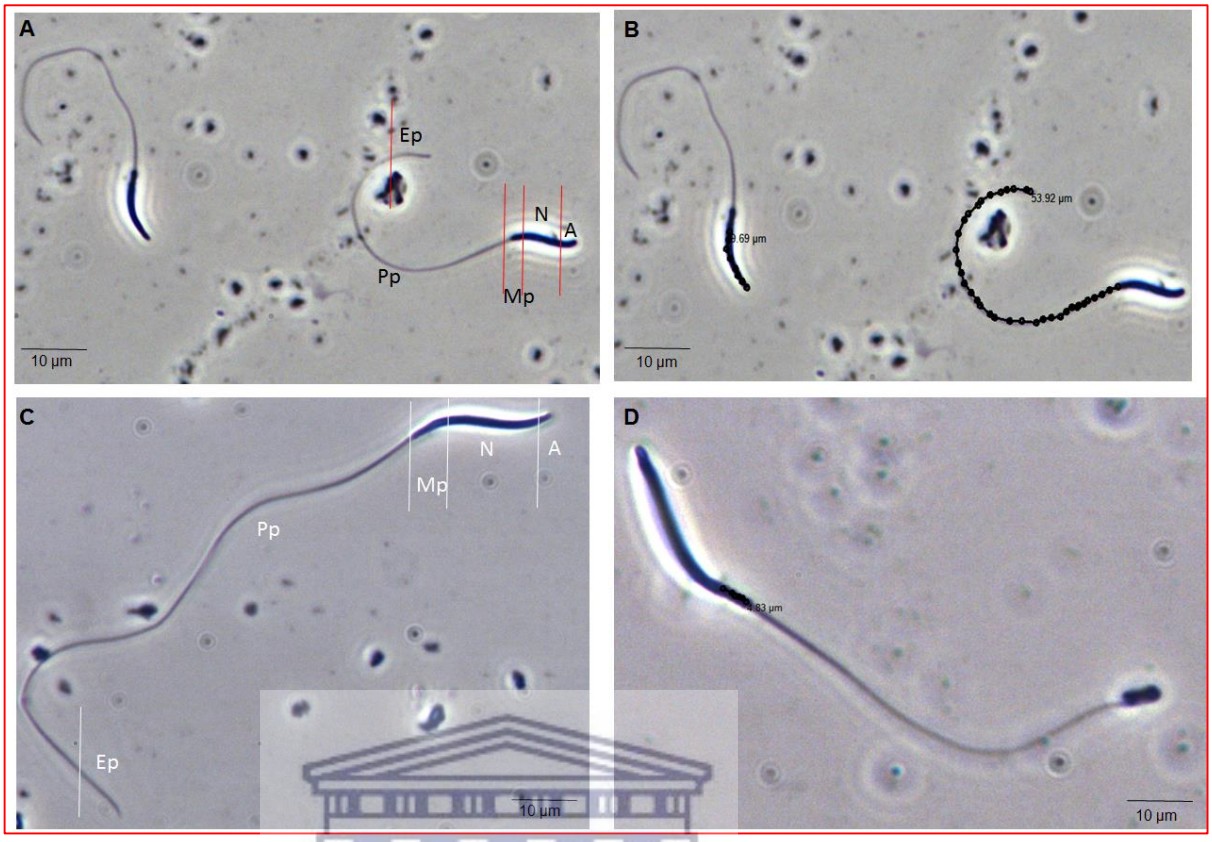

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Figure **4.28:** Phase contrast microscopy showing the various components of the African penguin spermatozoa. (A, B and C) stained with SpermBlue and analysed with brigthfield optics. The sperm head (H), mid-piece (Mp), tail (T), principal piece (Pp) and end-piece (Ep) could be distinguished and measured.

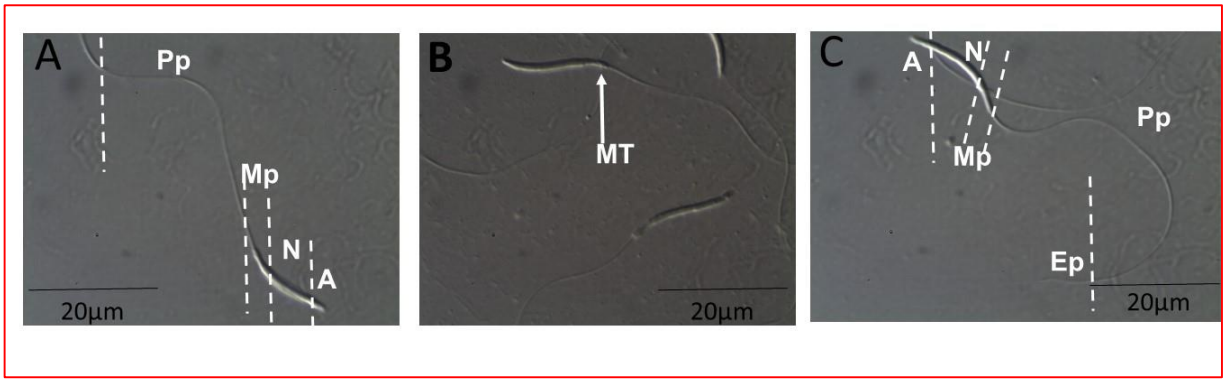

Figure 4.29: Nomarski differential interference contrast microscopy (NDICM) illustrating various African penguin sperm components. Namely the acrosome (A), nuclear region of the head (N), mid-piece (Mp), principal piece (Pp) and end-piece (Ep). Individual mitochondria (MT) are depicted in the mid-piece.

4.7. Sperm morphometry

Table 4.20 summarizes the morphometric values of the three major African penguin sperm components (head, mid-piece and tail) as well as the whole spermatozoon measured by the analySIS® FIVE imaging system (semi-automated). To measure the acrosome accurately at highest maginification (X100) used in this study was difficult. The sperm head measured 12.32 ± 1.64 µm in length (range 9.46 - 21.3 µm). The average lengths of the different parts of the tail were 2.28 ± 0.42 µm (range 1.36 - 3.23 µm) for the mid-piece, while the combined principal piece and the end-piece was 52.47 ± 5.91 µm (range 37.47 – 76.12 µm). The mean total sperm length was 67.07 ± 6.13 µm (range 53.85 - 67.07 µm). Based on the above morphometries, total head length was determined to be 12.32 µm and the total tail length 54.75 µm, giving a head to tail ratio: 1:4. The distribution of various sperm components are presented in Fig 4.30 and Fig 4.31, showing the dimensions of the various parts of the sperm which reflect the measurements for each penguin no differences between P1 and P2 was observed.

Table 4.20: Sperm morphometry measurements of head, mid-piece, tail and total sperm length (mean ± SD) for two African penguins.

	Penguin 1		Penguin 2		All	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Number of sperm measured	55		25		80	
Head length (µm)	12.32 ± 1.24	9.46 - 15.30	12.52 ± 2.32	9.79 - 21.37	12.32 ± 1.64	9.46 - 21.37
Mid-piece length (µm)	2.29 ± 0.41	1.64 - 3.23	2.26 ± 0.44	1.36 - 2.98	2.28 ± 0.42	1.36 - 3.23
Tail length* (µm)	52.65 ± 5.09	37.49 - 63.59	52.07 ± 7.59	39.57 - 76.12	52.47 ± 5.91	37.49 - 76.12
Total sperm length (µm)	67.19 ± 4.77	54.83 - 76.52	66.84 ± 8.63	53.85 - 94.00	67.07 ± 6.15	53.85 – 94.00

SD = standard deviation

* Tail length = principal piece + end-piece

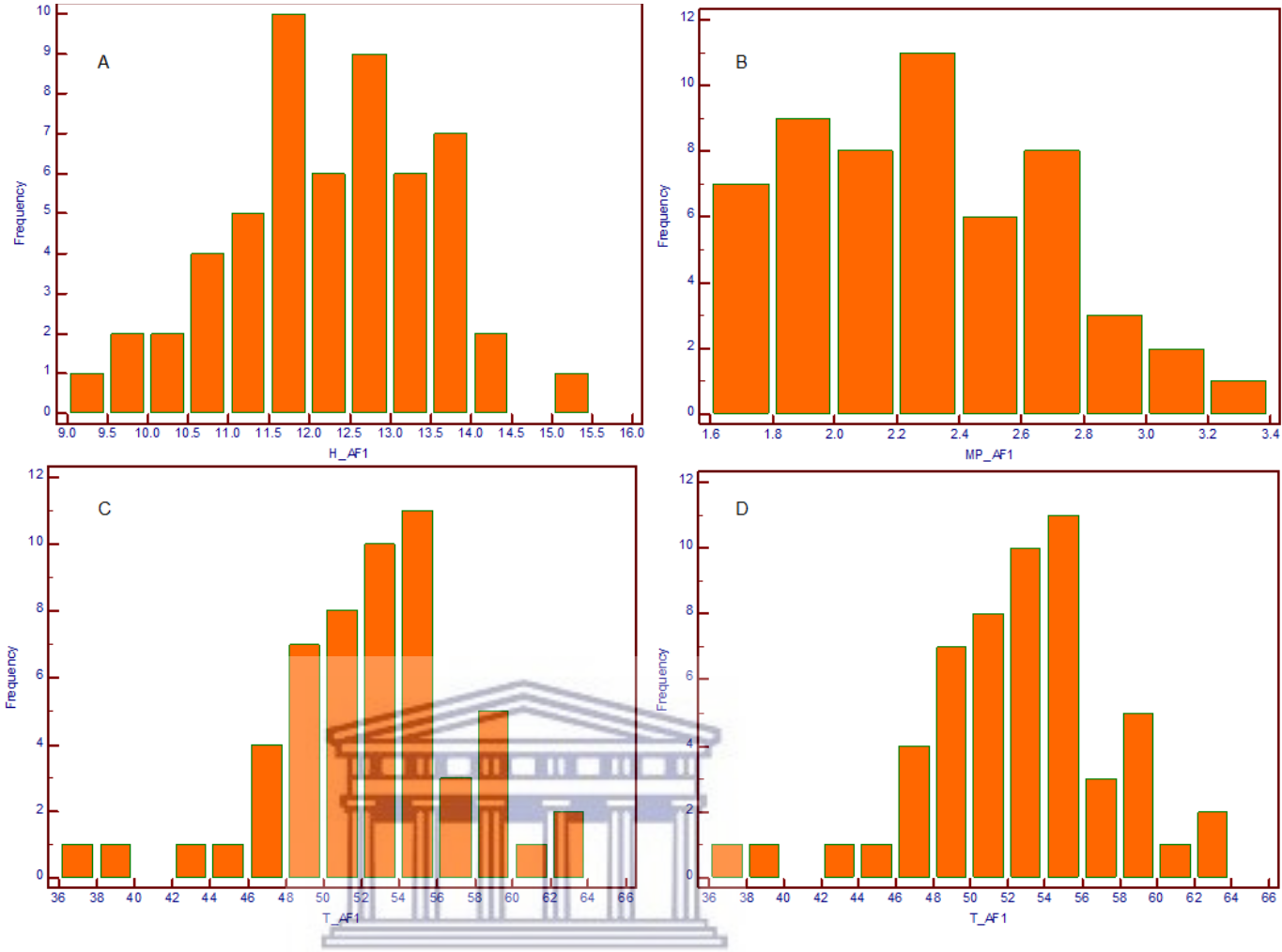

Figure 4.30: Frequency distribution of sperm components for Penguin.1.A) Head, B) mid-piece, C) tail and D) total sperm lengths (µm).

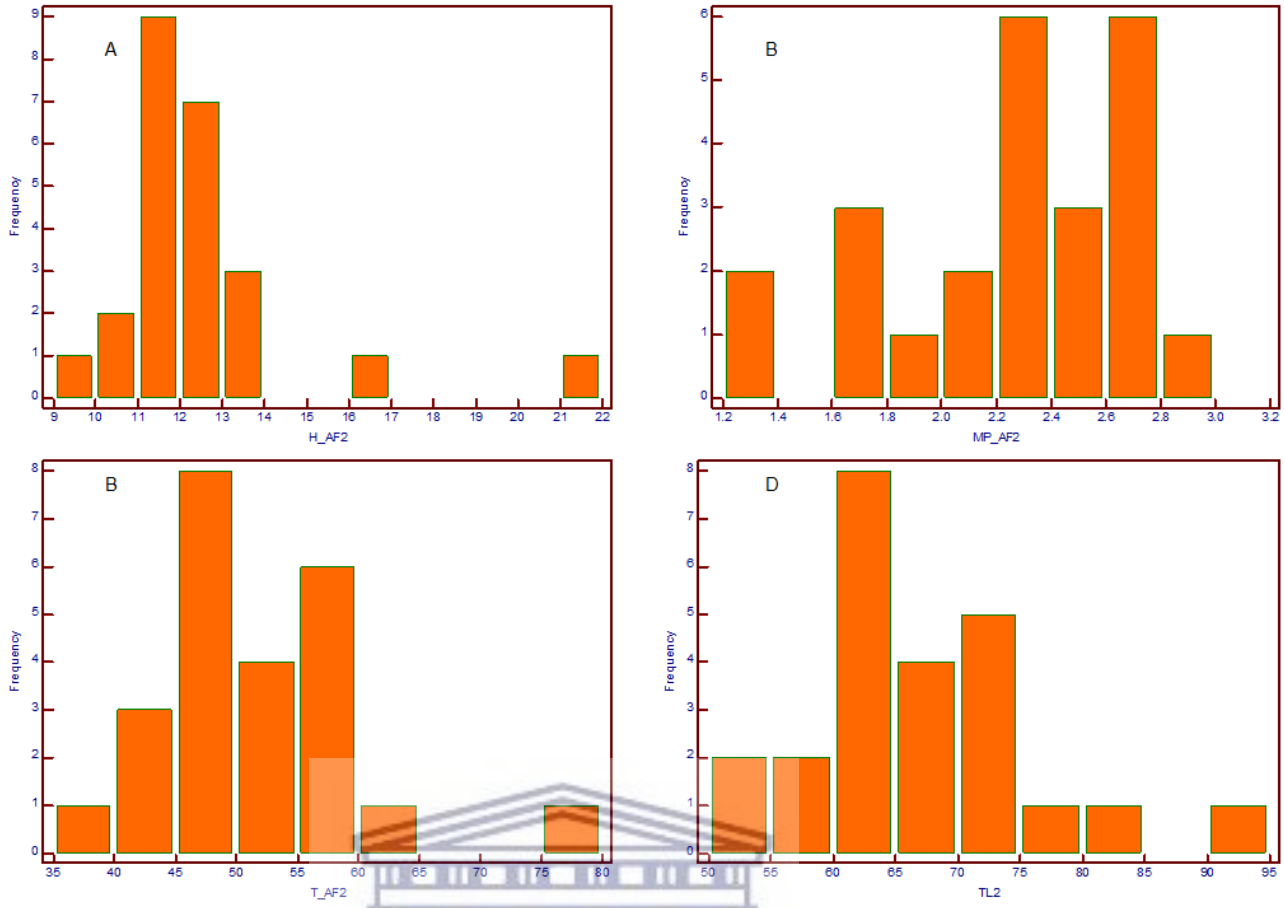

Figure 4.31: Frequency distribution of sperm components differentially and accordingly of Penguin 2. A) Head, B) mid-piece, C) tail and D) total sperm length (µm).

4.8. Sperm ultrastructure

As indicated in section 4.6, the spermatozoon of the African penguin includes a head and tail (flagellum). The tail is the longest part of the spermatozoon and includes the mid-piece, principal piece and end-piece. The sperm head is composed of the nucleus and acrosome. The sperm head is typically long, narrow and filiform-shaped with a distinct but small acrosome covering approximately 14% of the proximal part of the head (Fig 4.32A, B and C). The acrosome forms a cap-like structure, which is characterised by a small bump at the tip of the sperm head (Fig 4.32B). The acrosomal membrane is in close proximity to the nuclear membrane at the posterior border of the acrosome. A distinct part of the sperm head comprises the nucleus, stretching from

the acrosome and progresses to the proximal part of the neck. Transverse sections of the sperm head show the nucleus to be spherical (Fig 4.32B). The chromatin appears electron dense in mature spermatozoa.

The capitulum and the proximal centriole are located in the neck (Fig 4.32C), with two dominant cross-banded structures emerging from the capitulum and running longitudinally towards the mid-piece. The proximal centriole is situated near the base of the nucleus, whereas the distal centriole runs partly through the mid-piece and gives rise to the axoneme. The mid-piece contains approximately 13 spherical mitochondria with extensive cristae, which are arranged around the centrioles and axoneme in a single helix (Fig 4.32A and D).

The annulus (Fig 4.32D) is situated below the last row of mitochondria and in close association with the plasma membrane, indicating the boundary between the mid-piece and principal piece. Transverse sections of the flagellum show a typical 9+2 axoneme structure of nine outer microtubule doublets and a central pair (doublet) (Fig 4.33A). Dynein arms are attached to the outer microtubule doublets. Surrounding the principal piece outer doublets is an amorphous fibrous sheath and no outer dense fibres were identified. The principal piece is the longest part of the flagellum, followed by a short end-piece.

4.8.1. Multiple axonemes

As mentioned, the penguin sperm axoneme consists of two single microtubules surrounded by nine sets of double microtubules which is the typical structure in mammalian and avian species (9+2 pattern, Fig 4.33A). However, in mammalian and all internal fertilizers there is in addition nine solid outer fibers (Lacking in African penguin) and the typical flagella pattern is 9 + 9 + 2. TEM images also revealed that the axonome is present for the most part of the length of the flagellum. In addition, spermatozoa with multiple axonemes were observed in approximately 4% sperm of

tails evaluated from both African penguin males' semen. Double axonemes were most frequently observed, with triple and quadruple axonemes also noted, and bound together by the same plasma membrane (Fig 4.33D and G). These multiple axonemes aligned in both the horizontal and vertical planes, sometimes as mirror images of each other, with the central doublets always in line with outer doublets 3 and 8 (normal presentation, Fig 4.33B, E, H and K). The plane of bending is similar for all axonemes (Fig 4.33C, F, I and L). Accordingly axonemes can bend in the three to eight plane but not in the one to six plane. In the multiple axoneme conformation there appears to be synchrony that will theoretically allow sperm to still be motile by active sliding of microtubules.

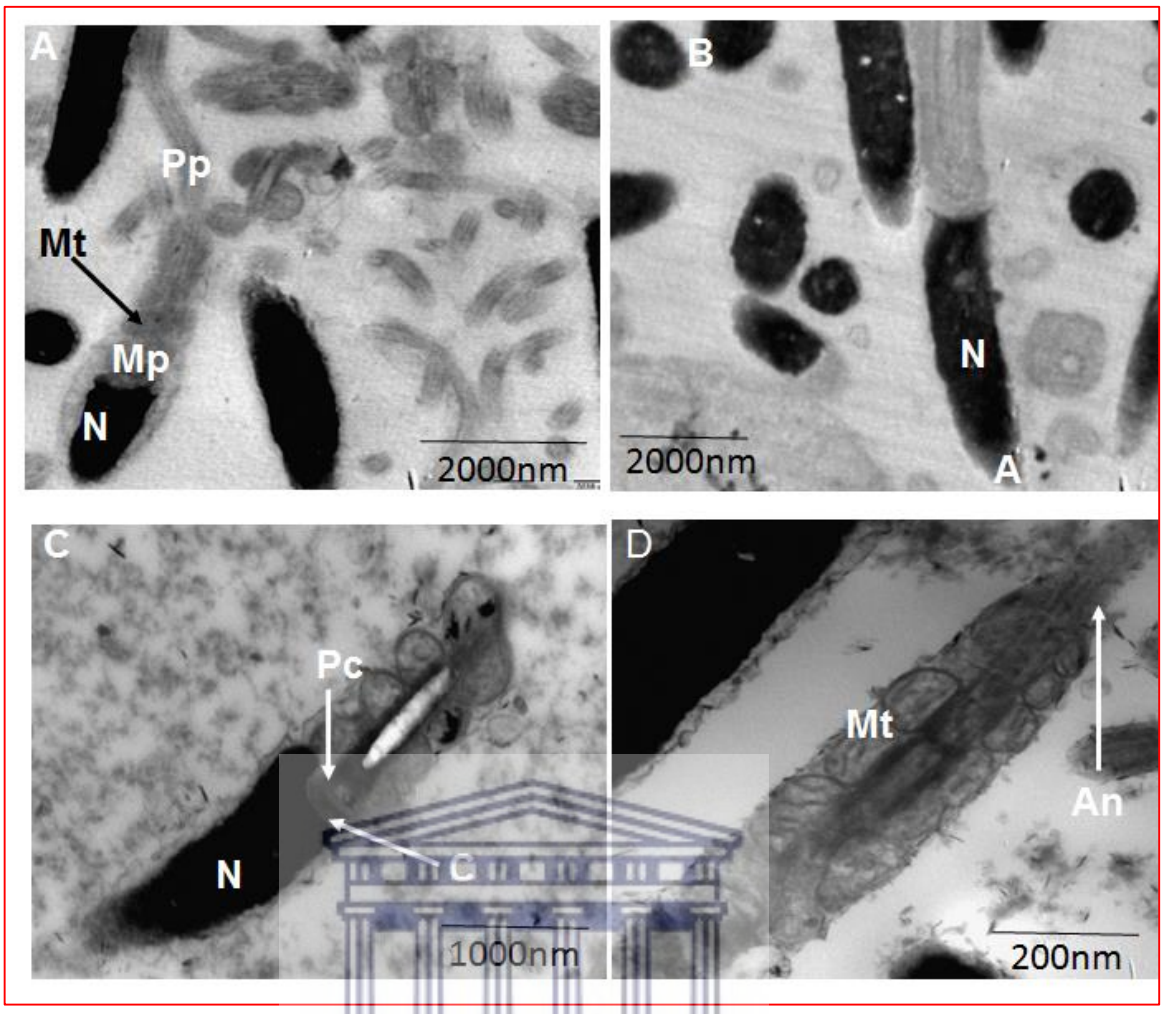

Figure 4.32: Ultrastructure of the spermatozoa of African penguin (*Spheniscus demersus*). A) Longitudinal section trough the head an mid-piece, nucleus (N), mid-piece (Mp), mitochondria (Mt), principal piece (Pp), B) Longitudinal, cross and transverse sections of the head showing that nucleus is spherical, acrosome (A), nucleus (N). C) Longitudinal section through nucleus and mid-piece showing, nucleus (N), capitulum (C), proximal centriole (Pc). D) Longitudinal section of the mid-piece showing mitochondria (Mt) with cristae, annulus (An).

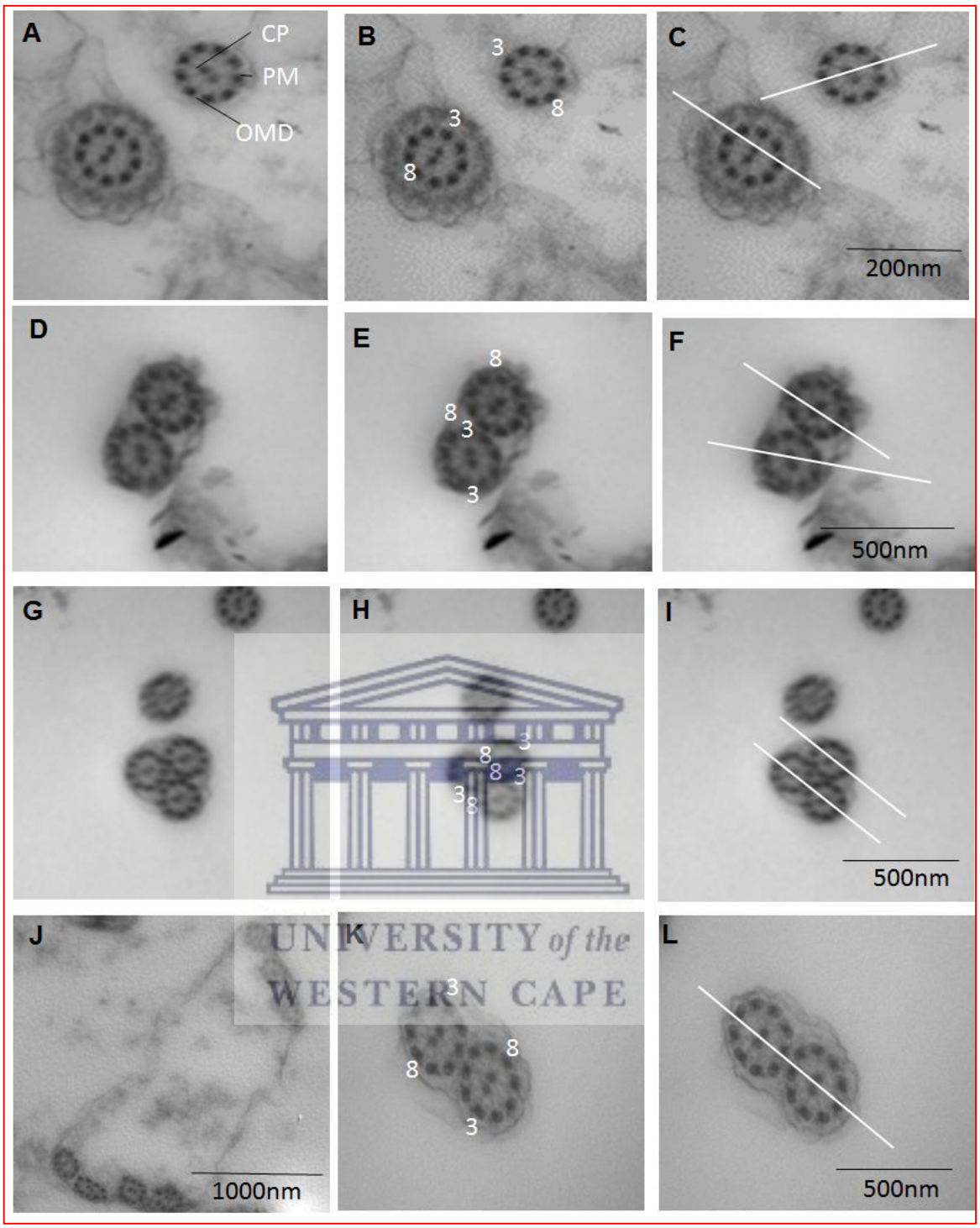

Figure 4.33: Cross section through the sperm flagellum illustrating the axonemes. A) Two typical and individual axonemes, D) double axoneme in one plasmalemma, G) three axonemes in one plasmalemma and j) four axonemes in one plasmalemma, cut at an oblique angle. B, E, H, K) Orientation of doublets 3 and 8. C, F, J, L) Display of the possible plane of bending. Central pair (CP), plasma membrane (PM), outer microtubule doublet (OMD).

Chapter 5:

Discussion and conclusion

5.1 Discussion

This study reports on the basic macro-anatomy, histology, hormone profiles and semen and sperm characteristics of the African penguin, with the use of different macroscopic and microscopic techniques. The lack of information on the reproductive biology of the African penguin, as well as other species of penguin, makes it difficult to compare features of African penguins within the order Sphenisciformes. Results from this study contribute new data on sperm biology and various aspects of the reproductive system of male and female African penguins. The collective findings of macro- and micro-anatomy, hormone levels and seminal traits can be integrated into management strategies for ensuring the long term survival of the African penguin in captivity and in the wild. Together these findings can assist in determing the reproductive and general health of penguin colonies and further aid in management of species.

To our knowledge, no substantial macroscopic and microscopic data on the reproductive organs of the African penguin has been published to date. A study of the reproductive system of the African penguin is important for the conservation of the species, and the testes, ducts and ovaries are the most important functional regions of this system. The establishment of a correlation between testicular shape, size and sexual maturity has been described for Iranian white rooster (Razi *et al*., 2010), but

such fundamental research has not been done in the order Sphenisciformes.

This study represents the first report for sperm motility characteristic for the African penguin, but the motility data for other penguin species using CASA has been described (O'Brien *et al*., 2014). This important information is valuable for constructing a species reproductive database, which in turn can help in the monitoring of *ex situ* populations and compare it to those in the wild.

There is currently only few published studies on sperm characteristics of penguin species (O'Brien *et al*., 1999, 2014; 2016; Waldoch *et al*., 2007), but there are no previous studies on African penguins. With regards to morphological studies of sperm ultrastructure, few reports on non-passerine bird's exists (Asa *et al*., 1986; Soley *et al*., 1994; du Plessis *et al*.,2011, 2012), however there are no previous reports on penguins and the occurrence of multiple axonemes within one sperm plasma membrane.

5.2. Macro- and micro-anatomy of the male and female reproductive structures in African penguin

There are several asymmetrical features of gonadal development in African penguins, and are present in both sexes while others are restricted to the female. Before the stage of gonadal sex determination, left and right gonads are commonly present in both males and females. After gonadal sex determination an irregularity is restricted to females; only the left gonad develops into a fully mature functional ovary. The phenomenon of the reproductive adaptation of only one ovary and oviduct in the female remains unclear but is the rule in avian species (Razi *et al*., 2010).

5.2.1. Male reproductive system

The male reproductive organs of birds have been studied (Calhim *et al*., 2007; Bull *et al*., 2007) but little attention has been paid to details in the order Sphenisciformes. The present study has been narrowed to basic morphological structural features of the male

reproductive tract. Like other avian species, the African penguin testes is oval shaped, but a vermiform shape was reported in swift (*Cypseloides spp*), (Marshall, 1961). We found that African penguin testes are asymmetrical, with the left testis always being larger than the right. This data corresponds with the findings of testis asymmetry in most bird species (Birkhead *et a*., 1992; Barrie, 2007; Gunn *et al*., 2008; du Plessis *et al*., 2012; Yu *et al*., 1998). In the study conducted in rooster (*Gallus domestica*), the left testis was larger than the right in 57% to 65.3% of the birds studied (Mimura, 1928; Marvan, 1969). Although the left organ is larger than the right organ, it is believed that these testes contribute equally to the production of androgen and, possibly, also spermatozoa (Malecki *et al*., 1998). The only contradictory results reported in birds were the testicular size in quails and sparrows which did not exhibit constant asymmetry (Yu *et al*., 1998). The reason for the generally observed difference in testis size, in favour of the left, is not clearly understood, especially given the variations that have been reported. However, one explanation is that of embryological study by Witschi (1935) displaying that the cortex of the right indifferent gonad loses its chemotactic attraction for primordial germ cells, in favour of the cortex of the left gonad.

The size of the avian testis differs considerably between phases of the reproductive cycle (Barrie, 2007). In this study, the size and the weight of the testis varied with the stage of the penguin breeding cycle, and may be due to males becoming sexually active when the testis enlarge (Bull *et al*., 2007). At the juvenile stage, testes are elongated and as the penguin reach the adult stage, both testes assumed the cylindrical shape. In most wild birds, the testis reaches maximum size for the species during the peak of the breeding season and becomes smaller, following regression, during the sexually inactive period of the reproductive cycle. Møller (1998) reported that during the active breeding season, testis size is positively related to sperm production. The size and the weight of the testes can be correlated with the age of a bird, hypothesising that older birds are more likely to be sexually active than the younger ones. Such data and hypothesis corroborate with ours, the testis size and

weight of African penguin varied based upon an annual testicular cycle. Like in other birds (Marshall, 1961), African penguin testis increased during the breeding season compared to juveniles and the non-breeding penguins. During the breeding season or period, active avian testes have a substantial amount of fluid content (Aire *et al*., 1979), resulting in the testis of breeding birds being soft to touch and quite fragile compared to the mammalian testis. Our understanding of these patterns across the annual testes cycle suggests that accurate measurements of reproductive organs can be related to sperm production.

The testicular volume provides an important criterion in the evaluation of male reproduction. Detailed measurement of testicular volume is necessary for evaluating testicular function, due to the fact that testicular volume represents the state of spermatogenesis (Lin *et al*., 2009). It was also suggested that accurate measurement of testicular size is required for precise monitoring of growth status (Behre *et al*., 1989; Lin *et al*., 2009). Calhim *et al*., (2007) reported the difficulty in measuring avian testes size, and further studies have relied on several assumptions for measurement of these organs. Such assumption will be the reliability of testes mass estimates and linear dimension of often only the larger combined testes mass. This indicates the lack of information of bird testes size and volume. Our results on volume of the African penguin testes is in agreement with the evidence indicating the left testis is larger in both size and volume compared to the right testis. Testicular function has a direct correlation with testes volume and size. Monitoring the size and the accurate measurements of testicular volume is important to define the changes in a testicular cycle and the evaluation from the juvenile age and also the difference between non-breeding and breeding penguin.

The epididymal region is remarkably small, flat and attached to the medial side of the testis. In the African penguin the epididymal region is similar to the general structure and composition as described in other avian species such as guinea-fowl (Lake, 1957),

turkey and Japanese quail (Aire, 1979). The avian epididymis is a small spindle-shaped structure that is attached intimately to the medial border of the testis. The tail end of the epididymis thins out and is connected to the ductus deferens, which becomes thick at its base where it is attached to the testis (Hodges, 1974). Gray (1937), described the epididymis as being about 1.0 mm in thick in Leghorn males. Unlike in mammals, avian species epididymis is not sub-divided into distinct anatomical regions (Bath *et al*., 2002), this is in agreement with the observation we made in the epididymis of African penguin. Hodges (1974) reported that in fowl a small anterior portion of the epididymis, which is not attached to the testis extends dorsally and is enclosed in the capsule of the adrenal gland. The ductus deferens leaves the caudal end of the epididymis in a slight wavy manner then become considerably longwinded. It is convoluted tube which runs posteriorly, along the midline, and parallel with the ureter.

5.2.2. Female reproductive system

The female reproductive system of the African penguin consisted of an unpaired ovary, developing on the left side, and an oviduct, all of which is in accordance with findings in other birds (Essam *et al*.,; 2016; Hanna *et al*., 2017; Mirhish *et al*., 2013; Sari *et al*., 2014; Vijayakamar *et al*., 2014.). The left and right ovaries are present during the early embryonic stages of presumably all birds (Barrie, 2007), but in African penguin species only a functional left ovary is maintained post-hatch and is in agreement with other investigations on birds (Hanna *et al*., 2017; Vijayakamar *et al*., 2014). A fully developed right ovary is reported to be rare in avian species; although it is present during embryogenesis, it will later degenerate during development in the adult penguins. In contrast, some species commonly maintain both gonads (e.g. kiwi and vultures), yet these functional ovaries may be asymmetrical in size, the left characteristically being the larger (Kinsky, 1971). Various other birds, including sparrows, gulls and doves, may maintain two ovaries through adulthood (Kinsky, 1971). The ventral surface of an

adult African penguin ovary had numerous ovarian follicles that varied in size. The change in follicle size during the development suggests the transition from a non-breeding to a breeding penguin, preparing the penguin for ovulation and oviposition. In avian species during maturation, follicles are generally categorized according to size and the presence and colour of the yolk (Mary *et al*., 1995). The number of follicles at each stage of development suggests ovarian activity and can be used to categorize an adult female penguin as non-breeding or breeding. These events proceed with significant support of hormonal changes in the African penguin.

The oviduct in the African penguin appears as a highly vascular and long tube in the female penguin reproductive system. Despite not being able to measure the length of the oviduct in this study, other studies on birds have reported that the length varies in different orders, with the magnum being the longest part (Hanna *et al*., 2017). The oviduct was reported to be divided into five segments (Anisur, 2013; Essam *et al*; 2016; Sari *et al*., 2014; Vijayakamar *et al*., 2014), in a sequence from the thin infundibulum, magnum, isthmus, uterus and thick-walled vagina, with each segment having a unique function. In Pegagan duck the oviduct was 45-50 cm (Sari *et al*., 2014), while in rhea the average length of the left oviduct, during egg laying season, was 122 cm (Parizzi *et al*., 2008). In the fowl the oviduct reached lengths of 60-70 cm, and in the duck was reported to be up to 60 cm (Blendea *et al*., 2012; Mohammadpour *et al*., 2012). The oviduct extends from the ovary to the cloaca (Mirhish *et al*., 2013) and transports the ovum after it is released from the ovary. After ovulation the ovum is engulfed by the funnel of infundibulum and transported along the duct until it reaches the uterus. Before the ovum is secreted out through the cloaca, the ova gradually matures in the oviduct through several maturity processes and then be transported to the cloaca for oviposition.

5.2.3. Micro anatomy of the male and female reproductive structures in African penguin

5.2.3.1. Histology of the African penguin testis

Histology reports show that compared to mammals the testicular capsule in birds is thin and solid, consisting predominantly of collagen fibers and few elastic fibers (Aire, 2007; Al-Tememy, 2010; Lake, 1957; Razi *et al*., 2010). The testicular capsule thickness has been reported for adult ostrich (578.1 µm), rooster (81.5 µm), Japanese qual (91.7 µm), drake (91.9 µm) (Al-Tememy, 2010) and domestic fowl (85.5 ± 13.7µm), but is reported to be five-fold lower in younger birds (Aire *et al*., 2007). The testicular capsule in the order Sphenisciformes has not been reported before, our observational results disclosed that the African penguin testicular capsule resemble that of other avian species being thin and became much more thicking between testis and epididymis. Furthermore, the testicular capsule contains a large quantity of highly contractile tissue, which probably assists with the transporting of testicular fluid into the excurrent ducts.

The African penguin testes contain two types of tissues, namely the interstitial tissue and the seminiferous epithelium. The interstitial tissue contains blood vessels and Leydig cells and is fairly dense (Aire, 1979).This compact structural feature, is common in birds except in the ostrich, showing loose connective tissue (Al-Tememy, 2010; Shil *et al*., 2015). Leydig cells are functionally and structurally similar to those found in mammals, therefore, they appear individually scattered in small groups found within the larger intertubular spaces in most birds (Aire, 1997). In Japanese quail, the smooth endoplasmic reticulum in the testes is not well developed as it is in mammals. Hodges (1974) reported that the Leydig cells for fowl were highly variable, depending mainly upon the size and shape of the space where they are situated. In the present study, the Leydig cells in adult breeding penguins contained lipid droplets which is most probably related to its function to produce and secrete testosterone.

The seminiferous tubules (ST) of avian species are dissimilar to those of mammals by forming a highly complex network of tubules (Hodges. 1974). In fowl, no true septa are present and only fine strands of connective tissue pass inwards from the tunica to separate the ST (Hodges, 1974). In accordance to the study by Aire (1997) which showed that the ST of gonadal non-breeding birds physiologically decrease in size, we also observe that juveniles ST were smaller in size compared to adults. The observation made in this study was that the relative volume of the testes was occupied by the ST. During the breeding season the diameter of the ST was larger compared to the non-breeding season. There were different cells types observed in the ST between juvenile, non-breeding and breeding penguins, where no spermatids were present at any stage in the juvenile's penguins. In adult male penguin all cell types were present during non-breeding and breeding seasons although we can predict that no active sperm were active during the non-breeding season, because we could not collect any semen during that season. The other difference was that of the lumen of the ST, in juveniles there was no distinct opening in the lumen while the non-breeding penguin at some stage showed the clear opening and the breeding penguin lumen was filled with different cell types of spermatids.

Examination of the seminiferous epithelium in African penguins revealed that the germ cells are not arranged at random but are structured into a well-defined cellular association. In a particular region of the seminiferous tubules, spermatogonia, spermatocytes and spermatids occur at given steps during spermiogenesis. These cellular associations succeed one another in any given area of the seminiferous tubules and the sequence repeats itself. For example in African penguin the group of spermatids at a given step of development will be always associated with certain groups of spermatocytes and spermatogonia, constituency form cellular association. The cellular association was not clearly classified into the limited number of types as in rats, where they were classified into 10 (Perry *et al*., 1961).

In the convoluted ST the different cells could be identified from the periphery towards the lumen - spermatogonia, primary spermatocytes, secondary spermatocytes and finally spermatids and maturing spermatozoa. The arrangement of the cellular association in African penguin is the same cellular association described in mammals. Generally, the spermatogonia are bordering on the limiting membrane of the ST. Their nuclei are slightly ovoid, with nucleoli usually attached to the nuclear envelope. The preleptotene spermatocytes produced as a result of mitotic division of the late spermatogonia; their nuclei is somewhat smaller than spermatogonia. Preleptotene spermatocytes are located at the periphery of the tubule and are normally in direct contact with basal lamina of the epithelium (Weiss *et al*., 1977). The preleptotene spermatocytes migrate away from the basal lamina as the cell progresses to pacyhytene and other prophase stages. As the cell enters pacythene, there is marked increased nuclear volume, the sperm cell become shorter and thicker and each one splits into two chromatids (Weiss *et al*., 1977). As this is where exchange of genetic material occurs between pairs of homologous chromosome, it is long stage in humans occupying about 16 days and they are most visible in the a cross section of the ST (Barrie, 2007), but this stage can be short in African penguins due to different process compare to that of humans. Like in other birds and in humans, secondary spermatocytes in African penguins are much smaller than primary spermatocyte and beside size difference they may be identified by their spherical nuclei containing granular chromatin and they are infrequently found in sections of seminiferous tubules. Our results were in agreement with Al-Tememy (2010), describing the process of spermatogenesis in birds, as in mammals, involving the process of division from spermatogonia to some mature spermatids, in which they undergo meiotic division.

The Sertoli cells (SER) are generally described as possessing supportive and nutrition functions in relation to the germinal cells, particularly the maturing spermatozoa. The histology of the fowl and quail described that the SER are tall, columnar and extending from the basal lamina to the luminal border of seminiferous epithelium. Our results

suggest these structures are the same in African penguin and they are similar to that described in mammals (Al-Tememy, 2010; Hodges, 1974; Cooksey and Rothwell, 1973). The SER cells had prominent nucleoli, and are situated close to the basal lamina as in most bird species (rooster and in Japanese quail).

5.2.3.2. Histology of the African penguin ovaries

The ovary of avian species consists principally of an outer cortex containing the ova which surrounds a highly vascular medulla composed primarily of connective tissue (Hodges, 1974). Although we did not observe a clear separation between these two areas in the African penguin ovary, it was reported that in birds these are distinctly separated, but as the ovary grows and matures, the division between the two layers becomes hidden and eventually lost (Hodges, 1974). Our study revealed that the ovary of the African penguin consists of an outer cortex which contained many ovarian follicles of different sizes and an internal vascular medulla, similar to descriptions reported for the chicken (Hanna *et al*., 2017). There are potentially a large number of primordial and primary follicles available in the ovary, but most do not reach maturity and only a selected few are ovulated.

The most noticeable part of an active African penguin ovary was the number of follicles passing through the different stages of maturation namely the primary, secondary and tertiary stages. In this study we used size to differentiate between different classes of follicles. Within the cortex there were numerous developing follicles varying from 8.98 µm to 42.46 µm in diameter, with primary stage averaging to 11.21 µm and secondary and tertiary averaging 16.25 µm and 25.17 µm, respectively. Each ovum contains a centrally-located nucleus encompassing one or two nucleoli and a diffuse chromatin network. In mammals there are eight stages that are involved in transition from primordial to Graafian follicles, but in our study we observed large differences compared to mammals in terms of the number of layers of granulosa cells, in penguins,

as in all birds, one layer of granulosa cells is evident (Eppig, 1978). Our observations were consistent with the findings for duck and fowl (Anisur, 2013). In this study, such developing follicles were between 5µm and 25 µm in diameter. Our findings agree with Pagagan ducks (Sari *at el*., 2014) where four layers of the follicle were described, namely granulosa cells, vitelline membrane and theca interna and externa.

Compared to the mammalian counterpart a unique morphological and functional aspect of the reproductive active avian ovary, is that follicles at all stages of development, from resting primordial and primary follicles to the fully differentiated preovulatory stage, exist even during egg laying. The information pertaining to cellular and molecular mechanism regulating follicle growth and differentiation is not well documented in penguins. This is because of the quantity and ready availability of tissues required for detailed studies. The most prominent features of a reproductively active ovary are the developing follicles that server to provide a structural support for growth of the large oocytes and release the oocyte in a timely fashion at ovulation (Eppig, 1978).

5.3. Testes, ovary size and reproduction activity

The analysis of the testis and ovary histological features such as structure, weight and size, can give a clear indication of breeding status. Møller (1988), stated that birds which have relatively large testes produced ejaculates with higher sperm concentrations. Testicular size related to the development of spermatogenic cells in breeding *versus* non-breeding birds. Accordingly, larger testes were associated with complete spermatocytogenesis/spermiogenesis compared to poorer development of any spermatogenic stage in the non-breeding stage. Such observation is common in avian species. The lack of spermatids in juvenile African penguins is consistent with what was found in pukeko (*Porphyrio porphyrio melanotus*) study, which shows a correlation between increasing testicular size and maturity of seminiferous tubules.

The histology and ultrastructure sections from juveniles, non-breeding and breeding penguins suggest that spermatogenesis had not yet commenced in juveniles, and this suggest that juveniles were incapable of breeding. We have found that microscopically, the testes of the adult non-breeding penguin resemble that of the breeding penguin, although there are difference depending when was the tissue collected. In the non-breeding seasons, the lining of these seminiferous epithelium consisted almost exclusively of Sertoli cells, spermatogonia, primary spermatocytes, secondary spermatocytes and few spermatids in this way resembled the early breeding state. The presence of Leydig cells and the increase in testes activity showed a close analogy and suggest the breeding season. We can speculate that the testes increased in size and weight is a result of the deposition of lipid by the Leydig cells and also as a result of the increase in diameter of the seminiferous tubules (Møller, 1998). The correlation observed between micro- and macro-anatomy could be because testicular development is dependent on the age and breeding season of the penguin.

The female reproductive tract of penguins is clear, in that they ovulate almost the whole breeding seasons, laying 2-3 eggs per breeding season. They have a longer period of time between ovulation and oviposition, which increase the time that eggs spend in the oviduct. The follicles develop into hierarchical arrangement from small visible follicle to the yellow visible yolk. Once the follicle has followed this hierarchy, the steroid hormones regulate the factors that modulate growth, development of the follicle and ovulation. These findings suggested that macro- and micro-anatomy of the testes and ovaries may be used to estimate both non-breeding and breeding seasons and hence the reproductive potential of the African penguin.

5.4. Hormone profiling

In African penguins breeding occurs throughout the year and the species is monogamous. The clutch size is usually two eggs, sometimes one and rarely three

(Crawford *et al.*, 2010, 2011). In South Africa the laying of eggs differs for different islands. On Dassen Island, eggs are laid through the year but mostly in December to June and most chicks are encountered in January to August (Borboroglu and Boersma, 2013). On Robben Island, the first clutches are laid in January to August, most (38%) in February, and 94% by the end of May (Borboroglu and Boersma, 2013). On St. Croix Island, egg -laying peaks are in January, with a second peak in March to April attributed to replacement laying after clutch failure and a third peak in June that results from second clutches or further replacement laying (Borboroglu and Boersma, 2013).

5.4.1. Change in plasma hormones in males and females

In penguins an increase frequency of blood collection may adversely affect the ovulatory and oviposition processes (O'Brien *et al.*, 2016). An understanding of basic hormonal profiling will help the developing plan to conserve the genetic pool of the African penguin species. Blood sampling was not frequent enough in the present study to determine whether in African penguin the same pattern is followed which is evident in many other avian species. Our data is not sufficient to make a full comparison but is reliable as a base study of increases and decreases during the breeding season and non-breeding seasons.

In this study we report the findings on the reproductive hormone levels underlying the distinction of breeding and non-breeding African penguins. Both male and female showed the highest level of blood plasma testosterone through the breeding seasons and lowest during the non-breeding seasons. This difference in testosterone levels is in agreement with results from studies on Great rhea, Humboldt penguins, Yellow-Eyed penguin and also with those in Emperor and Adelie penguins (Valdez *et al*., 2014; Otsuka *et al*., 2004; Cockrem *et al*., 1994). In Macaroni, Gentoo and King penguin the plasma levels of testosterone increased during nest building and copulation stages (Williams., 1991; Mauget *et al*., 1994), then declined and remain low for the rest of the

breeding cycle. The peak in testosterone level in Yellow-Eyed penguins male was reported during pre-egg laying phase and is consistent with the pattern observed in this study (Cockrem *et al*., 1994). The fluctuation of testosterone suggests the seasonal change on reproductive organs in accordance with their mating system. In this study where we only looked at the adult nesting penguins, our prediction will be that no fluctuation will be observed in the blood plasma testosterone levels in juvenile males and females.

A unexpected finding of this study is that female penguins had slightly higher plasma level of testosterone than males in the breeding seasons, in contrast with what is reported in Magellanic penguins and Humboldt penguins (Fowller *et al*., 1994; Otsuka *et al*., 2004) but in agreement with female Macaroni penguin (Williams., 1991). The increase in female testosterone of Humboldt penguins (*Spheniscus humboldti*) during the breeding seasons was also observed by Otsuka *et al*. (2004). An explanation for the high testosterone blood plasma levels may be that the nests of this species are highly exposed to predators, therefore, reproductive penguins require high testosterone to activate masculine copulatory and aggressive behaviour for protecting either their eggs or chicks as both sexes participate in nest guarding (Cockrem *et al*., 1994; Williams, 1991). These results provide compelling support for the claim that testosterone regulate dominance and aggressiveness in penguins. This observation in African penguin is in agreement with that of ratites (Valdez *et al*., 2014), with the exception of kiwi, in which the level of testosterone drop when incubation begins (Potter *et al*., 1992). Blood plasma level of testosterone in both sexes showed large individual variation during the different seasons. The study on hormonal response behaviour have been reported before (Fowler, 1999), reporting that penguins need to be exposed to a very high levels of human visitation in order not to respond to human presence. Literature indicates that dominance behaviour in different spceies is influenced by testosterone (Mehta *et al*., 2010). In animals testosterone levels are positively related to aggressive and especially when social status is threatened

(Anestis, 2006; Archer, 2006), thus we can suggest that the individual difference is caused by the mechanism of defense that penguins show during the breeding season. This is in agreement with the findings by Valdez *et al*. (2014), stating that the monogamous species exhibit high testosterone levels only during territorial establishment and mate selection, with low testosterone levels during incubation and parental care.

The DHT levels of both sexes did not fluctuate for both seasons as we observed in other steroid hormones. There is a general lack on information from the literature on the role of DHT in avian species. Females showed to secrete more DHT than males, but no different observed in both species for different seasons. These results correlate with testosterone levels. We can speculate that females secrete more DHT as they have to develop protective mechanism (aggressive behaviour) as early as nest building. In males DHT plays a sufficient role to support courtship behaviour (Wingfield *et al*., 1978).

The plasma concentration of estradiol in both sexes was potentially higher in breeding seasons than the non-breading season, but there was no significant difference observed. African penguins breed throughout the year, therefore the increase of steroid hormones is not the direct response to increase in daylight as it has been reported in many birds. Studies on other penguin species reported that estradiol together with testosterone always increase at the same sample period (Otsuka *et al*., 2004). In Macaroni penguins the plasma level of testosterone in males was higher relatively to estradiol at the beginning of the breeding seasons (Williams, 1991), in this study we couldn't test these results. An increase in steroids hormones early in the breeding seasons in this study are in agreement with studies in Adelie penguins, where high levels of testosterone and estradiol early in the breeding season was reported to be associated with competition for nesting site in the colony (Otsuka *et al*., 2004).

Testosterone and estradiol in a study of the white-crowned sparrow were reported to have a combination effect in promoting growth of the oviduct. The increase or the elevation of estradiol in penguin males is not known, but Fowler *et al*. (1994), suggest it may be associated with control of copulatory behaviour. The observations done in Great Rhea and Ratites, except in kiwi, showed that estradiol levels were higher during pre-egg laying and then decrease after the first egg laid (Valdez *et al*., 2014) and similar results were observed in Magellanic penguin and in Yellow-Eyed penguin (O'Brien *et al*., 2015). This increase in estradiol is known to influence the synthesis of yolk protein by the liver, as was the case in Macaroni and Adelie penguins. In our study no significant difference was observed between the two sexes which is in agreement with what was discovered in Macaroni penguin (Williams, 1991). In contrast, Fivizzani *et al*. (1986), reported that estradiol levels were four times higher in females than in males. Although other avian species show a similar pattern between estradiol and testosterone levels, this study observed elevated estradiol levels for both sexes, this however is more complex to explain. We can only speculate that the elevation of estradiol in African penguin indicate the beginning of folliculogenesis.

5.4.2. Change in faecal hormones in males and females

To our knowledge, the faecal measurements of steroid hormones in African penguin have never been reported on before. The information on androgen metabolites in avian species is limited and very few studies have measured the level of androgen metabolites in avian species (Ninnes *et al*., 2010; Goymann *et al*., 2002). To be able to reliably assess androgen metabolites in this species, we provided for the first time a base study using Enzyme Immunoassays (EIA). In this investigation testosterone in faecal samples was only limited to males, these results were similar to that reported for blood plasma levels in this study and to other penguin studies (Wingfield *et al*., 1978; Williams. 1991). In these cases a significant difference was observed in blood plasma levels but not in faecal testosterone metabolites during breeding or non-

breeding periods.

In females, faecal levels of estrogen and progestogen increased before egg laying similar to that in Magellanic penguin (O'Brien *et al*., 2015); this increase is suggested to activate female receptive parental care (Schilinger *et al*., 2001a). Schilinger *et al*. (2001a), also found that in adult females, estradiol is elevated when females are laying eggs, whereas progesterone is elevated in some species during incubation. The findings in Adelie penguins of plasma concentration of estrogens and androgens drastically decline from courtship to clutch completion and then remain stable (Ninnes *et al*., 2011). The increase in progesterone levels in females throughout the breeding season may be for maintenance of incubating the eggs.

5.4.3. Relationship between seasonal changes in hormone levels and reproductive anatomy

The increase in steroids hormones correlates with changes in macro-and micro-anatomy and also the presence of spermatids and mature follicles. This could be due to the seasonal change and age dependent in African penguin, but we could not test these hypothesis in this study. Although we did not investigate the seasonal changes in juvenile we suppose that male and female African penguin juveniles will not show seasonal changes in sex steroids throughout the year due to their sexual immaturity. In male penguins the presence of LC which secrete the testosterone in the breeding seasons is in analogy with the elevation of testosterone in male penguins. The fluctuations reveal the seasonal changes in gonadal function in accordance with penguin mating system. The important results in both male and female penguins are that the level of testosterone remains high during breeding season. This implies that even while incubating, penguins may still need to trigger defensive behaviour for protecting either their clutch or their brood, thus requiring testosterone levels to remain high (Valdez *et al*., 2014). The results indicated that the highest estradiol levels of the female African penguins coincided with the pre-egg laying period. The presence of

small follicles are indicative of the start of the breeding season, thereby agreeing with findings reported for the female ostrich by Degen *et al*., (1994).

5.5. Semen characteristics

Seminal traits in this study were characterised using 51 ejaculates from two African penguin males. The males were between four to six years old and were not paired during the semen collection period. Our results showed that the interval of sperm collection in the long breeding season was 14 days while in the short breeding season was 7 days, compared to 39 days in paired Magellanic penguins (O'Brien *et al*., 1999) and 21 days in unpaired males (O'Brien *et al*., 2016). African penguins had shorter sperm collection period than Magellanic penguin. Due to the use of unpaired males, egg production did not mark the termination of semen collection. Semen collection was terminated because penguin used in the study couldn't produce semen any more.

Semen collection was performed with the use of an abdominal massage technique, which is a cooperative and is a non-invasive procedure. The collection of semen samples was done by a familiar handler as presented previously (Waldoch *et al*., 2012), and which minimizes discomfort and stress in males. The semen was maintained close to body temperature to prevent a rapid change in temperature. It has been reported that sperm exposed to higher temperature would be functioning at a higher metabolic rate and would be more likely to deplete nutrients and build up metabolic waste (Garron, *et al*., 1952).

The seminal characteristics based on the two males in this study revealed that African penguin produced high quantity sperm. No difference could be made in colour for the two penguins. Ejaculate volume, sperm concentration and number of sperm in the ejaculate varied across the weeks and the seasons of semen collection with no clear indication when was the sperm quality at its peak. Ejaculate volume of the African penguins in this study (0.01-1.10 ml), was similar to that of Rockhopper (*Eudyptes*

chrysocome chrysocome) (0.01-1.10 ml) reported (Waldoch *et al*., 2007), but lower than the Magellanic and King penguins (0.356 ml and 0.360 ml respectively) (O'Brien *et al*., 2016; O'Brien *et al*., 1999; O'Brien *et al*., 2014). The mean sperm concentration in this investigation (3274.7 x 10^6/ml) was considerably higher than that of Magellanic penguin (114.6 x 10^7/ml) and King penguin (16.4 x 10^8/ml) (O'Brien *et al*., 2016; O'Brien *et al*., 2014). The total number of spermatozoa in African penguin ejaculates (164.0 x10^6) was higher than that found in Magellanic penguin (80.3 x 10^6), King penguin (5.6 x 10^6) and both the southern Rockhopper and northern Rockhopper penguin (1.7 x 10^7 and 16.3 x10^6) respectively (O'Brien *et al*., 2014; Waldoch *et al*., 2007 O'Brien *et al*., 2016; Mafunda *et al*., 2017). The number of sperm in the ejaculate was higher in African penguin than other species in the order sphenisciformes. The limitation in this study was the small sample size. However, we recommend that more individuals be assessed in future for determination of peak semen volume and sperm concentration values during the breeding season.

5.6 Sperm motility

This study compared the sperm selection techniques, media, assessment time, individuals and seasons in terms of motility traits recorded for freshly ejaculated African penguin spermatozoa. One of the basic steps in objective analysis of sperm motility is the subdivision of a motile sperm population into slow, medium and rapid categories based on their velocity. Motility is a common feature of spermatozoa, especially for flagellate spermatozoa, and is an essential sperm characteristic for both internal and external fertilizing species (Holt *et al.*, 2004). During sperm transport through the female reproductive tract, mammalian spermatozoa display two types of physiological motility, namely activated motility and hyperactivated motility (Suarez *et al*., 2003b). Activated motility is seen in freshly ejaculated spermatozoa, where the flagellum generates a symmetrical and low amplitude waveform that results in the spermatozoon swimming in a relatively straight-line. On the other hand, hyperactivated motility, is

commonly seen in spermatozoa recovered from the site of fertilization. The role of activated motility is currently proposed as necessary in propelling the spermatozoa from the site of semen deposition to the oviduct. Higher sperm motility has also been related to increase in vitro fertilization rates and pregnancy rates (Dhurvey *et al*., 2012; Sifer *et al*., 2006).

Sperm motility is one of several sperm traits that provide an indication of the overall viability and functionality of a semen sample. In the field of reproductive biology sperm motion is considered crucial in semen analysis because normally, only motile spermatozoa can move through the barriers of the female reproductive tract to fertilise an egg (Farooq *et al*., 2016). The main objective on sperm motility assessment in the current study was to develop techniques to sample semen and determine parameters that define high quality spermatozoa. Semen was treated or exposed to different media and the analysis was done over time and compared over different breeding seasons. Sperm functionality was evaluated by the measurement of the percentages of sperm motility classes and the sperm swimming characteristics using different techniques to select motile spermatozoa in African penguin. The discussion below will focus on motile spermatozoa.

The average percentage total motility of African penguin spermatozoa in this investigation (72.1%) was similar to that of Magellanic penguin (78.0%) (O'Brien *et al*., 2016), lower than that of King penguin (85.3%) (O'Brien *et al*., 2014), and higher than that of northern Rockhopper penguin (59.8%) (Mafunda *et al*., 2017) as well as the southern Rockhopper penguin (34.5%) (Waldoch *et al*., 2007). At this stage it is not clear why Rockhopper penguin sperm motility is considerably lower compared to other penguin species. The sperm kinematics parameters of African penguin (VCL 76.7 µm/s, VSL 45.3 µm/s and VAP 58.9 µm/s), were lower compared to that found in King penguin and Magellanic penguin (O'Brien *et al*., 2014; O'Brien *et al*., 2016). Moreover, this study highlighted the importance of reporting subpopulations of motile sperm, such

as rapid swimming sperm, rather than using only the total motile population.Subpopulation swimming spermatozoa had higher mean values for all sperm kinematics parameters compared to the mean kinematics of all motile spermatozoa. The use of subpopulation compared to mean average favours the selection of the most motile spermatozoa in a sample and gives an indication of highly motile spermatozoa that are potentially closer to the point of undergoing capacitation (Boshoff *et al*., 2018).

The use of a swim-up technique to separate or isolate populations of highly motile sperm is well described in previous studies (Jameel 2008; Mehmood *et al*., 2009). A disadvantage of this technique is that it is time consuming. This may have a negative impact on sperm viability, therefore, there is a needs for the development of other separation techniques. In this study the flush technique is used as a complimentary technique, to evaluate sperm motility very rapidly due to the simplicity of the technique (Boshoff *et al*., 2018).

Comparing the swim-up and flush technique the swimming speed classes and most of the kinematics parameters obtained with the swim-up resulted favorably to those obtained using the flush technique. The percentage total motility, swimming speed classes (rapid and medium) and sperm kinematic parameters (VCL, VSL and VAP) indicated higher values when using the swim-up technique. Therefore, in this study it can be suggested that the swim-up technique gives a better motile sperm sample than the flush technique. The swim-up can be preferred because of its ability to maximize motile sperm recovery. Our results are in agreement with the study by Jameel (2008), in that the swim-up technique not only recovers motile sperm but also the fast forward-swimming motile spermatozoa. The percentage of actively motile sperm observed in this study ensures the reliability and efficiency of both techniques, TM and VCL had the mean of >60 % motile sperm. These techniques omit the initial centrifugation like in double density centrifugation gradient while separating the sperm and that help in

avoiding sperm damage. In flush technique spermatozoa are gradually exposed to the medium present and it is more realistic approach to studying spermatozoa without adding another variable. These techniques reduce the risks without affecting the outcomes as our results displayed improved sperm motility as shown by the swim-up technique. The results in this study indicated that swim-up as a technique need less expertise to separate motile sperm and it removes the non-motile spermatozoa. The fast swimming sperm are good for insemination as they have a better ability to fertilize (Pasqualatto *et al*., 1999). Although swim-up technique had higher values for certain motility parameters, flush technique allow for a more rapid assessment of sperm motility. Motility analyses by using samples subjected to the flush techniques can possibly contribute to shortening the time lapse from collection to the recording of sperm motility. The flush technique can be used as an alternative to the more time consuming swim-up technique in the preparation of sperm samples for motility analysis in African penguin.

The chemical environment and the physical environment are reported to have a profound effect on sperm motility (Zhou *et al*., 2015). The sperm movement pattern is therefore depending upon the environment it is exposed to (Suarez *et al*., 2006). Hyperactivation of sperm is characterized by a vigorous movement, playing a vital role in penetration of the zona pellucida (Suarez *et al*., 2003a). Hyperactivated motility sperm become extremely active as they gain the ability to fertilize oocyte in vitro (Yanagimachi, 1981). In this study we used three different media to potentially induce sperm hyperactivation, and to observe the effect over time.

The hyperactivation of sperm has been reported in mammalian species (Suarez. 2008), but there are no studies reporting on hyperactivation in avian species. In this study we revealed that all three media used appeared to not induce hyperactivation at the concentrations of chemicals used. African penguin sperm stimulated with all the media including those that traditionally cause hyperactivation; Hams F10 showed no

significant difference for all the sperm kinematics. Studies on human (Sharma *et al*., 1996), indicate that 5 mM caffeine to induce the sperm kinematics but in our current study with a higher concentration (8 mM) no hyperactivation was observed and no changes in sperm kinematics were observed. Procaine stimulates hyperactivation in mammalian spermatozoa, but in this study no hyperactivation observed. It is assumed that the role of procaine is to increase the plasma membrane's permeability to calcium (Suarez, 2008). Since the hyperactivated motility appears to be critical to the process of fertilization *in vivo*, African penguin sperm seems not to hyperactive. This might be because the mid-piece in avian sperm do not seem to undergo bending like in mammals and appears stiff and inhibiting mid-piece bending.

The effect of time and different seasons on sperm motility was evaluated in order to determine if there was a difference between the two African penguins in this study and to suggest the standardized procedures for future studies. The effect of time and different seasons on sperm motility showed no differences between the sperm kinematic parameters captured at 0 min, 15 min, 30 min, 45 min and 60 minutes. The trend increase value observed was that of TM, VCL, VSL and VAP after 30 minutes.

5.7. Cryopreservation

The conservation efforts and natural breeding efforts in combination with assisted reproductive technologies (ART) such as semen cryopreservation and artificial insemination (AI) have the potential to enlarge the genetic pool of captive African penguin populations, ensuring sustained healthy wild population. These techniques have been successfully used primarily in breeding programs of rare and endangered species and in the production of hybrid falcons for the sport of falconry (Samour, 2004). Furthermore, it has been proven that the semen stored from founder members of a population can be used to maintain genetic diversity and equalize their genetic contribution to the gene pool after numerous generations. A protocol for

cryopreservation of African penguin has not yet been optimized. The freezing methodology developed in this study provides a foundation for sperm banking and facilitate future research towards developing artificial insemination in the African penguin We recommend that different cryprotectant media as well as a higher sample size be used in future for studies in cryopreservation. The semen extender sustains sperm viability for several hours. The most commonly accepted method of semen preservation is cryopreservation in liquid nitrogen at −196ºC for prolonged storage. Glycerol, commonly used as the main cryoprotectant for the preservation of the sperm in most animals, including the stallion, ram and the fowl (Mphaphathi *et al*., 2016), proved to hamper fertilization in poultry (Herrera *et al*., 2005).

The study show that African penguin sperm can be stored for at least two hours in liquid nitrogen using triladyl as a cryoprotectant. Although the addition of triladyl decrease the TM and all the sperm kinematics, it can still be used as a cryoprotactant for African penguin semen (Rekha *et al*., 2016). The TM of sperm decreased from 60.0% before freezing to 27.1% after thawing in penguin 1, while in penguin 2 TM decreased from 61.9% before freezing to 16.8% after thawing (Table 4.16). The semen can therefore be transported if is prepared and chilled, making the management of this endangered species easier. The freezing of spermatozoa followed the protocol made by Waldoch *et al*., (2007), suggesting that temperature must be slowly lowered to 5-10ºC after the diluent has been added and then proceeds with freezing in liquid nitrogen.

In this study approximately one-third to half of the initial population of semen survived the deleterious effects of cryopreservation, a result comparable with those previously observed in fowl (Chalah *et al*., 1999). In general during cryopreservation spermatozoa undergo membrane damage which may induce earlier apoptosis (Graham, 2001). Our findings reveal that after cooling but before freezing, swimming sperm classes and sperm kinematics parameters were higher compared to after freezing. The

maintenance of high sperm quality at 0 minutes (37ºC) and 30 minutes (5 ºC) than at two hours at -196ºC, reflects similar results for chicken (Clarke *et al*., 1982) and Emu sperm (Sood *et al*., 2012), where progressive motility were higher after short storage and at low temperature. This observation was also reported by Ciereszko *et al*. (2010) where the percentage of motile sperm in semen stored for short storage did not change, however, longer storage of four hours resulted in significant decrease in the number of motile sperm. All the kinematics, with the exception of BCF showed similar pattern in decrease over time. The current study showed that the addition of cryodiluent and freezing process significantly impacted on sperm characteristics. Both penguins indicated that up to 30% of spermatozoa display collective losses in motility during the freeze thawing process. The current study confirmed the results on King penguins and Magellanic penguin and also in turkey (O'Brien *et al*., 2014; O'Brien *et al*., 2016; King *et al*., 2000), that liquid nitrogen of avian semen at 5ºC is typically associated with decrease compared to non-stored semen. Donoghue *et al*. (1999) also reported that during chilled storage, turkey sperm resulted in a decrease from 88% to 73% after 24 hr. storage. A study on *Alectoris rafu* clearly indicated that sperm kinematics (VCL, VSL, VAP, LIN, STR, and WOB), were negatively affected when extender was added (Santiago-Moreno *et al*., 2017). This results in a decrease in plasma membrane integrity and subsequent degeneration of the sperm (Waldoch *et al*., 2007). In poultry sperm the cellular activity of the sperm that ceases at cryopreservation, resumes after thawing and freezing and have been found to damage or kill in sperm (Madeddu *et al*., 2010). The changes in ATP levels and pH after sperm has been exposed to different temperatures reduce motility and viability (Sood *at el*., 2012). Therefore, this study supports the findings that with subsequent storage, sperm quality was strongly influenced by the temperature with adverse outcomes evident after thawing (Mphaphathi *et al*., 2016). Alternative cryoprotectants used for avian semen have been dimethyl acetamide and DMSO and these cryoprotectants were selected in terms of their low molecular weight and toxicity when used at low temperatures (Mphaphathi *et*

al., 2016).

In African penguin, the motile spermatozoa assessed after cryopreservation probably have the ability to penetrate the vitelline membrane, as CASA showed kinematics parameters similar to some avian species before cooling (Clarke *et al*., 1982; Santiago-Moreno *et al*., 2017). The variation between two penguins maybe because we sampled during the different seasons, but the other methods in this study showed that there was no significant different in both seasons. A high percentage of motile sperm was observed at 0 and 30 minutes after adding triladyl.

Our findings in the African penguin are encouraging as they indicate that semen cryopreservation after two hours still show fairly good sperm kinematics parameters. This was in agreement with the findings in Venda chickens, Masindi *et al*., 2016, who reported no loss in the fertilisation capacity of semen stored *in vitro*, at 2–5 º C. We can hypothesize that a population of frozen-thawed spermatozoa remain fully functional after thawing, and are able to survive in the female reproductive. It will be interesting to monitor the thawed semen and do AI in order to demonstrate the egg production, ultimately production of chicks and compared with reports from naturally breeding African penguin. This similar study was done on King penguin were they successfully used frozen to freeze avian semen to and produce offspring after AI using cryodiluents containing glycerol (O'Brien *et al*., 2014).

5.8. Sperm vitality

The integrity of the sperm membrane is a vital prerequisite for cell survival. The plasma membrane defines the cell boundary around the sperm cytoplasm while maintaining the ability of sperm to interact with its surroundings. An intact sperm plasma membrane is required for sperm viability and function (Salisbury *et al*., 1978). Avian sperm vitality is typically assed after eosin-nigrosin staining and preparation of sperm smears (Bask *et al*., 1997). The sperm viability was maintained in both African penguins used in the

present study and also during the different seasons of the reproductive cycle. Overall, we concluded that a longer or shorter breeding season does not have an effect on viability of sperm with sperm samples evaluated recorded high percentages of spermatozoa with the plasma membrane intact. The low percentage of dead sperm in the study indicate the high viability of spermatozoa irrespective of their motile effectiveness during motility analysis. The intact plasma membrane provides a good suggestion of sperm quality and only viable sperm can undergo capacitation (Yanagimachi, 1981). As previously describe for many species (Salisbury *et al*., 1978; Sood *et al*., 2012), eosin-nigrosin staining staining proves to be very useful for evaluating viable/vitality sperm and the results obtain can be correlated with total sperm motility in order to have an idea of how much percentage can be used for insemination.

5.9. Sperm morphology

Morphology is an important criterion for evaluating the quality of a semen sample. Sperm morphology is thought to be involved in post-copulatory selection during sperm competition and fertilization (Møller *et al*., 2008). There is relatively little data available on sperm morphology and ultrastructure in the order Sphenisciformes, as well as other non-passerine birds, which makes it difficult to compare morphological components of penguin spermatozoa within the group or to other avian groups. Despite the few published studies on sperm ultrastructure of non-passerine birds, with most recorded for roosters, turkeys, and quails (Asa *et al*., 1986; du Plessis *et al.,* 2011; 2012; Grigg *et al*., 1949; Korn *et al*., 2000; Marqueze *et al*., 1975; Soley and Roberts, 1994), there are no previous reports on the occurrence of multiple axonemes within one sperm plasma membrane. Sperm morphology and morphometry are believed to be important in sperm evaluation since artificial reproduction techniques were first developed (Santiago-Moreno *et al*., 2016). Numerous studies have found a close relationship between sperm morphology and fertility, with positive correlations reported between

percentage normal sperm morphology or size and fertilization success (Enginsu *et al*., 1991; Łukaszewicz *et al*., 2008; Møller *et al*., 2008).

Sphenisciformes spermatozoa are morphologically similar to that of other non-passerine birds, with light microscopy revealing a filiform sperm structure as observed in ostrich and turkey (Bertschinger *et al*., 1992; Soley, 1992). The spermatozoa of non-passerine birds are of the so-called sauropsid type, as found in reptiles, being plain-surfaced and elongated (Tom, 2014). This study shows that African penguin spermatozoa consist of five components, namely the head (acrosome and nucleus), a neck and a tail consisting of the mid-piece, principal piece and end piece, in agreement with what was reported in the study of *Gallus domesticus* (Grigg *et al*., 1949). Light microscopic observations displayed filiform-shaped head structures and a distinct but small acrosome covering the anterior part of the nucleus. The head length of the African penguin (12.32 µm) similar to that of other species of Sphenisciformes, including Rockhopper penguin (11.57 µm), Gentoo penguin (12.5 µm) and King penguin (13.8 µm), (Mafunda *et al*., 2017; Santiago-Moreno *et al*., 2016) spermatozoa and falls within the range of tinamou and ostrich (10.95 µm), but are longer than that observed in the turkey (7-9 µm) (Soley *et al*., 1994; du Plessis *et al*., 2014). The sperm mid-piece of African penguins is noticeably similar to that of Rockhopper penguins (2.28 µm and 2.06 µm respectively) (Mafunda *et al*., 2017), but shorter than that of rhea, tinamou and emu (3.16 µm), as well as that of chicken, duck and turkey (4.0 µm, 3-4 µm and 4.8 µm respectively) (Soley *et al*., 1994; Asa., *et al*., 1985). The shorter mid-piece of African penguin (Nothling *et al*., 2008) compared to Emu (du Plessis *et al*., 2014) seem to be related to number of mitochondria it contains (du Plessis *et al*., 2014, 39). Mitochondria in the penguin sperm mid-piece could be clearly distinguished at light microscopic level, and were arranged in a single helical row around the proximal and distal centriole. The principal piece formed the longest segment of penguin spermatozoa, typical for non-passerine birds and similar to mammalian spermatozoa (Asa *et al*., 1985).

The total length of bird spermatozoa is reported to range between 30-300 µm (Santiago-Moreno *et al*., 2016). Even though there are only limited comparative data available for non-passerine birds, spermatozoa from these species appear to be similar in overall size**.** There are, however, slight species variation in total sperm length for ostrich (69.58 µm), rhea (65.00 µm), emu (67.64 µm), Rockhopper penguin (64.57 µm) and now found in African penguin (66.31 µm). In addition, the dimensions of each sperm component of non-passerine birds demonstrate uniformity, with sperm head length ranging between 11.57-13.60 µm, mid-piece length 2.06-3.16 µm and tail length 47.22-53.57 µm based on light microscopic measurements and electron micrographs (du Plessis *et al*., 2014, Mafunda *et al.*, 2017).

While ultrastructural features of normal and abnormal spermatozoa in several non-passerine birds have been described (du Plessis *et al*., 2011; du Plessis *et al*., 2014c; Asa *et al*., 1985; Maretta *et al*., 1979), no such information is available for the spermatozoa of African penguin. With only two African penguin birds included in this study and limitted sample size, it was not feasible to determine the percentage morphologically normal spermatozoa in each ejaculate. However, with light and electron microscopy we did identify spermatozoa with coiled tails, similar to a tail abnormality described by du Plessis and Soley (du Plessis *et al*., 2011).

Penguin sperm ultrastructure revealed an elongated head structure narrowing gradually at its most anterior aspect, with the acrosome sitting on top of the nucleus. The sperm tail of the penguins was clearly divided into three regions, namely, mid-piece, principal piece and short end-piece. The neck contained a capitulum which gives rise to banded columns. The neck region consists of a pair of centrioles and nine segmented columns (connecting piece) that appear to merge with nine outer dense fibers of the rest of the tail. Proximal and distal centrioles could be clearly discerned in both species and aligned at 90º to each other. The sperm mid-piece in most non-passerine birds is short in length and mitochondria are elongated and arranged around

the proximal and distal centrioles of the sperm tail in a helical fashion (Asa *et al*., 1985). In the African penguin, sperm mitochondria contained flattened cristae arranged in a helical pattern in the mid-piece. The mid-piece ended at the annulus which was identified as a distinct dense ring in the inner part of the cell membrane. The sperm principal piece started at the annulus and is surrounded by a fibrous sheath, as was also described in the tinamou, rhea, ostrich, domestic fowl, Japanese quail, and duck (Wooley, 1995; Asa *et al*., 1985; Jamieson, 2007; van der Horst *et al*., 2011; 2014). One function of the fibrous sheath is to give structural strength during flagella beating in a viscous medium as encountered in the female reproductive tract (van der Horst *et al*., 2014). The sperm end piece consisted of the axoneme covered only by the plasma membrane.

The penguin sperm flagellum, contained the axoneme comprising of the typical 9+2 microtubular configuration surrounded by a fibrous sheath. The three functional components observed in the mammalian axoneme, as suggested by (Lindemann *et al*., 1992) for rat spermatozoa, are probably also operational in penguin spermatozoa: i) a medial 3-central-8 partition formed by the central pair linked to outer doublets 3 and 8; ii) the doublets 9, 1 and 2 that slide as a group through the interaction of doublet 2 with doublet 3 of the central partition; and iii) the 4, 5-6, 7 doublet group that slide as a group on the central partition. The 3 and 8 arrangement gives one plane of the flagellum maximal stiffness to produce the coordination of multiple axonemal structures that beat as a unit (du Plessis *et al*., 2011). Our results show a similar arrangement of the penguin sperm axoneme and potentially the limited sliding to only certain doublets during each of the beating cycles.

A significant finding not previously reported for penguins and in birds, is the presence of multiple axonemes in 4% of spermatozoa in all the African penguin samples studied. Due to the general lack of detailed descriptions of avian spermatozoa with regards to multiple axonemes, it is difficult to compare this finding to similar structural anomalies

found in other birds. In a study on emu spermatozoa, it was reported that there are two axonemes within one cell membrane at the anterior end of the flagellum which splits up to form a biflagellate tail (Asa *et al*., 1985). Although the incidence of multiple or double tails has also been reported in a subsequent study on the emu (du Plessis *et al*., 2011), there is no information on double, triple or quadruple axonemes surrounded by one plasma membrane in the entire length of the sperm tail as described in the current study. It is impossible to state whether such multiple axonemes may give spermatozoa an advantage to swim faster. A more expanded description of avian spermatozoa through ultrastructural studies in relation to double axonemes is therefore required in support of this unusual finding.

We speculate that the multiple axonemes discovered in this study may be due to one of the following: i) a mutation that has no effect on sperm function, ii) a mutation that may give spermatozoa an advantage to swim faster due to multiple axonemes or iii) the lack of sperm competition in these penguin species. The fact that the double, triple and quadruple axonemes were arranged in a manner which would theoretically still allow for the tail to bend in unison (similar arrangement of doublets 3 and 8 as indicated in Fig 4.33 B, E, H, K) could increase the force generated by the flagellum for forward motility and possibly result in faster swimming speeds than for normal single axoneme flagella. Faster swimming spermatozoa may eventually reach the oocyte first and thus ensure fertilization. On the other hand, the multiple axonemes might not reflect any advantage to individual spermatozoa and its presence could be a result of the penguin's breeding strategy. Sphenisciformes individuals are monogamous and are therefore considered to have a low risk for sperm competition. Santiago-Moreno *et al*. (2016) reported that the spermatozoa of monogamous species usually exhibit high sperm abnormality. According to a review by van der Horst *et al*. (2014), it will be extremely unlikely for spermatozoa from males of monogamous species to compete for fertilization of the oocyte and these species often display abnormal sperm features that could possibly be due to the lack of sperm competition.

5.10. Conclusion

This study provides previously unknown values for testicular volume, size and weight in the African penguin. With the availability of technologies which can accurately determine the sizes of reproductive structures, it is now possible to measure the sizes and weight of the testes and ovaries of African penguin. Within the breeding season among males and females, there usually was a good relationship between the rise of testosterone levels and the observation of the Leydig cells and the presence of spermatogenic cells as well as the mature follicles. The study reveals information on reproductive biology and on hormonal-sperm relationships. The increase in reproductive organs and increase in steroids hormones relates with sperm production. We can conclude that there will be greater spermatogenic availability leading to larger testes in African penguin breeding birds during breeding season.

This study reports on the semen characteristics and basic sperm structure of African penguins using various microscopic techniques. Sperm characteristics may be used as biological markers of environmental influences since germinal cells are very sensitive to them (Santiago-Moreno *et al*., 2016). This study, for the first time described the morphology, morphometry and ultrastructure of African penguin spermatozoa, which were found to be largely similar to that of ratites and other non-passerine birds. Ultrastructural sperm analysis revealed that 4% of sperm had multiple axonemes of spermatozoa in both penguins assessed.

This study shows that scientific intervention not only furthers our knowledge and understanding of ART, but can act as an intervention to halt the endangerment of species. Illustrated in Fig 4.34 are various intervening techniques, which will ultimately aim at promoting the gene pool and further reduce the mortality rate of the African penguin. The quick decline in the population of African penguin in the recent years may lead to the species extinction.

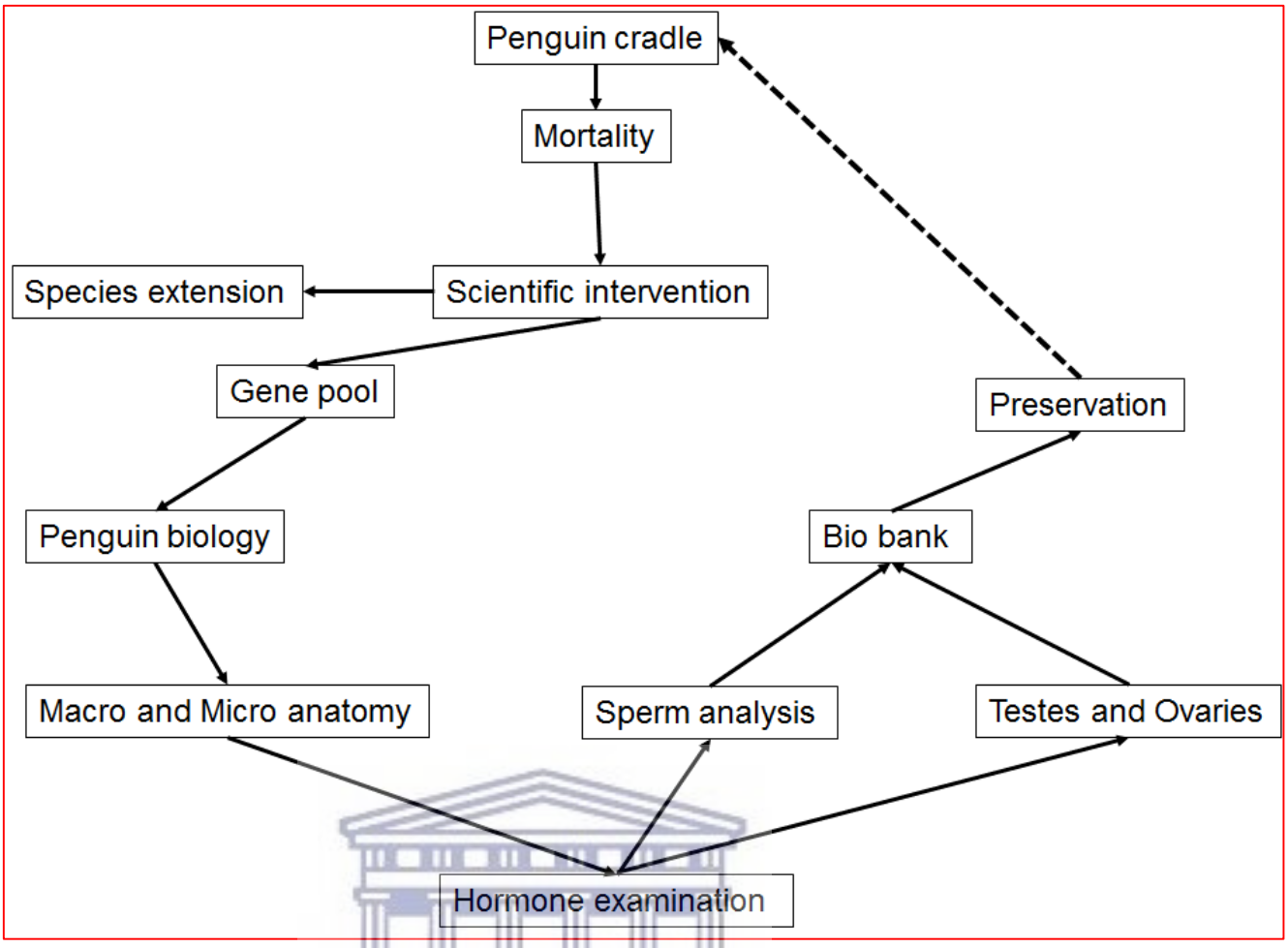

Figure 4.34: General summary and conclusion of the study.

Chapter 6: Future studies

The management approach to species conservation suggested by the BMP aimed to develop conservation strategies and action plans. This investigation present a new set of semen and sperm inception values that can be used as baseline values for future investigation in penguins. Furthermore, this study adds some meaningful insight into the reproductive biology of African penguin and some potential for the use of ART in conservational programs. The improved experimental protocols gained in this investigation should be used in future studies on other vulnerable species. The collective data can be used to further test the correlation between the aspects of reproductive biology (Endocrinology, macro and micro anatomy). To ensure the future success of African penguin populations combined with ART requires improvement and the collecting of basic reproductive data without delay. The routine application of such technologies is still not available for African penguin. To date no ART innovations have been made in African penguin mainly due to the lack of basic information; however, successes have been achieved during ART interventions in other species of penguin (O'Brien *et al*., 2014; O'Brien *et al*., 2016).

The semen samples from additional males are required to make quantitative species comparison, but the current study indicate that African penguin had a high quality of sperm. In an effort to understand species reproductive biology it is important to take into thought the normal morphology and functions of the species' reproductive system. Techniques used in the current study to analyse the semen parameters in African penguin should be used in avian studies to identify any abnormalities. Since a great deal of baseline information has been provided on African penguin reproduction, the focus should now incorporate factors such a food availability and other environmental

factors that may threaten breeding and survival of this unique species.

Chapter 7: Reference

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APPENDIX

Appendix A

REPORT **ScPGC2013/6R**

University of the Western Cape

FACULTY OF SCIENCE
POST GRADUATE COMMITTEE
REPORT TO SHD OF A MEETING OF THE ABOVE-MENTIONED COMMITTEE THAT WERE HELD ON THURSDAY 18 JULY 2013 AT 13H15 IN THE NEW LIFE SCIENCE BUILDING 5TH FLOOR, DEAN'S COMPLEX.

1 APPOINTMENT OF SUPERVISORS AND REGISTRATION OF THESIS/MINI-THESIS/RESEARCH REPORT TITLE

1.1	**Student name:**	**Ms Shana Sparks** **ScPGC2013/06/01**
	Student number:	2514123
	Degree:	MSc
	Department/Institute:	BCB
	Title of thesis/Mini-thesis:	Blood cell histology and haemoparasites of *Homopus areolatus*; effects of season and cohort.
	Keywords:	Differential counts, erythrocytes, erythropoiesis, histology, leukocytes, thrombocytes, tortoises.
	Supervisor:	Prof MD Hofmeyr
	Co-supervisor:	N/A
Faculty recommendation:		**That the thesis title be registered.**
1.2	**Student name:**	**Mr Isreal Olonade** **ScPGC2013/06/02**
	Student number:	3371729
	Degree:	PhD
	Department/Institute:	BTY
	Title of thesis/Mini-thesis:	Development of a high level throughput method for identifying phage-host pairs in an extreme environment.
	Keywords:	Bacteriophage, phage-host interactions, microbial diversity, metaviromics.
	Supervisor:	Prof Marla Tuffin
	Co-supervisor:	N/A
Faculty recommendation:		**That the thesis title be registered.**
1.3	**Student name:**	**Mr Walter Nevondo** **ScPGC2013/06/03**
	Student number:	3165907
	Degree:	PhD
	Department/Institute:	BTY
	Title of thesis/Mini-thesis:	Development of a high throughput cell-free metagenomic screening platform.
	Keywords:	Metagenomes; cell-free protein synthesis, *in vitro* compartmentalisation, Lignocellulose enzymes.
	Supervisor:	Prof Marla Tuffin

Appendix B

National Research Foundation | NZG National Zoological Gardens of South Africa

PO Box 754
Pretoria, 0001
South Africa
Tel: 012 328 3285
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Int. Code: +27
info@nzg.ac.za

www.nzg.ac.za

NZG/ P13/07

11 November 2013

Mr Siyambulela Patrick Mafunda
University of the Western Cape
Private Bag X17
Bellville
7535

Dear Mr Siyambulela Patrick Mafunda

APPROVAL OF RESEARCH PROPOSAL

This letter serves to inform you that your research proposal P13/07 "Aspects of reproduction of male and female African penguin (*Spheniscus demersus*) with special reference to sperm biology and cryopreservation" has been approved by the NZG Research Ethics and Scientific Committee (RESC) on October 30, 2013 with the following provisos:

1. The following changes need to be made and the proposal re-submitted:
   - There were deleted citations, this is not the correct way of fixing the document.
   - The quality of the application in terms of grammar and spelling is not good hence language editing is recommended.

Thank you for making use of the NZG as a research platform.

Yours sincerely

Prof Antoinette Kotze
Chair: NZG Research Ethics & Scientific Committee

Appendix C

Invalid without attached conditions

STANDING PERMIT

(Issued in terms of the provisions of the National Environmental Management: Biodiversity Act 2004, Act 10 of 2004)

PERMIT NUMBER: S 02507

NAME OF ISSUING AUTHORITY	
NAME	Dept. of Environmental Affairs
ADDRESS	P/Bag X 447
	Pretoria 0001
PROVINCE	National

	PROVINCIAL DEPARTMENT	NATIONAL DEPARTMENT	
	PROTECTED AREA MANAGEMENT AUTHORITY	VETERINARIAN	
X	REGISTERED CAPTIVE BREEDING OPERATION	REGISTERED SCIENTIFIC INSTITUTION	X
	REGISTERED SANCTUARY	REGISTERED REHABILITATION FACILITY	
X	REGISTERED COMMERCIAL EXHIBITION FACILITY	REGISTERED GAME FARM	
	REGISTERED WILDLIFE TRADER	REGISTERED NURSERY	

UNIQUE REGISTRATION NUMBER M34 | 530115 5053082

DETAILS OF PERMIT HOLDER		
NAME	National Zoological Gardens of South Africa	ID NO.
SURNAME	Biodiversity Conservation Centre (Pretoria)	PASSPORT NO.
	POSTAL ADDRESS	RESIDENTIAL ADDRESS
ADDRESS	P.O. Box 754	232 Boom Street
ADDRESS		
ADDRESS		
TOWN	Pretoria	Pretoria
POSTAL CODE	0001	0002
PROVINCE	Gauteng	Pretoria

PROPERTY WHERE RESTRICTED ACTIVITIES WILL BE CARRIED OUT	
NAME AND SURNAME OF APPLICANT	Mr. Eugene Morais - National
NAME AND SURNAME: RESPONSIBLE PERSON	Zoological Gardens of SA
NAME AND SURNAME OF AGENT	
PHYSICAL ADDRESS OF FACILITY	
PHYSICAL ADDRESS OF FACILITY	232 Boom Street
DISTRICT	Pretoria
PROVINCE	Gauteng
REGISTERED NAME AND NUMBER (in the case of game farm)	

DETAILS OF SPECIES INVOLVED

SPECIES		SEX (if known)	QUANTITY	MARKING (if applicable)
COMMON NAME	SCIENTIFIC NAME			
Refer to Annexure 1 to this permit				

DETAILS OF RESTRICTED ACITIVIES INVOLVED

Breeding, capture, keeping (permanent or temporary on loan), selling, buying, donate, receive as donation, darting, euthanasia, conveying, import, export

PERMIT VALIDATION		
PERIOD OF VALIDITY	FROM: 19/12/2011	TO: 18/12/2014
RECEIPT NUMBER	No payment required	
SIGNATURE OF ISSUING OFFICER	DEPT. OF ENVIRONMENTAL AFFAIRS PRIVATE BAG X 447	SIGNATURE PERMIT HOLDER
DATE STAMP:	19 -12- 2011	

Appendix D

National Research Foundation | NZG National Zoological Gardens of South Africa

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11 September 2014

To Whom It May Concern,

CAPENATURE RESEARCH PERMIT APPLICATION TO COLLECT BLOOD, SEMEN AND TISSUE SAMPLES FROM SEABIRDS ADMITTED TO SANCCOB REHABILITATION CENTRE

The attached permit application follows correspondence with Ms Danelle Oelofsen in August 2014. As a postdoctoral researcher at the National Zoological Gardens (NZG) Centre for Conservation Science, I am conducting conservation genetic research on threatened seabirds in South Africa, and am collaborating with researchers from the University of Cape Town (UCT), the NZG and SANCCOB. Ms Portia Mothebe is a PhD student based in Pretoria at the NZG and Mr Siya Mafunda is a PhD student based at the University of the Western Cape (UWC) and registered with NZG. Their applications are merged with mine, as we are using many of the same samples and are likely to share these in the future. Many of the samples required have already been collected by Dr. Nola Parsons at SANCCOB, and we are requesting to utilize those samples towards different, but connected, research goals (Please see attached letter provided by Dr. Parsons below). Some of the necessary blood and semen samples will be collected from captive African Penguins and Rockhopper Penguins at the Two Oceans Aquarium (TOA).

Additional sampling that we are requesting will be carried out during 2014/2015, and involves seabirds that are admitted to SANCCOB during that time, and captive birds at the TOA and NZG. Because we cannot predict the exact numbers and species of birds that will be admitted to SANCCOB, we have stated "all birds admitted to SANCCOB" under section 2.5a (if you wish to place an upper limit on the samples collected there, I estimate about 100 African Penguins and ten of each of the other species). It is my understanding that SANCCOB staff routinely take blood samples from birds to monitor their health, so we are requesting that 0.1ml of this blood be stored in appropriate buffers to be used in our parasite and pathogen research. We will liaise with SANCCOB staff about the logistics of collecting, storing, sharing and transporting the samples. Additional applications for transport permits are attached, as some samples will need to be transported between the various institutions involved in this research.

Thank you for your time and for considering this application.

Sincerely

Lisa Nupen

SANCCOB
saves seabirds

Association incorporated under Section 21
Registration Number: 2001/026272/08
PO Box 11116, Bloubergrant, Cape Town 7443, South Africa
Physical Address: 33 Upper Orms, Table View, 7441
Telephone: +27 21 557 6155, Fax: +27 21 557 6804
Email address: info@sanccob.co.za Website: www.sanccob.co.za

23 April 2014

To Whom it may concern,

REGARDING THE TRANSPORT OF SEABIRD BLOOD SAMPLES BETWEEN SANCCOB AND UCT FOR RESEARCH PURPOSES

The samples in Table 1 below were collected by myself (Dr Nola Parsons) at SANCCOB under the following CapeNature permit numbers:

AAA007-00007-0035 (2006)
AAA004-000120-0035 (2007)
AAA007-00040-0035 (2008)
AAA004-00508-0035 (2010)
AAA007-00047-0056 (2013).

This letter serves to grant permission to Lisa Nupen to transport and utilize the samples for research purposes, as her name does not appear as a collaborator on the original permit.

For any queries, please do not hesitate to contact Dr Parsons (nola@sanccob.co.za) or Ms Nupen (lisanupen@gmail.com).

Species	Number
African Penguin	40
Reed Cormorant	2
Crowned Cormorant	2
White-breasted Cormorant	2
Bank Cormorant	1
Cape Cormorant	1

Thank you for your time.

Kind Regards,

Nola Parsons, BVSc., PhD.

SANCCOB Researcher

1. PARTICULARS OF APPLICANT: Lisa Nupen

1.1

Surname: NUPEN	**Postal Address:** Percy FitzPatrick Institute,
	Office 2.17, John Day Zoology Building,
ID Number: 8402100481084	University Avenue, Upper Campus,
First names: LISA JANE	University of Cape Town, Private Bag X3,
	Rondebosch, 7701
Citizenship: SOUTH AFRICAN	**Residential Address:** 35 Chelmsford Road,
	Vredehoek, Cape Town
	8001
Fax No. (w): +27 (0) 12 323 4540	Fax No. (h): n/a
Tel No. (w): 021 650 3300	Tel No. (h): 0725122143
URL/Website: http://www.fitzpatrick.uct.ac.za	E-mail: lisanupen@gmail.com
University / Institution / Department affiliated to: Centre for Conservation Science at the National Zoological Gardens (NZG, Pretoria) and the DST/NRF CoE at the Percy FitzPatrick Institute, University of Cape Town)	
Current occupation: Postdoctoral researcher	
Highest Qualification: PhD	

Degree(s)	Field of Study	Period
MSc	Conservation Biology	2006 - 2008
PhD	Zoology	2009 - 2013

1.2 Experience to date:

1.3 Details of **previous** research / collecting in the Western Cape Province*

Name of employee / institution	Capacity and nature of work	Period
Area(s) Stony Point, Boulders Beach, Robben Island, Jutten Island, Malgas Island	Research assistant	2007 - 2008
SANBI	Research Assistant	2008 - 2009
NZG	Contract staff / Postdoc	2013 - present
Species collected African Penguin		
When (year/years) 2009 - 2011		
Permit number(s) and date(s) of issue: AAA-004-00520-0035		
*Should this application be for a new permit or for the renewal of a previous permit then a list of **ALL**		

1. PARTICULARS OF APPLICANT: Portia Motheo

1.1

Surname: MOTHEO	**Postal Address:** 232 Boom Street
	Centre for Conservation Science
ID Number: 8210250647085	National Zoological Gardens of South Africa
First names: MAMOFALALI PORTIA	Pretoria
	0001
Citizenship: SOUTH AFRICAN	**Residential Address:** 402 Angelina Flat,
	Leyds Street, Sunnyside
	Pretoria, 0001
Fax No. (w): +27 (0) 12 323 4540	Fax No. (h): n/a
Tel No. (w): 012 339 2792	Tel No. (h): 0839375763
URL/Website: http://www.nzg.ac.za	E-mail: mmotheo10@gmail.com

University / Institution / Department affiliated to:

Centre for Conservation Science at the National Zoological Gardens (NZG, Pretoria) and the Department of Zoology and Entomology, University of the Free State, Qwaqwa Campus)

Current occupation: Doctoral Fellow

Highest Qualification: MSc

Degree(s)	Field of Study	Period
MSc	Medical Immunology	2010-2012
BSc Honors	Zoology	2005-2006

1.2 Experience to date:

Name of employee / institution	Capacity and nature of work	Period
Department of Zoology and Entomology, University of the Free State	Research assistant	2005
Sci-Enza (University of Pretoria)	Volunteer (Science Communicator)	2008 – 2009
Medical Research Council/ University of Pretoria	Research assistant	2010-2011
NZG	Contract staff / Doctoral Fellow	2012 - present

1.3 Details of **previous** research / collecting at the National Zoological Gardens of South Africa*****

Area(s): NZG BioBank
Species collected African Penguin, Rosy Flamingo, Scarlet Ibis, Red Billed Oxpecker, Waldrapp Ibis, Spotted Dikkop, Southern Ground Hornbill, Jandaya Conure, Cockatoos
When (year/years) 2013
Permit number(s) and date(s) of issue: NZG Research Project Number P10/16
*****Should this application be for a new permit or for the renewal of a previous permit then a list of **ALL** the material collected (including; the species name, the number collected, the collection date and the locality) with any **previous** permit **MUST** be submitted with this application, unless said list has not already been submitted.Should a report or other publication of any kind have arisen from a previous permit then a copy thereof must also be included. **ANY APPLICATION THAT IS NOT ACCOMPANIED BY A COPY OF SAID LIST OR REPORT WILL NOT BE PROCESSED**
FOR OFFICIAL USE ONLY To what extent have permit conditions been complied with:

1. PARTICULARS OF APPLICANT: Siyambulela Mafunda

Surname: Mafunda	**Postal Address:** Department of Medical Bioscience.
	New life science Building 4th floor.
ID Number: 8508295811081	University of the Western Cape
First names: Patrcik Siyambulela	Private Bag X17, Bellville 7535,
Citizenship: SOUTH AFRICAN	**Residential Address:** 32 Aurecle Crescent
	High bury Park, Kuilsriver 7580
Fax No. (w):	Fax No. (h):
Tel No. (w): 072 610 4372	Tel No. (h): 072 610 4372
URL/Website:	E-mail: siya.85mafunda@gmail.com

University / Institution / Department affiliated to:		
Centre for Conservation Science at the National Zoological Gardens (NZG, Pretoria) and the University of the Western Cape		
Current occupation: PhD. PDP research candidate		
Highest Qualification: MSc		
Degree(s)	Field of Study	Period
B Sc. Medical Bioscience (Honours)	Medical Microbiology	2009
MSc	Neurobiology)	2011-2012

1.1

1.2 Experience to date:

Name of employee / institution	Capacity and nature of work	Period
University of the Western Cape	Laboratory practical coordinator	January 2010 to June
Medical Research Council	Environmental Enrichment	May2008 to December 2008
NZG	PhD. PDP research candidate	May 2013- to date

1.3 Details of **previous** research / collecting at the National Zoological Gardens of South Africa*

Area(s):
Species collected
When (year/years)
Permit number(s) and date(s) of issue:
***Should this application be for a new permit or for the renewal of a previous permit then a list of **ALL** the material collected (including; the species name, the number collected, the collection date and the locality) with any **previous** permit **MUST** be submitted with this application, unless said list has not already been submitted.Should a report or other publication of any kind have arisen from

a previous permit then a copy thereof must also be included. **ANY APPLICATION THAT IS NOT ACCOMPANIED BY A COPY OF SAID LIST OR REPORT WILL NOT BE PROCESSED**

FOR OFFICIAL USE ONLY

To what extent have permit conditions been complied with:

2. DETAILS OF PROPOSED COLLECTING / RESEARCH PROJECTS

2.1 **Title of project (attach full project proposal / details):**

PROJECTS: Avian parasite and pathogen research and penguin reproductive biology studies being conducted by staff and students at the NRF Centre for conservation Science at the National Zoological Gardens in Pretoria. Various projects discovering and describing avian parasites and pathogens (including avian malaria) and investigateing genetics and reproductive parameters in captivity and in the wild, with a focus on the African Penguin. The birds to be sampled are those admitted to SANCCOB for rehabilitation and those in captivity at the Two Oceans Aquarium and National Zoological Gardens..

Three project proposals are involved: Dr Nupen's, Mr Mafunda's and Ms Motheo's. Proposals are attached. Below are combined objectives, motivations, results and observations outlined in bullet form (Section 2.2).

2.2 Objectives and motivation (proposed results or observations to be made):

Lisa Nupen

- Assessing the genetic diversity at genes associated with parasite and pathogen resistance in African Penguins (and other seabirds) and identifying spatial patterns that might be correllated with pathogen environments.
- Assessing spatial patterns in the infection (intensity and prevalence) of wild African Penguins by various parasites and pathogens, and comparisons with those held in captivity
- Developmental staging study to investigate embryonic development (opportunistic sampling of non-viable eggs incubated at SANCCOB)
- Many of these results have implications for future management of captive and wild African Penguins, especially with regards to any future reintorduction program, as well as being of considerable academic interest.

2.3 Institution that sponsors and controls project:

Name of institution: Centre for Conservation Science at the National Zoological Gardens (Pretoria)

Contact: Lisa Nupen Tel: 0725122143

Name of institution: Southern African Foundation for the Conservation of Coastal Birds (SANCCOB)

Contact: Nola Parsons Tel: 27 (0)21 557 6155

2.4 **South African** University or Institution that endorses your project:

Name: University of Cape Town

Contact : Dr Jacqueline Bishop Tel: +27 21 6503631

Portia Motheo

• To develop and optimize PCR, quantitative real-time PCR (qPCR) and LAMP assays for detection and differentiation of haemoprotozoan parasites from several bird species, including African Penguins in South Africa.

• To determine the prevalence and the phylogenetic relationships of haemoprotozoan parasites in avian samples using the developed assays.

2.3 Institution that sponsors and controls project:

Name of institution: Centre for Conservation Science at the National Zoological Gardens (Pretoria)

Contact: Portia Motheo Tel: 0839375763

Contact: Dr Sibusiso Mtshali Tel: +27 12 339 2822

2.4 **South African** University or Institution that endorses your project:

Name: University of the Free State (QwaQwa Campus)

Contact : Associate Professor Oriel Thekisoe

Tel: +27 58 7185331

Siya Mafunda

The main aim of this investigation is to evaluate techniques for the collection of semen in African penguin, to define sperm quality in order to cryopreserve sperm that may subsequently be used for IVF purposes in captive and later on applied in the wild/free ranging populations.

- Objectives:
    - To describe the macro-anatomy and histology of the male and female reproductive systems of Spheniscus demersus and relate these aspects to breeding status.
    - To collect semen and blood during the breeding season and correlate this with hormonal profiles.
    - To develop techniques to sample semen/sperm and determine parameters that defines high quality sperm.

- To develop cryopreservation procedures by testing the best extenders and cryoprotectants.

2.3 Institution that sponsors and controls project: Centre for Conservation Science at the National Zoological Gardens (Pretoria),

Contact: Siya 0726104372, Prof A. Kotze Tel: +27 (0) 12 3392795

2.4 **South African** University or Institution that endorses your project: University of the Western Cape and National Zoological Gardens of South Africa.

Contact: Siya 0726104372, Prof A. Kotze Tel: +27 (0) 12 3392795, Prof Gerhard van der Horst Tel: 021-959 2183, Dr Liana Maree Tel: (021) 959 2917.

2.5 Species to be collected (**SEE ALSO FOLLOWING PAGE**)

2.5a Species requested for **examination** only (**NO permanent removal from habitat**):

Species (***Fauna X**)	Temporarily or permanently scarred*	Marked only*	Region / Farm / Property name / Conservation Area (Nature Reserve, State Forest etc. see
African Penguin *Spheniscus demersus*	X Blood sample (0.1ml) 2 Semen samples, 12 Faecal samples, 12 blood samples	All birds admitted to SANCCOB	SANCCOB and Two Oceans Aquarium
Cape Cormorant *Phalacrocorax capensis*	X Blood sample (0.1ml)	All birds admitted to SANCCOB	SANCCOB
Cape Gannet *Morus capensis*	X Blood sample (0.1ml)	All birds admitted to SANCCOB	SANCCOB
Crowned Cormorant *Microcarbo*	X Blood sample (0.1ml)	All birds admitted to SANCCOB	SANCCOB
Bank Cormorant *Phalacrocorax neglectus*	X Blood sample (0.1ml)	All birds admitted to SANCCOB	SANCCOB

Reed Cormorant *Microcarbo africanus*	X Blood sample (0.1ml)	All birds admitted to SANCCOB	SANCCOB
Other birds for rehab	X Blood sample (0.1ml)	All birds admitted to SANCCOB	SANCCOB

(*Indicate with X)

*In the case of **fauna** the method of capture must be mentioned (i.e. net, trap etc.)	Method of capture: Birds are those admitted to SANCCOB or kept in captivity at the TOA. They may be captured by various means, unknown to us.

2.9 Number of persons engaged in the research project (**names and status** if possible)

Dr Nola Parsons, Researcher, SANCCOB

Dr Jacqueline Bishop, Lecturer, University of Cape Town (UCT)

Dr. Lisa Nupen, Postdoctoral Researcher, UCT/NZG (8402100481084)

Dr Desire Dalton, National Zoological Gardens (NZG)

Portia Motheo, PhD Student NZG (8210250647085)

Mr Siyambulela Mafunda, PhD student (NZG/UWC) 85085811081

Mr Vincent Naude Honours student (UCT)

Lisa Nupen - samples collected since 2009 (Western Cape)

Please note that some samples were collected under the permits of other researchers and subsequently shared with me

Species Name	Number sampled	Collection Dates	Locality	Collaborators
African Penguin	25	March to June 2009	Dassen Island	Dr Nola Parsons
	10	May to July 2009	Stony Point	Dr Viviane Barquete
	10	February to March 2010	Robben Island	Dr Nola Parsons
	8	June to July 2010	Boulders Beach	Dr Viviane Barquete
	15	July to August 2010	Dyer Island	Dr Nola Parsons
	10	June to July 2010	Jutten Island	Dr Tim Cook

Portia Motheo - samples collected since 2013 (Pretoria)

Please note that all samples were collected under the permits of other researchers and subsequently shared with me

Species Name	Number sampled	Collection Dates	Locality	Collaborators
African Penguin	5	September 2013	NZG	Dr Emily Lane
Rosy Flamingo	9	September 2013	NZG	Dr Emily Lane
Scarlet Ibis	9	September 2013	NZG	Dr Emily Lane
Cockatoos	10	September 2013	NZG	
Red Billed Oxpecker	7	September 2013	NZG	Dr Emily Lane
Waldrapp Ibis	14	September 2013	NZG	Dr Emily Lane
Spotted Dikkop	8	September 2013	NZG	Dr Emily Lane
Southern Ground Hornbill	9	September 2013	NZG	Dr Emily Lane
Jandaya Conure	5	September 2013	NZG	Dr Emily Lane

Appendix E

CapeNature

HEAD OFFICE

postal	Private Bag x29 Gatesville 7766
physical	PGWC Shared Services Center cnr Bosduif & Volstruis Streets Bridgetown 7764
website	www.capenature.co.za
enquiries	Lee-Anne Benjamin
telephone	+27 21 483 0120
fax	086 556 7734
email	lbenjamin@capenature.co.za
reference	1/2/1/6/5/F6
date	20 October 2014

Dr LJ Nupen
University of Cape Town
Private Bag X3
RONDEBOSCH
7701

Dear Dr Nupen

APPLICATION TO COLLECT FAUNA SPECIMENS FOR SCIENTIFIC RESEARCH

I refer to your application to collect specimens in the Western Cape Province.

Attached are permit No.'s: **AAA007-00136-0056** dated **20 October 2014** to collect specimens in the Western Cape Province. Please take special note of the standard conditions attached to the permits. I specifically draw your attention to permit condition (i). **It is imperative that you make contact with the Reserve Manager BEFORE you intend collecting on any nature reserve, conservation area, wilderness area and / or state forest**. No deviation is allowed from the fore-mentioned conditions without the prior written approval of the Chief Executive Officer: Western Cape Nature Conservation Board.

Please also take note of the *pro forma* (copy attached), which must please be used when submitting your collection / distribution records to CapeNature as per the conditions to your permit. Please feel free to add columns for extra data to the *pro forma* but no columns should be deleted. This pro forma is also available electronically from CapeNature.

Should you have any queries please do not hesitate to contact this office.

Yours faithfully,

CapeNature
FAUNA • FLORA • HUNTING • CITES

CHIEF EXECUTIVE OFFICER

Western Cape Province

Telephone No: (027) 021 483 0000
EMail: permits.fax@capenature.co.z
PGWC Shared Services Centre
cnr Bosduif and Volstruis Streets
Bridgetown
7764

CapeNature

Facsimile No: (027)0865567734
Internet: www.capenature.co.za
Private Bag X29
Gatesville
7766

PERMIT TO HUNT WITH
PROHIBITED HUNTING METHOD OF WILD ANIMALS - RESEARCH PURPOSES

(Issued in terms of the provisions of the Nature Conservation Ordinance 1974, (Ord 19 of 1974)Section29&33)

Not Transferable

Holder

Full Name	Dr L Nupen	**Identity No.**	8402100481084
Trade Name	University of Cape Town	**Registration No.**	AAA004-00995
Postal Address	University of Cape Town Private Bag X3	**Physical Address**	Percy FitzPatrick Institute, Office 2.17, John Day Zoology Building, Unversity Avenue
Suburb\Town	Rondebosch	**Suburb\Town**	Rondebosch
Province\State	Western Cape	**Province\State**	Western Cape
Country	South Africa	**Country**	South Africa
Postal\Zip Code	7701	**Longitude**	.0000
		Latitude	.0000

In terms of and to the provisions of the abovementioned Ordinance and the Regulations framed thereunder, the holder of this permit is hereby authorised to Hunt (capture/disturb/stampede/kill) the protected wild animal(s) specified below on the property mentioned on this permit. See conditions on last page:

Details

Permit/Licence No	**0056-AAA007-00136**	*Stamp:* CapeNature FAUNA • FLORA • HUNTING • CITES
Expiry Date	29/02/2016	
Date Issued	01/11/2014	
Amount Paid	R 0.00	
Reference	NO COST	
File Code	1/2/1/6/5/F6	

Description	Property
Organization	University of Cape Town
Person	Nupen L Dr
ID	8402100481084
Properties	Two Oceans Aquarium & SANCCOB
Physical Address	Sampling / collection areas.
District	NA
Province/State	Western Cape
Country	South Africa
Longitude	.0000
Latitude	.0000

Species(Scientific Name)	Qty	Note
A) Note(NA)	0	Conditions apply, note special conditions.
African Penguin(Spheniscus demersus)	0	samples: blood,2 semen,12 faecal,non-viable eggs
Bank Cormorant(Phalacrocorax neglectus)	0	0.1ml blood sample.
Cape Cormorant(Phalacrocorax capensis)	0	0.1ml blood sample.
Cape Gannet(Morus capensis)	0	0.1ml blood sample.
Crowned Cormorant(Phalacrocorax coronatus)	0	0.1ml blood sample.
Reed cormorant(Microcarbo africanus)	0	0.1ml blood sample.

Issued by:	**Approved on Behalf CEO**	**Effective Date**	**Signature of Holder**
Lee-Anne Benjamin	Western Cape Nature Conservation Board	01/11/2014	I acknowledge, accept and understand fully the permit conditions as described

21/10/2014 07:53:38AM

software by: www.u-rad.com

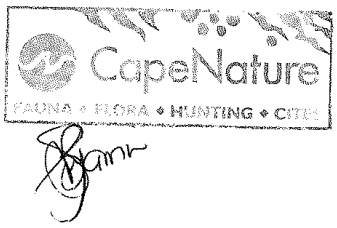
Standard Conditions

1. When the holder of this permit *kills/captures/collect any wild animal in terms thereof, he shall, before leaving the above-mentioned property, or if he does not leave it, after each day's *hunt/capture/collection, record the particulars regarding the date, species and number of each sex of each species, or if it is impossible to distinguish the sex, the total number of each species of such wild animals which he had *killed/capture/collected.

2. The holder of this permit shall return it to the Chief Executive Officer: Western Cape Nature Conservation Board, Private Bag X29, Gatesville, 7766, within 14 days of the date of expiry thereof.

3. THIS PERMIT IS SUBJECT TO THE ADDITIONAL CONDITIONS AS SET OUT IN THE ADDENDUM HERETO.

Special Conditions

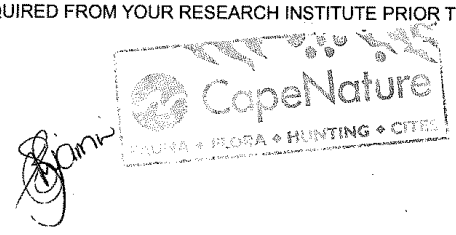


THIS PERMIT IS ISSUED SUBJECT TO THE FOLLOWING SPECIAL CONDITIONS:

a) People involved in this project:
i. Dr Nola Parsons, Researcher, SANCCOB
ii. Dr Jacqueline Bishop, Lecturer, University of Cape Town (UCT)
iii. Dr. Lisa Nupen, Postdoctoral Researcher, UCT/NZG (8402100481084)
iv. Dr Desire Dalton, National ZoologicalGardens (NZG)
v. Portia Motheo, PhD Student NZG (8210250647085)
vi. Mr Siyamulela Mafunda, PhD student, NZG/UWC (S5085S11031)
vii. Mr Vincent Naude Honours student (UCT)
b) The study area: The birds to be sampled are those admitted to SANCCOB for rehabilitation and those in captivity at the Two Oceans Aquarium.
c) Methods and Sample No: 0.1ml blood samples using capillary tubes and needles (all birds admitted to SANCCOB); semen samples (2 African penguin samples); faecal samples (12 African penguin samples); additional 12 blood samples for RNA extraction (African penguin)
d) Species: African penguin (Spheniscus demersus), Cape cormorant (Phalacrocorax capensis), Cape Gannet (Morus capensis), Crowned Cormorant (Microcarbo coronatus), Bank Cormorant (Phalacrocorax neglectus), Reed Cormorant (Microcarbo africanus), other species in rehab.
e) For samples that need to be transported out of South Africa, an export permit is required.
CONDITIONS APPLICABLE TO RESEARCHERS UNDERTAKING RESEARCH OR OTHER COLLECTING WORKS ON PROVINCIAL CONSERVATION AREAS AND / OR PRIVATELY OWNED LAND IN THE PROVINCE OF WESTERN CAPE:

1. THE MANAGER OF THE RELEVANT CONSERVATION AREA(S) (IF ANY) MUST BE INFORMED TIMEOUSLY BEFORE ANY CONSERVATION AREA IS ENTERED FOR COLLECTING OR RESEARCH PURPOSES AND THE MANAGER'S WRITTEN PERMISSION TO ENTER SUCH RESERVE MUST BE ACQUIRED BEFOREHAND. THIS PERMIT DOES NOT GRANT THE PERMIT HOLDER AUTOMATIC ACCESS TO ANY NATURE RESERVE, CONSERVATION AREA, WILDERNESS AREA AND / OR STATE FOREST. ANY OTHER / FURTHER CONDITIONS OR RESTRICTIONS THAT THE MANAGER MAY STIPULATE AT HIS / HER DISCRETION MUST ALSO BE ADHERED TO. THIS PERMIT MUST BE AVAILABLE TO BE SHOWN ON DEMAND.
2. The owner of any other land concerned (be it privately or publicly owned land) must give WRITTEN consent allowing the permit holder to enter said property to collect flora / fauna. This written permission must reflect the full name and address of the property owner (or of the person authorised to grant such permission), the full name and address of the person to whom the permission is granted and the number and species of the flora / fauna, the date or dates on which such flora / fauna may be picked / collected and the land in respect of which permission is granted. Copies of this written permission must be made available to The Western Cape Nature Conservation Board upon request.
3. Type-specimens of any newly described / discovered species or other taxon collected must be lodged with a recognised South African scientific institution / museum / herbarium (preferably within the Province of Western Cape) where such material will be available to other researchers. For every flora specimen collected on a Western Cape Nature Conservation Board nature reserve, one additional (extra) herbarium specimen must be forwarded to the Western Cape Nature Conservation Board Herbarium at Jonkershoek (c/o MJ Simpson, Private Bag X5014, Stellenbosch 7599).
4. A list of all collected specimens / material including the; species name, the number collected, the collection date and the precise locality of the collection must be submitted within 14 days from the date of expiry of your permit to The Chief Executive Officer: CapeNature, Private Bag X29, Gatesville, 7766
5. The maximum number of specimens per species specified in the permit (if at all) may not be exceeded without the prior permission of The Chief Executive Officer: Western Cape Nature Conservation Board.
6. For projects of more than one year's duration a progress report must be submitted to The Chief Executive Officer: Western Cape Nature Conservation Board before 31 December of each year.
7. One copy of all completed reports, publications, or articles (including books, videos, CDs, DVDs etc.) resulting from the project/collection must be submitted to The Chief Executive Officer: Western Cape Nature Conservation Board free of charge.
8. Should a report, publication, article or thesis arise from this project/collection, an acknowledgement to Western Cape Nature Conservation Board must be included.
9. The Forest Act 1984 (Act 122 of 1984) and regulations, the Nature Conservation Ordinance, 1974 (Ordinance 19 of 1974) and all regulations in terms of the Ordinance must be adhered to.
10. Should it be envisaged to export any material / specimens across the boundaries of the Western Cape Province, an export permit will be required in respect of certain species and a further application form will have to be completed. The permit holder must confirm with the Western Cape Nature Conservation Board whether an export permit is required BEFORE exporting any material / specimens from the Western Cape Province.
11. No species that appear on the Red Data List or species listed as endangered in terms of the Nature Conservation Ordinance, 1974 (Ordinance 19 of 1974) may be collected, except for those mentioned on the permit.
12. Unless otherwise specifically indicated in writing, no material or specimens collected with this permit or material or specimens bred or propagated, from material or specimens collected with this permit, may be donated, sold or used for any commercial purpose by any party.
13. IF APPLICABLE, ETHICS CLEARANCE MUST BE ACQUIRED FROM YOUR RESEARCH INSTITUTE PRIOR TO COLLECTION.

CHIEF EXECUTIVE OFFICER
WCNCB



Import format for SOB data

The data should be saved in a Microsoft Excel spreadsheet. The later the version the better.

The spreadsheet should contain the following columns with the column headings in row 1:

Column	Description
Date	Enter full date e.g. 25/9/1994
LocalityName	Name of locality e.g. town name, reserve name, farm name etc.
DegreesSouth	Number of Degrees South of 0 degrees South
MinutesSouth	Number of minutes South
SecondsSouth	Number of seconds South
DegreesEast	Number of Degrees East of 0 degrees East
MinutesEast	Number of minutes East
SecondsEast	Number of seconds East
QuarterDegree	Enter if degrees, minutes, seconds are unknown
Species	Full scientific name eg. Panthera pardus or Cacosternum nanum parvum
Record Type	Type of record: Specimen, Observation, Photograph, Audio, Literature, Scat
Collector	Name of the person that collected the record
LodgingCode	The unique number that each institution assigns each record eg. PEM-00348 (must have a dash before the number)
Contact	The name of the contact person at the Institution
Institution	The Institution where the record is lodged
Remarks	Any associated remarks or notes that you wish to include with the record