

**A protocol for liquid storage and cryopreservation of
ostrich (*Struthio camelus*) semen**

by

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DECLARATION

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ABSTRACT

The aim of this study was to develop a species-specific protocol for short (liquid) and long (cryopreserved) term storage of ostrich semen. Effective storage followed by artificial insemination (AI) could assist to overcome the current industry limitations, such as poor egg fertility as well as low embryo and chick survival. Initially, factors influencing sperm storage in other avian species were considered to identify the steps needed to develop an ostrich-specific protocol. Secondly, it was necessary to adapt a set of *in vitro* functions to quantify and classify sperm of the ostrich, even before storage processing could commence. This approach allowed the evaluation of ejaculates and detected differences in sperm function caused by semen processing.

The classification of ostrich sperm characteristics through *in vitro* sperm functions of sperm motility, viability and membrane integrity allowed for ostrich semen to be assessed for quality. Males differed from one another in terms of motility, progressive motility, sperm swimming speed and the linearity of swimming. Males may be screened using these traits to determine storage suitability and inclusion in an AI programme to optimize fertility. Semen dilution immediately upon collection is a prerequisite for the further assessment of sperm. An ostrich specific diluent (OS1) based on macro mineral composition and concentration of ostrich seminal plasma maintained sperm function during short-term storage for 24 hours at 5 °C without a significant loss in sperm quality. The maintenance of sperm function and specifically swim quality over extended storage periods of 48 hours depended upon a dilution medium (OS2) balanced for macro and micro minerals in ostrich seminal plasma. However the rate at which the semen is diluted as well as the dilution temperature is vital for optimum sperm function. An interpolated rate of 1:6 (semen: diluent) together at a dilution temperature of ≥ 21 °C gave the best results. Slow cooling (1 °C/minute) after dilution to 5 °C reduce metabolic activity and maintained sperm function after 24 hour short term storage for AI. Fertilized eggs were obtained after AI with semen in liquid storage proving that short term storage of ostrich semen is feasible. The semen of certain ostrich males were more resistant to processing stressors associated with short-term storage than others.

A suitable cryoprotectant (CP) at the best inclusion level was needed for the indefinite cryopreservation storage of ostrich semen. Dimethylacetamide (DMA) was the CP of choice. Ostrich semen could be equilibrated for storage in liquid nitrogen (LN) without a significant reduction in normal sperm morphology at an inclusion level of 16 % DMA. Fast freezing in a programmable freezer at 10 °C/minute to -30 °C or in a LN Styrofoam box with a plate set at 3.5 cm LN sustained sperm motility best after thawing. The utility of the latter cryopreservation protocol was supported by an *in vivo* fertility assessment of the cryopreserved semen after AI. Further research on female factors as well as the refinement of the AI regime is needed for optimizing both the *in vitro* and *in vivo* protocols for the ostrich.

OPSOMMING

Hierdie studie se oogmerk was om 'n spesie-spesifieke werkswyse vir die lang- en korttermynberging van volstruissemen te ontwikkel. Doeltreffende berging, gevolg deur kunsmatige inseminasie (KI) kan daartoe bydra dat bedryfsbeperkings soos swak vrugbaarheid van eiers sowel as swak embryo- en kuikenoorlewing die hoof gebied kan word. Faktore betrokke by die stoor van semen in ander spesies is aanvanklik oorweeg ten einde die stappe benodig vir die ontwikkeling van 'n spesie-spesifieke werkswyse vir die lang- en korttermyn berging van volstruissemen te identifiseer. Verder is die *in vitro*-funksies vir die kwantifisering en klassifisering van semen aangepas vir volstruise voordat berging in aanvang geneem het. Hierdie benadering het dit moontlik gemaak om ejakulate te evalueer en verskille in spermfunksie as gevolg van die proses te beskryf.

Dit was moontlik om in die *in vitro*-funksies beweeglikheid, lewenskragtigheid en membraan-integriteit van volstruisperme vir gehalte aan te slaan. Die semen van volstruismannetjies het verskil ten opsigte van sperm beweeglikheid, doelgerigte beweeglikheid, swemspeed en reglynige beweging. Mannetjies kan op grond van hierdie eienskappe tot 'n KI-program vir verbeterde vrugbaarheid toegelaat word. Die verdunning van semen direk na kolleksies 'n voorvereiste vir die verdere aanslag van semen. Inligting oor die makro-mineraalsamestelling van seminale plasma is bekom en gebruik om 'n verdunningsmengsel spesifiek vir volstruise saam te stel (OS1) om sperme vir solank as 24 uur by 5 °C te berg, sonder om sperm kwaliteit prys te gee. Spermfunksie en swemgehalte is behou vir solank as 48 uur deur verdere ontwikkeling van hierdie verdunningsmengsel deur mikro-minerale ook in ag te neem (OS2). Die vlak van verdunning sowel as die temperatuur van die verdunningsoplossing is egter belangrik om spermfunksie te behou. 'n Ge-interpoleerde verdunningstempo van 1:6 (semen:verdunningsmengsel) by 'n verdunningstemperatuur van ≥ 21 °C het die beste gevaar in terme van semen kwaliteit. Stadige afkoeling (1 °C/minuut) na verdunning tot by 5 °C het die metaboliese aktiwiteit van sperme laat afneem en dit moontlik gemaak om semen vir tot 24 uur voor KI te berg. Daar is bevrugte eiers gekry na KI met semen in vloeibare berging, wat daarop dui dat die korttermynberging van volstruissemen uitvoerbaar is. Sommige volstruis mannetjies se sperme was meer weerstandbiedend teen proseseringstremmings, met korttermynberging, as andere.

'n Aanvaarbare vriesbeskermingsmiddel (CP) teen die beste toedieningsvlak is ondersoek vir die onbeperkte berging van volstruissemen. Dimetielasetamied (DMA) was die verkose CP. Volstruissemen kon vir berging in vloeibare stikstof (LN) ge-egaliseer word sonder 'n betekenisvolle verlaging in normale spermorfologie by 'n insluitingspeil van 16 % DMA. Versnelde bevriesing in 'n programmeerbare bevriesingsapparaat teen 10 °C/minuut tot by -30 °C of in 'n polistireen houër met LN op die bodem met 'n bord op 'n hoogte van 3.5 cm bokant die LN het sperm-beweeglikheid die beste na ontdooiing behou. Die toepaslikheid van laasgenoemde bevriesingswerkswyse is bevestig deur 'n *in vivo* vrugbaarheidsaanslag na die KI van wyfies met bevrore semen. Verdere navorsing op wyfie-faktore sowel as die verfyning van die KI-protokol word benodig om beide *in vitro*- en *in vivo*-werksywes te optimaliseer.

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CHAPTER I

GENERAL INTRODUCTION

1. Background

Increased commercial production of ostriches, *Struthio camelus*, for their skin, meat, fat and feathers has resulted in pressure on producers for improved output and focused attention on constraints like a poor egg fertility, embryo and chick survival (Bunter and Cloete, 2004; Cloete *et al.*, 1998, 2008b; Malecki *et al.*, 2008). The reproductive efficiency on commercial ostrich farms directly affects the profitability, as it depends on the number of healthy chicks hatched and surviving to slaughter (Cloete *et al.*, 1998, 2008b). Quantitative reproduction traits in ostriches are highly variable, with variation coefficients exceeding 50 % (Deeming, 1996; More, 1996; Van Schalkwyk *et al.*, 1996; Bunter and Graser, 2000; Bunter *et al.*, 2001; Cloete *et al.*, 2008b). Poor sperm supply has been stated as one of the primary reasons for low fertility in ostriches due to male-female incompatibility and/or poor sperm output (Bertschinger *et al.*, 1992; Hemberger *et al.*, 2001; Ya-jie *et al.*, 2001; Malecki and Martin, 2003). The observed poor fertility is partially to blame on the current reproduction system that relies solely on natural reproduction in a free-range system with a 6:10 male to female ratio dominating in colonies, while the birds are also kept in pairs or trios (Lambrechts, 2004). The colony system does not allow the identification of sub-fertile individuals and it is impossible to record individual female reproductive performance as well as chick pedigrees (Cloete *et al.*, 2008b). It is also impossible to select for commercially important traits (productive and reproductive), since superior males can only mate with a few females in one season, hence compromising selection differentials on the male side. Although genetic variation for most economic traits in experimental flocks has been documented by Bunter and Cloete (2004) and Cloete *et al.* (2008a), this potential is not realized due to a lack of performance records with identifiable parentage and the difficulty in increasing the selection differentials on an industry level. In pair-bred flocks (which allows pedigree recording) other problems arise due to female and male genetic effects that are confounded if only a few generations of data are available (Cloete *et al.*, 1998, 2008b). Significant genetic improvement in chick production has been achieved during the last 10-15 years (Cloete *et al.*, 2008b), although a trait like egg production only averages 40 to 50 eggs per 8-month breeding season (May to December), which is far below the theoretical maximum of 120 eggs with an egg being produced every second day. Production and variable fertility rates needs to be

addressed and increased significantly to maximize profitability of the industry to remain competitive (Deeming, 1999). The latter may be improved by implementing a structured breeding program, with specific focus on selective breeding and artificial insemination (AI) technology, which is currently lacking in most of the ratite industries compared to other livestock industries like poultry, swine, dairy cattle, beef cattle and sheep. Successful AI protocols depend on reliable methods for semen collection, handling and processing for storage (short- and long-term), as well as the accurate assessment of male and female fertility (Holt, 2000; Malecki *et al.*, 2008).

Although collection and semen handling methods have been investigated to some extent in the ostrich (Bertschinger *et al.*, 1992; Rybnik *et al.*, 2007; Bonato *et al.*, 2011) knowledge about semen preservation is limited, with suboptimal results (Ya-jie *et al.*, 2001; Malecki *et al.*, 2008). Processing semen for both short-term (liquid/chilled) and long term (frozen/cryopreserved) purposes exerts multiple effects on the sperm cell due to changes in their microenvironment for which they are not adapted. A certain degree of sperm function sperm loss is expected even under ideal *in vitro* conditions. A standard rate of 50 to 70 % loss in sperm function has been described as the norm when semen has been processed for *in vitro* storage with cryopreservation of semen inflicting the highest rate of functional loss (Watson, 2000; Blesbois *et al.*, 2008; Partyka *et al.*, 2012). The storage methods empirically developed in the 1950's are still fundamentally those in use today. These methods are associated with a highly variable response across species and individuals (Schaffner *et al.*, 1941; Lake and Stewart, 1978; Lake and Ravie, 1979; Wishart, 1984, 1989, 2007; Malecki and Martin, 2004; Blesbois and Brillard, 2007; Moce *et al.*, 2010; Waberski *et al.*, 2011). The degree of sperm function loss is not only dependent on storage processing procedures, but also on the species, breed and individual's uniqueness in terms of sperm specific composition and properties which determine processing resistance or sensitivity. This variable response to processing has been shown both in avian (Wilcox *et al.*, 1961; Blanco *et al.*, 2000; Blesbois *et al.*, 2005) and mammalian species (Holt, 2000; Waberski *et al.*, 2011). Wilcox *et al.* (1961) demonstrated marked differences among fowl breeds during *in vitro* storage although they did not differ in storing ability *in vivo* as measured by fertility using fresh semen. In avian species, storage protocols are different from those used in mammals due to specific biological features of avian sperm (Blesbois and Brillard, 2007). Avian sperm has some distinctive anatomical and physiological characteristics that include a cylindrical head with

little cytoplasm and a very long tail that is almost eight times the length of the head (McFarlane, 1962; Humphreys, 1972; Soley, 1993; Donoghue and Wishart, 2000). The latter is in part responsible for generally poorer storage and freezing success compared to mammalian sperm (Donoghue and Wishart, 2000). Factors affecting avian semen storage include dilution rate and temperature (Sexton, 1977), storage medium composition and associated osmotic and pH properties (Sexton and Fewlass, 1978; Lake and Wishart, 1984; Wishart, 1989; Blanco *et al.*, 2000, 2008), storage time and temperature (Clarke *et al.*, 1982), type of cryoprotectant (CP) and its concentration (Tselutin *et al.*, 1999), equilibration time and temperature of CP addition, cooling/freezing rates (Sexton, 1980) and thawing rates (Blesbois and Brillard, 2007). To quantify the suitability of each of these processing factors and the success of the storage protocol on sperm survival and potential fertilizing ability, a series of *in vitro* sperm functions and *in vivo* fertility assessment techniques were suggested, with great predictability of true sperm survival in both avian and mammalian species (Donoghue and Wishart, 2000; Malecki and Martin, 2003; Graham and Moce, 2005; Malecki *et al.*, 2008; Blesbois *et al.*, 2008; Moce *et al.*, 2010; Brand *et al.*, 2014).

Even with extensive research specifically focused on short- and long-term storage of domestic (Sexton, 1977, 1980; Sexton and Fewlass, 1978; Sexton *et al.*, 1980; Clarke *et al.*, 1982; Chalah *et al.*, 1999; Tselutin *et al.*, 1999; Blanco *et al.*, 2000; Donoghue and Wishart, 2000; Blesbois *et al.*, 2005, Blesbois, 2011) and non-domestic (Gee *et al.*, 1985, 2004; Blanco *et al.*, 2000; Sontakke *et al.*, 2004; Barna *et al.*, 2010; Sood *et al.*, 2011; Váradi *et al.*, 2013) avian species very little progress has been made in the ostrich (Yajie *et al.*, 2001; Malecki *et al.*, 2008). It can be confidently stated that the methodology for *in vitro* storage developed for one breed or species cannot necessarily be applied with equal success to other breeds or species. Hence, sperm storage, both short- and long-term protocols need to be optimized for each species for acceptable fertility *in vitro* and *in vivo* (Ashrafi *et al.*, 2011).

2. Rationale

The first step to assisted reproduction has been established by the release of a reliable stress-free semen collection method for ostriches (where the male can be trained to ejaculate into an artificial cloaca using a teaser or dummy female) (Rybnik *et al.*, 2007). This method has been implemented with great success for regular collection of good

quality ejaculates. Suitable especially for avian males that has the extended potential to produce large quantities of sperm within a given period of time, because of the relatively short interval of time required for avian B-type spermatogonia to evolve into sperm (De Reviere, 1968; Noirault *et al.*, 2006; Blesbois and Brillard, 2007). The proficiency of the male, together with a suitable method for collection, has enabled the recent expansion of male reproductive studies. A semen collection frequency of twice daily has been established by Bonato *et al.* (2011) to maximize sperm output, while still maintaining semen viability and male libido. Collected semen quality in terms of high volume, motility and sperm numbers has been found optimal during the spring (October to November) when studied in South Africa in the Southern Hemisphere (Bonato *et al.*, 2014b).

Recent advances on the female side made the possibility of *in vivo* testing through artificial insemination possible. Firstly the selection and establishment of a female only flock to produce eggs in the absence of males has been proven feasible and invaluable to further reproductive studies (Bonato *et al.*, 2014a). Malecki *et al.* (2004) established a 4-week fertile period after the last copulation, which suggested exceptional sperm storage capability in ostrich females. The ability to store sperm is possibly due to the relatively large utero-vaginal region, containing long sperm storage tubules (SST), a characteristic feature of many avian species with specific reference to the turkey (Lake, 1975; Wishart, 1985; Birkhead, 1988; Bakst *et al.*, 1994; Malecki *et al.*, 2004; Holm *et al.*, 2000). The prolonged storage ability of semen in the ostrich SST offers the possibility of less frequent inseminations using good quality semen and using relatively low insemination dosages from genetically superior males. However, the widespread application of AI for commercial production also depends on proper optimized protocols for short- and long-term semen storage, as well as the reliability of AI techniques that result in acceptable fertility. Species-specific studies are necessary for successful storage and the subsequent facilitation of assisted breeding (Blanco *et al.*, 2000; Blesbois and Labbe, 2003; Saint Jalme *et al.*, 2003; Blesbois and Brillard, 2007). Preliminary studies on ostrich sperm suggest that although the quality of the fresh semen is good, a considerable loss of sperm quality occurs during storage *in vitro*. Methodology for storage processing is thus far from optimal and needed to utilize this technology to its fullest potential in future (Ya-jie *et al.*, 2001; Malecki *et al.*, 2008; Malecki and Kadokawa, 2011; Ciereszko *et al.*, 2010).

AI with both short- and long-term stored semen will enable breeders to utilize the known advantages of these assisted reproduction technologies. AI could thus potentially overcome poor sperm supply, by eliminating the obstacle of male-female incompatibility during natural mating and ensure enough acceptable quality sperm. Male numbers can be reduced, since semen from genetically superior males can be routinely collected and stored. The excess males can be replaced by additional females that would automatically increase profit margins by an increased production of eggs and chicks. It has been demonstrated in other avian sectors that AI can generally improve egg hatchability with fewer genetically superior males being kept for breeding, resulting in reduced expenses with increased profitability (Omparakash *et al.*, 1992). Assisted reproductive technologies, specifically cryopreservation, offers the potential for conservation of genetically superior and diverse lines through germ plasm preservation, also seen as “semen banking”, as it is not feasible to cryopreserve or transfer embryos to recipients in avian species. Conservation of germplasm from domestic and endangered avian species is essential for long-term sustainability of wild and farmed bird stocks and has already been seen as a scientific priority for decades (Blesbois and Brillard, 2007; Blesbois *et al.*, 2005). Conservation of unique genetic resources is especially of high importance in species like the ostrich where the recurrent episodes of Avian Influenza could potentially lead to the loss of very important genetic production lines. The loss of genetic lines and future overbreeding of superior lines on a commercial level may reduce the fairly large effective population size for the ostrich (Fair *et al.*, 2012). However by implementing *ex situ* management of the genetic resources, inbreeding and drift in small populations can be counteracted (Dresser, 1988; Wildt *et al.*, 1993; Karow and Critser, 1997; Meuwissen and Sonesson, 1998; Sonesson *et al.*, 2002). Classification of ostrich semen to developed *in vitro* sperm function assays would allow breeders and farmers to screen males and ejaculates with the potential to predict fertilization ability and storage suitability. In certain species like equines it has been established that only 20 % of males produce superior quality semen suitable for cryopreservation purposes, while 60 % is more suitable for chilled storage, while the remaining 20 % is only suitable for immediate insemination purposes without processing for storage (Vidament *et al.*, 1997). Prior knowledge of the male’s potential in terms of semen quality would enable immediate elimination of sub-fertile males from a breeding population. Cryopreserved semen would allow international retail of superior ostrich genetics unique to South Africa, thus enabling breeders to earn foreign currency in a depressed economic climate. Retail of chilled semen on a national

level would provide the exploitation of superior genetics that are easily accessible and fairly cost-effective compared to cryopreserved semen. Local farmers and breeders can benefit from increased productive and reproductive efficiency through increased fertilization rates when ostrich females are inseminated during high egg production months (July/August) when males produce semen of inferior quality by inseminating females with cryopreserved semen collected and processed during months with high semen output and/or quality (Lambrechts, 2004; Bonato *et al.*, 2014b). Data recording and genetic evaluation schemes would be feasible through better pedigree recording offered by AI without the additional management of a pair-bred flock. AI would allow the estimation of genetic parameters and breeding values for the measured traits with the added bonus of genetic trends over time in a resource population. These records will allow the estimation of breeding values facilitating increased selection intensity of males as well as females for superior reproductive traits specific applicable to assisted reproduction (Cloete *et al.*, 2002a, b). Holsberger (1998) showed that selection of the appropriate avian male on the basis of superior semen traits can benefit female fertility and reproductive efficiency. The use of elite males will improve the genetic structure of a population by optimizing genetic diversity and maximizing the rate of genetic improvement (Gowe, 1993; Chambers, 1990; Pollock, 1999; Foote, 2002), while reducing the effective population size (Fair *et al.*, 2012). Ostrich reproductive traits are moderately to highly heritable (12 to 29 %), while it is also linked to high levels of phenotypic variation (Cloete *et al.*, 2008b). Annual genetic responses in chick production have thus been worthwhile at 2.3 to 3.1 % of the overall phenotypic mean (Cloete *et al.*, 2004, 2008a). It should be noted that a small increase in a trait, like hatchability for instance, would enable higher profit margins, as shown in other poultry species (Pollock, 1999; Gowe, 1993; Chambers, 1990).

The ostrich industry with its current limitations would be able to gain appreciably from assisted reproduction technology as proven in all other livestock industries. With the knowledge and methodologies already established in a previously set-up research flock (Cloete *et al.*, 2008a, b), immediate expansion to assisted reproduction techniques, including effective short- and long-term semen storage protocols seem to be implementable in the immediate future.

3. Study Objectives

The main objective of this study is to develop an effective, species specific protocol for short (liquid) and long (cryopreservation) term storage of ostrich semen that would guarantee good fertility after AI.

The general hypotheses tested are:

1. Ostrich sperm survival during short-term storage will depend on extender composition, dilution rate and temperature, cooling rate, storage temperature and storage time.
2. The success of the ostrich sperm cryopreservation will depend on the extender composition, dilution rate and temperature, cooling rate, cryoprotectant type and concentration, cooling/freezing and thawing rate.

A stepwise procedure was adopted to achieve success in one facet before moving to the next. To obtain viable results within a manageable time frame, and a cost-effective budget, would depend on prior knowledge of sperm physiology and the factors that might influence sperm cell function during short- and long-term storage. Hence, a biochemical understanding of the sperm and seminal plasma was necessary to establish suitable handling and storage environments. Appropriate evaluation of ostrich sperm is crucial to quantify and classify semen before and after processing procedures to detect differences in sperm function cause by the processing thereof.

The overall success of both short- and long-term storage protocols was quantified by *in vivo* fertility trials measuring the number of fertile eggs following AI. However, *in vivo* fertility was problematic since such a test may be considered a sperm fertilizing ability test and would indicate the efficiency of the hen's oviducts at accepting and retaining sperm exposed to storage processing. At present, there is no standard measure for fertilizing ability of ostrich sperm *in vivo*, which is independent of the female environment. Hence, when females are inseminated with equivalent doses of fresh, liquid or cryopreserved semen, the ratio of their apparent fertilizing abilities will vary, depending on the particular dose chosen and the female oviduct environment. In applied work such a limitation is often overlooked in favour of obtaining "good" fertility. Therefore, the success of both short- and long-term sperm storage needs to be tested in two ways during the development of the protocols: firstly a series of sperm function tests *in vitro* to set up

a baseline for ostrich sperm characterization and to assess the impact of storage processing factors on individual sperm; and secondly *in vivo* tests by means of insemination trials to assess the ability of sperm to reach the sperm storage sites in the female oviduct, the acceptance of sperm by the female reproductive tract and the ability of sperm to fertilize the eggs. These insemination trials ultimately tested the true fertilizing ability of sperm, which could be used in further improvements of the protocols at the laboratory level, as well as to provide selective breeding programs with a number of male and female fertility measures.

The first study in this dissertation (Chapter II; Classification of ostrich sperm characteristics) was conducted to develop a set of ostrich sperm function tests that could be implemented as an objective evaluation tool to establish a good foundation of sperm characteristics for the study. This would allow the evaluation and comparison of males, as well as ejaculates to ultimately improve semen processing for storage. The second study (Chapter III; Development of an ostrich specific storage extender) was conducted to formulate a medium consistent to ostrich seminal plasma (the natural medium of ostrich sperm) to maintain sperm *in vitro* for storage and evaluation purposes. The third study (Chapter IV; Prolonged storage of ostrich semen at 5 °C through intermediate dilution) was conducted to establish the most appropriate dilution procedures (rate and temperature at which the diluent should be set) for sperm with the newly formulated ostrich specific diluent to maintain sperm function at an optimum level during storage. The fourth study (Chapter V; The response of ostrich sperm to cooling at 5 °C storage and *in vivo* application through artificial insemination) was conducted to establish the chilling sensitivity of ostrich sperm by exposing them to different cooling rates. The fifth study (Chapter VI; Sensitivity of ostrich sperm towards cryoprotectant toxicity) was conducted to identify the most suitable cryoprotectant and its level of usage for optimal sperm function maintenance with the least deleterious effects, after its addition and equilibration that could be used during cryopreservation of ostrich semen for indefinite storage. The sixth study, (Chapter VII; Cryopreservation of ostrich sperm through fast freezing and artificial insemination of frozen-thawed semen), was conducted to determine the most appropriate freezing rate during controlled and uncontrolled freezing for indefinite sperm storage at sub-zero temperatures.

These processing steps are related. Short-term storage protocols are needed as steps during pre-processing for cryopreservation. *In vivo* tests were conducted only after *in vitro* results confirmed that ostrich semen could be successfully stored over the short-term as well as the long-term.

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CHAPTER II

CLASSIFICATION OF OSTRICH SPERM CHARACTERISTICS

The success of assisted reproduction techniques is dependent on a good foundation of sperm characteristics to evaluate, compare and improve semen processing. This study offers a descriptive basis for ostrich semen quality in terms of sperm function characteristics (SFC) that include motility, (measured by computer assisted sperm analysis CASA (SCA®)), viability (SYBR14/PI) and membrane integrity (Hypo-osmotic swelling test). Relationships between these SFC's were explored and described in terms of correlations and regressions. Certain fixed effects that include the dilution of semen, season, year and male effects associated with semen collection were interpreted for future applications. The seasonal effect on sperm samples collected throughout the year suggested the prudence of restricting collections to spring and summer, when SFC's and sperm concentration are maximized, compared to winter when they are suppressed. Dilution of ejaculates helped to maintain important SFC's associated with the fertilization success. SFC's and sperm concentration varied among males, with certain males displaying higher values for the percentage of motile (MOT) and progressive motile (PMOT) sperm, as well as sperm velocity (VCL, VSL, VAP) and linearity (LIN) traits. Males may thus be screened on these traits for inclusion in an artificial insemination (AI) programme to optimize fertility success rates.

1. Introduction

Variation in semen quality in terms of functionality within and between species, males as well as ejaculates, has been well documented (Songsasen and Leibo, 1997; Blanco *et al.*, 2000; King *et al.*, 2000; Yu *et al.*, 2002; Blesbois *et al.*, 2005; Chaveiro *et al.*, 2006; Roca *et al.*, 2006; Leahy and Gadella, 2011). Because of inter- and intra-male variation in ejaculate quality, semen samples should be evaluated before processing for storage and AI. The initial ejaculate quality is of the utmost importance for successful semen processing since sperm cells are irreparable (Graham and Moce, 2005; Blesbois *et al.*, 2005). Damage that is likely to occur during processing will lead to a decrease in sperm function after storage, manifested more explicitly in cryopreserved semen than in chilled or neat semen. A 40 to 70 % reduction in different sperm functions has been reported in the literature for cryopreserved sperm of both domestic and non-domestic avian species, emphasizing the importance of an ejaculate with good initial quality (Parks and Graham, 1992; Donoghue and Wishart, 2000; Watson, 2000; Gee *et al.*, 2004; Malecki *et al.*, 2008; Moce *et al.*, 2010). However, semen processing technology can be technical, costly and time consuming and should thus not be wasted on a poor semen sample. Assessing semen throughout the processing protocol can also give an indication of the type and

amount of damage exerted on the cell during the different steps and can be used as a basis for protocol optimizations.

Poor sperm production and supply has been noted as one of the primary reasons for low fertility in the ostrich industry and has stressed the importance of effective male fertility evaluation (Bertschinger *et al.*, 1992; Hemberger *et al.*, 2001; Malecki and Martin, 2003; Malecki *et al.*, 2008). The evaluation and selection of males for semen quality and potential fertility is a very important factor to consider before allowing a male into a breeding (natural or artificial reproduction, stored or non-stored) scheme. Knowledge of the capacity of an ostrich male to contribute to an artificial insemination (AI) programme would allow the timely exclusion of unfit males. As the maintenance of a resource population for AI is a costly and hazardous exercise that includes many facets. The ratio of males to females kept in a natural reproduction scheme, where a colony breeding system still dominates, can also be reduced (Lambrechts, 2004). The latter will potentially increase overall profitability by increasing chick numbers, while maintaining fewer males with a high sperm functional quality.

Recent advances in ostrich semen collection by means of the dummy-female method developed by Rybnik *et al.* (2007) facilitated obtaining true representative biological ejaculates, suitable for evaluation. Ejaculate quality was not compromised at a collection frequency of two times per day (Bonato *et al.*, 2011). Ejaculate quality can therefore be assessed in terms of different sperm functional tests developed and adapted specifically for the ostrich by Smith (Chapter II). Sperm functional tests have been well correlated with sperm survivability after storage and good fertility after artificial insemination in most other species, including man (Mahmoud *et al.*, 1998), bull (Ericsson *et al.*, 1993; Farrell *et al.*, 1998; Kasimanickam *et al.*, 2006), fowl (Wishart and Palmer, 1986) and turkey (King *et al.*, 2000). Subjective visual measures of conventional semen parameters (commonly used to evaluate sperm traits in various livestock industries) is not highly repeatable or reliable when predicting fertility and is thus not recommended (Linford *et al.*, 1976; Neuwinger *et al.*, 1990; Hoflack *et al.*, 2005; Moce and Graham, 2008). Sperm function variation can therefore be used to develop an objective, cost effective, time efficient and reliable classification system method for objective evaluation of ostrich ejaculate and male screening. The aim of this chapter is thus to describe the variation of functional sperm traits within and among ostrich ejaculates.

2. Material and methods

2.1. Animal population

Ten South African Black (SAB) (*Struthio camelus var. domesticus*) males, aged between 3 and 7 years, were allocated to the study over a period of 5 years (2011 to 2015), although ejaculates collected in 2013 and 2014 were primarily used. Three hundred and twenty six ejaculates were collected from these males using the dummy female method (Rybnik *et al.*, 2007) during winter (June to August), spring (September to November) and summer (December to February). Males in the resource population were screened from the commercial ostrich breeding flock maintained at the Oudtshoorn Research Farm on the basis of behavioural attributes rendering them suitable for AI (referred as desirable behaviour as described by Bonato *et al.*, 2013). The origin of the ostrich flock and the general management procedures implemented therein were described previously (Van Schalkwyk *et al.*, 1996; Bunter and Cloete, 2004).

2.2. Semen preparation

Ejaculates were diluted 1:1 (Malecki and Kadokawa, 2011; Sood *et al.*, 2012) after collection with the ostrich specific diluent (OS1) developed by Smith (Chapter III). OS1 diluent configuration was based on the macro mineral composition of ostrich seminal plasma.

Sperm concentration was obtained by means of spectrophotometer (Spectrawave, WPA, S800, Biochrom). The transmittance values were used to calculate sperm concentration using regression equation previously developed using the actual sperm counts from a haemocytometer. Neat and diluted samples were evaluated for sperm specific functions that included motility, viability and membrane integrity.

2.3. In vitro sperm function evaluation

2.3.1. Motility

Sperm was captured using the Sperm Class Analyzer® (SCA) version 5.3 (Microptic S.L., Barcelona, Spain) with a Basler A312fc digital camera (Basler AG, Ahrensburg, Germany), mounted on an Olympus BX41 microscope (Olympus Optical Co., Tokyo,

Japan), equipped with phase contrast optics. The neat and diluted samples were evaluated for sperm motility traits at different storage intervals over 48 hours. All sperm motility recordings were made after re-suspension of neat sperm as well as treated sperm in a standard motility buffer using Sodium Chloride (150 mM) and TES (20 mM) with male specific seminal plasma (2 %), to a final sperm concentration of 20×10^6 sperm cells/mL. After re-suspension, the tube was placed in a 38 °C water bath for 1 minute. For motility recording 2 μ l of diluted semen was placed onto a pre-warmed slide covered gently with a cover glass (22 x 22 mm) and allowed to settle for 20 seconds prior to recording. Seven to nine different fields were captured until at least 500 motile sperm tracks were obtained. The fields were captured randomly to eliminate bias towards higher sperm concentration or motility. Sperm motility traits included motility (MOT, %), progressive motility (PMOT, %), curve-linear velocity (VCL, μ m/s), straight-line velocity (VSL, μ m/s), average path velocity (VAP, μ m/s), amplitude of lateral head displacement (ALH, μ m), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), and beat-cross frequency (BCF, Hz).

2.3.2. Viability evaluation

Sperm viability was measured using the LIVE/DEAD® Sperm Viability Kit from Life technologies, that contain the SYBR® 14 and Propidium Iodide (PI) fluorescent stains. All sperm viability recordings were made after re-suspension of neat sperm, as well as treated sperm in the standard ostrich diluent, pH7 to a final sperm concentration of 20×10^6 sperm cells/mL. The SYBR® 14 working solution was prepared in a HEPES/NaCl medium to a 1:49 concentration (v/v) of SYBR® 14 to HEPES/NaCl solution. Sperm suspension aliquots of 250 μ l were re-suspended with 1.5 μ l membrane-permeant SYBR® 14 working solution and incubated for 10 minutes in a temperature controlled environment of 38 °C. After incubation 2 μ l of the next fluorescent stain, Propidium Iodide (PI), was added and incubated for 10 minutes whereafter the cells were evaluated. For evaluation of viable (green) and non-viable (red/or green with red) sperm a 2 μ l droplet was placed on a glass slide and covered with a cover glass (22 x 22 mm) and allowed to settle for 20 seconds, prior to recording. The fluorescent sperm was observed and photographed under 10x microscopy with an Olympus BX41 epifluorescent microscope (Olympus Optical Co., Tokyo, Japan), equipped with a filter camera (ColorView Illu Soft Imaging System) and software package (analysis FIVE, Olympus Soft Imaging Solutions GmbH, Münster) to count viable and non-viable sperm. Nine to ten different fields were randomly captured until at least 500 spermatozoa were recorded. Distorted fields as well

as fields that included drift or debris or clumps of sperm were excluded. The SYBR® 14 nucleic acid stain labels live sperm with green fluorescence, and membrane-impermeable PI labels the nucleic acids of membrane-compromised sperm with red fluorescence.

2.3.3. Membrane integrity evaluation

Sperm membrane integrity was measured using the Hypo-osmotic swelling test (Jeyendran *et al.*, 1984), adapted specifically for the ostrich by means of preliminary experimental exploration. The neat sperm samples for the hypo-osmotic swelling resistant sperm (HOS, %) were prepared at the same time as that of sperm motility evaluation. All sperm membrane integrity recordings were made after re-suspension of neat sperm and treated sperm in a standard salt (NaCl/H₂O) solution adapted to 25 mOsm to a final sperm concentration of 20×10^6 sperm cells/mL. For HOS recording 2 μ l of diluted semen was placed onto a pre-warmed slide, using a heated stage set at 38 °C, covered gently with a cover glass (22 x 22 mm) and allowed to settle for 20 seconds prior to recording. Sperm was captured using the Sperm Class Analyzer® (SCA) version 5.3 (Microptic S.L., Barcelona, Spain) with a Basler A312fc digital camera (Basler AG, Ahrensburg, Germany), mounted on an Olympus BX41 microscope (Olympus Optical Co., Tokyo, Japan), equipped with phase contrast optics. Seven to nine different fields were captured randomly until good representation (500 sperm) was reached and to eliminate biasness to higher sperm concentrations. Distorted fields as well as fields that included drift or debris or clumps of sperm were excluded.

2.4. Statistical analyses

Sperm traits as percentages, and with skewed distribution (as determined by the Shapiro-Wilk test: $P < 0.05$) were transformed using the arc sine of the percentage mean square-root ($\text{degree} \cdot \arcsin \sqrt{\%}$), while the sperm concentration was transformed to natural logarithms. Analyses included a distribution analysis and summary statistics to obtain variance parameters and graphs, describing the sperm traits. The total number of records, means, standard deviation, minimum, maximum and coefficient of variation (CV) was determined for each sperm trait. The contribution of each FE to a particular SFC was evaluated by expressing the sum of squares for such an effect, as a percentage of the total corrected sum of squares (TCSS) (Leighton *et al.*, 1982; Smith, 2010). General Linearized Mixed Models (GLMM) were performed to evaluate the influence of factors

such as dilution rate (D), season (S), year (A) and sperm concentration (C; as a linear covariate) affecting the different sperm traits, with the inclusion of male (M) as random effect to account for the repeated sampling of the same males. General Linear Models (GLM) were applied to assess the specific effect of variation between males and its interactions with other fixed effects (D, S, A and C). GLM were also applied to evaluate sperm concentration (C) as the response variable in analyses that included the fixed effects of M, S, D and A. Sperm quality characteristics or traits (SFC) included motility traits derived from CASA (SCA®), viability (LIVE/DEAD®) and membrane integrity (HOS) that were fitted individually to each model, as the dependent variables. Motility traits included sperm motility (MOT, %), progressive motility (PMOT, %), curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), beat-cross frequency (BCF, Hz). Viability included the percentage live and dead sperm, while membrane integrity included the number of hypo-osmotic resistant and non-resistant sperm. Least squares means, standard errors (S.E) and variation coefficients (CV) were calculated and subjected to Tukey's multiple range tests to investigate differences between least squares means. Correlations (Pearson) and regressions (linear and non-linear) were applied to investigate significant ($P < 0.05$) relationships between traits. Statistical Analysis System (SAS, version 9.3) was used for analyses performed.

An example of the GLMM fitted with Y being the dependent sperm characteristic is provided below:

$$Y_{ijkl} = \mu + M_i + D_j + S_k + A_l + b_0(C)_{ijkl} + e_{ijkl}$$

Where: Y_{ijkl} = Sperm trait under assessment
 μ = population mean
 M_i = random effect of the i^{th} male ($i = 1, 2, 3, 4, 5, 6, 7, 8, 9, 10$)
 D_j = fixed effect of the j^{th} dilution rate ($j = 1, 2$)
 S_k = fixed effect of the k^{th} season ($k = 1, 2, 3$)
 A_l = fixed effect of the l^{th} year ($l = 1, 2, 3, 4, 5$)
 C_{ijkl} = sperm concentration fitted as a linear covariate
 b_0 = regression coefficients of Y_{ijkl} on sperm concentration (C)
 e_{ijkl} = random error

3. Results

3.1. Descriptive statistics

Across the whole analyses sperm concentration (mean \pm SE) was $3.26 \times 10^9 \pm 3.27 \times 10^7$ sperm cells / mL, with a minimum of 1.73×10^9 and a maximum of 4.73×10^9 / mL. LIVE = 83.69 ± 0.70 %, HOS = 79.53 ± 0.86 %, PMOT = 48.03 ± 1.03 %, MOT = 82.55 ± 0.67 %, VCL = 67.68 ± 0.78 $\mu\text{m/s}$, VSL = 40.72 ± 0.79 $\mu\text{m/s}$, VAP = 54.54 ± 0.88 $\mu\text{m/s}$, ALH = 2.44 ± 0.02 μm , LIN = 59.27 ± 0.64 %, STR = 73.77 ± 0.60 %, WOB = 79.45 ± 0.52 % and BCF = 8.77 ± 0.08 Hz being recorded. Fewer records, 102 and 97 respectively, were obtained for LIVE and HOS, compared to motility traits due to 2014 year limitation, with relatively small variation coefficients (8.75 and 10.66 % respectively). Variation coefficients for motility and kinematic sperm traits extended from 11.10 to 36.52 %. The total number of records, means, standard deviation, minimum, maximum and coefficient of variation (CV) was recorded for each sperm trait and is presented in Table 1.

Table 1. Description of sperm viability and motility traits for ten ostrich males over a period of two years.

Sperm trait	Records	Mean	S.D	Minimum	Maximum	CV (%)
Live (%)	102	83.69	7.32	66.36	94.19	8.75
Hypo-osmotic swelling resistant (%)	97	79.53	8.48	58.42	90.67	10.66
Progressive motile (%)	292	48.03	17.54	6.30	95.40	36.52
Motile (%)	292	82.55	11.43	23.80	98.20	13.85
Curve-linear velocity velocity($\mu\text{m/s}$)	292	67.68	13.30	37.50	100	19.65
Straight-line velocity ($\mu\text{m/s}$)	292	40.72	13.48	7.20	81.50	33.10
Average path velocity ($\mu\text{m/s}$)	292	54.54	15.08	15.30	94.10	27.65
Amplitude of lateral head displacement (μm)	292	2.44	0.40	1.60	4.00	16.39
Linearity (%)	292	59.27	11.07	18.00	87.200	18.68
Straight (%)	292	73.77	10.20	28.90	97.00	13.83
Wobble (%)	292	79.45	8.82	38.10	93.90	11.10
Beat-cross frequency (Hz)	292	8.77	1.36	4.60	13.50	15.51

S.D: Standard deviation; CV: Coefficient of Variation

The contribution of each fixed effect (FE, %) with its degrees of freedom (DF), R-square value and coefficient of value and coefficient of variation (CV, %) is presented in Table 2 for LIVE, HOS, PMOT and MOT, while/a: not applicable since these sperm traits were not recorded in 2014; TCSS: Total corrected sum of squares; CV: Coefficient of variation; *P < 0.05; **P < 0.01; ***P < 0.001

Table 3: The source of variation given by the fixed effect of sperm concentration, season, dilution rate and year with their variation contribution (FE, %) and degrees of freedom (DF) on sperm kinematic traits, namely curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat-cross frequency (BCF, Hz) kinematic sperm traits (VCL, VSL, VCL, VAP, ALH, LIN, STR, WOB and BCF) are presented in Table 3. The R^2 values indicate variable response for the different SFC's ranging from 7 (% live spermatozoa) to 80 % (beat-cross frequency) of the variation explained by the fixed effects fitted. The low R^2 values associated with LIVE and HOS can partially be explained by not being able to fit year as a fixed effect when compared to analyses on motility and kinematic traits that had data available in both years analysed. The least squares means for the different SFC's are presented in Table 4 and Table 5. The latter is indicative of the variation each fixed effect exerted on the SFCs. Pearson's correlation coefficients between all sperm function traits and sperm concentrations are presented in Table 6. The FE contribution of male effect is given in Table 7 and Table 8 while categorization of ostrich males according to their sperm traits in relation to quality is given in Table 9.

Table 2: The source of variation given by the fixed effect of sperm concentration, season, dilution and year with their variation contribution (FE, %) and degrees of freedom (DF) on sperm viability (LIVE, %), membrane integrity (HOS, %), progressive motility (PMOT, %) and motility (MOT, %).

Variation source	DF	LIVE	DF	HOS	DF	PMOT	MOT
Concentration	1	0.71	1	5.86**	1	1.12	1.96
Season	1	3.01 *	1	0.23**	2	23.72***	6.30***
Dilution	1	3.85*	1	4.80*	1	2.04**	0.27
Year	n/a	n/a	n/a	n/a	1	2.40	0.58
TCSS	108	3418.55	96	3389.41	291	33646.05	20358.37
Error mean square	105	3160.58	93	3017.07	286	21181.80	18420.72
R^2		0.07		0.11		0.37	0.10
CV (%)		8.23		8.97		19.62	12.11

n/a: not applicable since these sperm traits were not recorded in 2014; TCSS: Total corrected sum of squares; CV: Coefficient of variation; *P < 0.05; **P < 0.01; ***P < 0.001

Table 3: The source of variation given by the fixed effect of sperm concentration, season, dilution rate and year with their variation contribution (FE, %) and degrees of freedom (DF) on sperm kinematic traits, namely curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat- cross frequency (BCF, Hz) being indicated.

Variation source	DF	VCL	VSL	VAP	ALH	LIN	STR	WOB	BCF
Concentration	1	0.40	0.16	0.23	0.02	0.01	0.01	0.03	0.02
Season	2	13.97***	24.82***	18.24***	4.76***	24.24***	12.17***	9.60***	1.47**
Dilution rate	1	6.71***	3.82***	5.19***	2.11**	0.68	0.00	1.18*	0.82
Year	1	3.18	1.84	1.86	0.02	0.03	0.03	0.65	3.60***
TCSS	291	51526.27	52884.94	66181.98	154.51	12945.99	13825.66	11079.01	549.94
Error mean square	286	35151.25	29705.61	41190.67	138.25	8235.79	11271.46	7368.44	506.11
R ²		0.32	0.44	0.38	0.11	0.36	0.18	0.33	0.80
CV (%)		16.38	25.03	22.01	7.76	10.63	10.53	7.99	7.74

TCSS: Total corrected sum of squares; CV: Coefficient of variation; *P < 0.05; **P < 0.01; ***P < 0.001

Table 4: The least square mean (\pm S.E) of sperm concentration, season, dilution and year on sperm viability (LIVE, %), membrane integrity (HOS, %), progressive motility (PMOT, %) and motility (MOT, %).

Variation source	LIVE	HOS	PMOT	MOT
Concentration	P > 0.05	**	P > 0.05	P > 0.05
Season	*	**	***	***
Winter	83.36 \pm 1.11 ^a	78.25 \pm 3.33 ^a	38.91 \pm 2.93 ^a	81.91 \pm 1.96 ^a
Spring	90.72 \pm 3.67 ^b	89.54 \pm 7.85 ^b	41.61 \pm 3.36 ^a	78.81 \pm 2.35 ^a
Summer	n/a	n/a	59.60 \pm 3.08 ^b	85.63 \pm 2.11 ^b
Dilution rate	*	**	***	P > 0.05
Neat_0	88.31 \pm 2.09 ^a	85.53 \pm 3.72 ^a	43.82 \pm 2.99 ^a	82.81 \pm 2.04 ^a
Diluted_1:1	85.78 \pm 2.09 ^b	82.27 \pm 3.72 ^b	49.60 \pm 2.86 ^b	81.43 \pm 1.91 ^a
Year	n/a	n/a	P > 0.05	P > 0.05
2013	n/a	n/a	46.06 \pm 2.98 ^a	81.56 \pm 2.04 ^a
2014	n/a	n/a	47.36 \pm 2.99 ^a	82.68 \pm 2.03 ^a

n/a: not applicable since these sperm traits were not recorded in 2014; *P < 0.05; **P < 0.01; ***P < 0.001; Means with different superscripts differ (P < 0.05)

Table 5: The least square means (\pm S.E) of sperm concentration, season, dilution and year on the sperm kinematic traits, namely curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat- cross frequency (BCF, Hz).

Variation source	VCL	VSL	VAP	ALH	LIN	STR	WOB	BCF
Concentration	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Season	***	***	***	***	***	***	***	**
Winter	61.54 \pm 1.91 ^a	31.92 \pm 1.86 ^a	46.18 \pm 2.01 ^a	2.51 \pm 0.05 ^a	52.32 \pm 1.36 ^a	68.55 \pm 1.90 ^a	74.57 \pm 1.30 ^a	8.86 \pm 0.19 ^a
Spring	63.29 \pm 2.33 ^b	39.37 \pm 2.22 ^b	51.53 \pm 2.48 ^b	2.36 \pm 0.07 ^{ba}	61.76 \pm 1.75 ^b	76.42 \pm 2.19 ^b	80.16 \pm 1.56 ^b	8.51 \pm 0.26 ^{ba}
Summer	72.95 \pm 2.07 ^c	49.29 \pm 1.99 ^c	61.89 \pm 2.18 ^c	2.29 \pm 0.06 ^b	66.92 \pm 1.50 ^c	78.76 \pm 2.02 ^b	83.84 \pm 1.40 ^c	9.26 \pm 0.22 ^a
Dilution	***	***	***	**	P > 0.05	P > 0.05	*	P > 0.05
Neat_0	62.05 \pm 1.99 ^a	37.22 \pm 1.93 ^a	49.27 \pm 2.10 ^a	2.32 \pm 0.06 ^a	74.74 \pm 2.00 ^a	82.81 \pm 2.04 ^a	78.60 \pm 1.35 ^a	9.01 \pm 0.21 ^a
Diluted_1:1	69.80 \pm 1.85 ^b	43.16 \pm 1.81 ^b	57.02 \pm 1.94 ^b	2.45 \pm 0.05 ^b	74.41 \pm 1.86 ^a	81.43 \pm 1.91 ^a	80.45 \pm 1.26 ^b	8.75 \pm 0.19 ^a
Year	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	***
2013	64.56 \pm 1.99 ^a	39.94 \pm 1.92 ^a	51.53 \pm 22.0 ^a	2.42 \pm 0.06 ^a	61.11 \pm 1.45 ^a	75.82 \pm 2.00 ^a	78.85 \pm 1.34 ^a	8.51 \pm 0.21 ^a
2014	67.29 \pm 1.98 ^a	40.44 \pm 1.92 ^a	54.77 \pm 2.08 ^a	2.35 \pm 0.05 ^a	59.56 \pm 1.42 ^a	73.33 \pm 2.00 ^a	80.20 \pm 1.34 ^a	9.24 \pm 0.21 ^b

*P < 0.05; **P < 0.01; ***P < 0.001; Means with different superscripts differ (P < 0.05)

3.2. *The effect of season on sperm traits*

All SFC's, namely LIVE, HOS, motility and kinematic characteristics were influenced ($P < 0.05$) by season of collection. The contribution of seasonal variation to the overall variation ranged from 0.23 to 24.82 % across SFCs with motility and kinematic parameters being more dependent ($P < 0.001$) on season, compared to LIVE and HOS. It is evident from TCSS: Total corrected sum of squares; CV: Coefficient of variation; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 4 that there was no LIVE and HOS sperm data obtained during the summer season and this may be partially responsible for the lack of marked seasonal effects on the variation in LIVE and HOS. Spring yielded the highest ($P < 0.05$) LIVE (mean \pm SE = 90.72 ± 3.67 %) and HOS (mean \pm SE = 89.54 ± 7.85 %) compared to winter. Summer was significantly better than winter and spring for most sperm motility traits e.g. PMOT (mean \pm SE = 59.60 ± 3.08 %) and MOT (mean \pm SE = 85.63 ± 2.11 %) and kinematic traits of VCL (mean \pm SE = 72.95 ± 2.07 $\mu\text{m/s}$), VSL (mean \pm SE = 49.29 ± 1.99 $\mu\text{m/s}$), VAP (mean \pm SE = 61.89 ± 2.18 $\mu\text{m/s}$), LIN (mean \pm SE = 66.92 ± 1.50 %), WOB (mean \pm SE = 83.84 ± 1.40 %). STR was the highest in both summer (mean \pm SE = 78.76 ± 2.02 %) and spring (mean \pm SE = 76.42 ± 2.19 %), with no difference ($P > 0.05$) between the two seasons for this trait. However this was significantly higher than the STR in the winter (mean \pm SE = 68.55 ± 1.90 %). BCF was the highest in summer (mean \pm SE = 9.26 ± 0.22 Hz) and differed ($P < 0.05$) from spring (mean \pm SE = 8.51 ± 0.26 Hz) that was the lowest, but was not significantly different from winter (mean \pm SE = 8.86 ± 0.19 Hz). ALH was highest in winter (mean \pm SE = 2.51 ± 0.05 μm) and did not differ ($P > 0.05$) from spring (mean \pm SE = 2.36 ± 0.07 μm), but was significantly different from summer that yielded the lowest ALH (mean \pm SE = 2.29 ± 0.06 μm). Medium positive Pearson's correlations ($P < 0.001$) were recorded between season and PMOT ($r = 0.55$), VCL ($r = 0.44$), VSL ($r = 0.61$), VAP ($r = 0.53$), LIN ($r = 0.59$), STR ($r = 0.42$) and WOB ($r = 0.53$) suggesting linear relationships. The linear relationships of season with each of the SFC's reported above have been validated by linear regressions. Figure 1 illustrates the significant ($P < 0.001$) linear regressions and R^2 values obtained for PMOT, VCL, VSL, VAP, LIN, STR and WOB. The R^2 values indicate that the linear regressions fitted explain 18 to 35 % of the variation for the different SFC's. The difference between winter and summer collections for an important trait like PMOT can be as much as 22 % taking into consideration that PMOT could increase with 10.88 % for each season, from winter to summer.

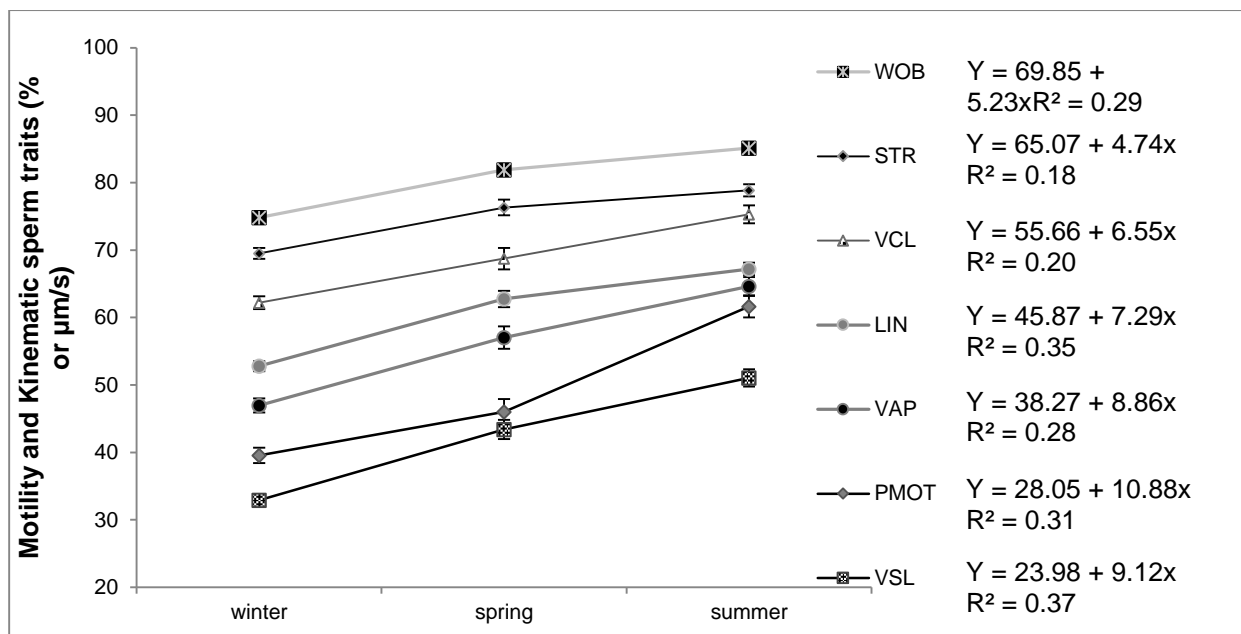


Figure 1: Influence of season on ostrich sperm function traits, namely progressive motility (PMOT, %), curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), linearity (LIN, %), straightness (STR, %) and wobble (WOB, %). Standard errors are indicated by vertical bars at the means.

3.3. The effect of dilution on sperm traits

Diluting the ejaculate after collection affects ($P < 0.05$) most SFC's measured, except ($P > 0.05$) MOT, LIN, STR and BCF. Dilution contributed to a lesser extent to the variation explained by the FE % ranging from 2.04 to 6.71 (Table 2 and Table 3). Furthermore dilution had a marked effect ($P < 0.0001$) on some of the more important sperm velocity traits associated with fertilizing ability, namely VCL, VSL and VAP. Dilution at 1:1 decreased ($P < 0.05$) LIVE and HOS by $\sim 3\%$ whereas PMOT, VCL, VSL, VAP, ALH, WOB increased ($P < 0.05$). Dilution enhanced PMOT (mean \pm SE = $49.60 \pm 2.86\%$), VCL (mean \pm SE = $69.80 \pm 1.85\ \mu\text{m/s}$), VSL (mean \pm SE = $43.16 \pm 1.81\ \mu\text{m/s}$), VAP (mean \pm SE = $57.02 \pm 1.94\ \mu\text{m/s}$), ALH (mean \pm SE = $2.45 \pm 0.05\ \mu\text{m}$) and WOB (mean \pm SE = $80.45 \pm 1.26\%$). The effect of dilution displayed in Figure 2 with exact values between undiluted and diluted samples for the different SFC's set out in Table 4 and Table 5.

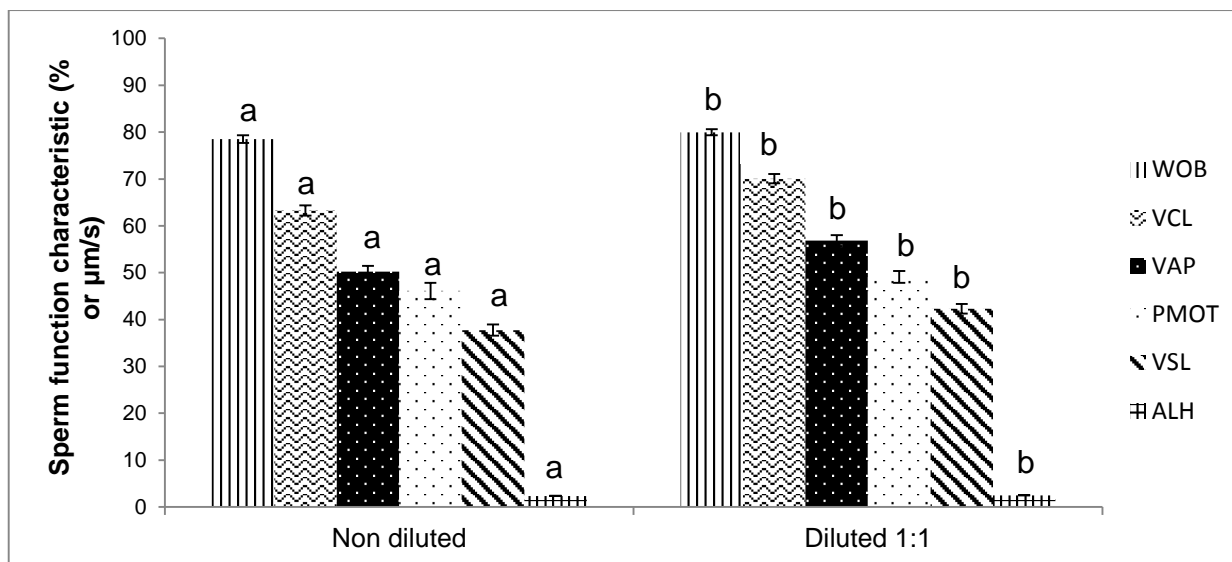


Figure 2: The effect of dilution on ostrich sperm function traits, namely wobble (WOB, %), curve-linear velocity (VCL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), progressive motility (PMOT, %), straight-line velocity (VSL, $\mu\text{m/s}$) and amplitude of lateral head displacement (ALH, μm). Standard errors are indicated by vertical bars at the means. Means with different superscripts differ ($P < 0.05$).

3.4. The effect of sperm concentration and year on sperm traits

Sperm concentration was influenced ($P < 0.01$) only by HOS with a FE contribution of 5.86 %. A negative correlation ($P < 0.05$) was recorded between HOS ($r = -0.27$) and sperm concentration (Table 6). A non-linear quadratic equation ($Y = \alpha + \beta_1X + \beta_2X^2$; $P < 0.01$) was applied (Figure 3) to quantify the relationship ($Y = 40.20 + 2.94 \times 10^{-8}X - 527 \times 10^{-20}X^2$) between HOS and sperm concentration. The R^2 value shows that 10 % of the variation in HOS was explained by the quadratic regression. The threshold point ($X = -(\beta_1/2\beta_2)$) where HOS would be maximized (81.20 %) is given by $X = 2.79 \times 10^9$ where after which HOS will decrease with an increase in sperm concentration.

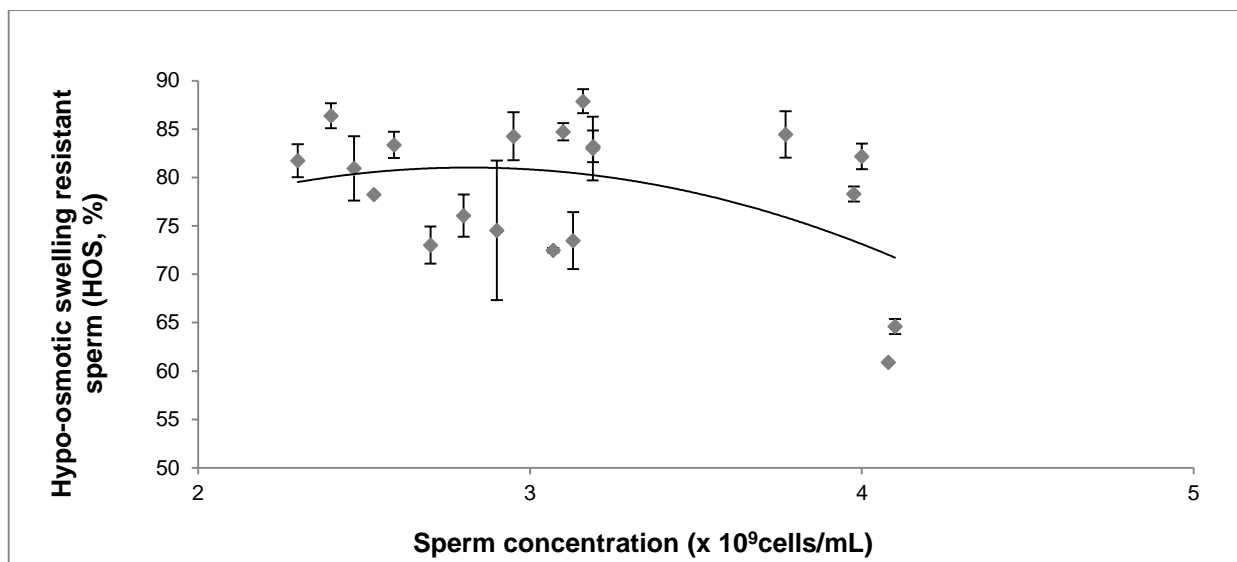


Figure 3: The quadratic relationship between membrane integrity (HOS, %) and sperm concentration. Standard errors are indicated by vertical bars at the means.

BCF was the only SFC influenced ($P < 0.001$) by year, with year contributing 3.60 % to the observed variation in this trait. BCF increased ($P < 0.05$) from 2013 (mean \pm SE = 8.51 ± 0.21 Hz) to 2014 (mean \pm SE = 9.24 ± 0.21 Hz).

3.5. Relationships among sperm traits

Relationships among sperm traits calculated as Pearson correlation coefficients are set out in Table 6. PMOT showed strong positive correlations ($P < 0.001$) with sperm motility (MOT) and kinematic traits (VCL, VSL, VAP, LIN, STR, WOB) that ranged from 0.52 to 0.83, but no correlation ($P > 0.05$) with LIVE, HOS, ALH and BCF. LIVE was positively correlated ($P < 0.05$) to MOT, VSL, VAP, LIN and WOB, correlations ranging from 0.22 to 0.41 and negatively correlated to ALH ($r = -0.18$; $P < 0.01$) and BCF ($r = -0.26$; $P < 0.05$). HOS could only be correlated ($P < 0.05$) to ALH ($r = 0.28$). The correlations ($P < 0.001$) among traits reflecting motility, namely MOT and PMOT, VCL, VSL, VAP and WOB were moderately to highly positive. VCL, VSL and VAP were highly positively correlated with each other, as well as with PMOT, MOT, LIN, STR and WOB. ALH was negatively correlated ($P < 0.05$) with VSL, LIN, STR, WOB and BCF and displayed a slight positive ($P < 0.01$) correlation with VCL.

Table 6: Pearson's correlations among ostrich sperm traits, namely progressive motility (PMOT, %), sperm viability (LIVE, %), membrane integrity (HOS, %), motility (MOT, %), curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat-cross frequency (BCF, Hz).

Sperm trait	PMOT	LIVE	HOS	MOT	VCL	VSL	VAP	ALH	LIN	STR	WOB	BCF	CONC
PMOT	1	0.14	-0.02	0.60***	0.76***	0.83***	0.77***	-0.04	0.66***	0.52***	0.61***	0.07	0.10
LIVE	0.14	1	-0.05	0.22*	0.05	0.26*	0.22*	-0.34	0.35**	0.08	0.41***	-0.26*	-0.07
HOS	-0.02	-0.05	1	0.15	0.18	0.10	0.09	0.28*	-0.15	0.00	-0.12	0.16	-0.23**
MOT	0.60***	0.22*	0.15	1	0.48***	0.38***	0.49***	0.00	0.19	0.02	0.41***	-0.04	-0.03
VCL	0.76***	0.05	0.18	0.48***	1	0.86***	0.96***	0.17**	0.47***	0.19**	0.66***	-0.07	0.09
VSL	0.83***	0.26*	0.10	0.38***	0.86***	1	0.91***	-0.14*	0.84***	0.61***	0.80***	0.17**	0.15
VAP	0.77***	0.22*	0.09	0.49***	0.96***	0.91***	1	-0.07	0.60***	0.27***	0.84***	-0.04	0.13
ALH	-0.04	-0.34**	0.28*	0.00	0.17**	-0.14*	-0.07	1	-0.39***	-0.28***	-0.46***	-0.18**	-0.09
LIN	0.66***	0.35**	-0.15	0.19	0.47***	0.84***	0.60***	-0.39***	1	0.82***	0.74***	0.36***	0.19
STR	0.52***	0.08	0.00	0.02	0.19**	0.61***	0.27***	-0.28***	0.82***	1	0.35***	0.52***	0.10
WOB	0.61***	0.41***	-0.12	0.41***	0.66***	0.80***	0.84***	-0.46***	0.74***	0.35***	1	0.03	0.16
BCF	0.07	-0.26*	0.16	-0.04	-0.07	0.17**	-0.04	-0.18**	0.36***	0.52***	0.03	1	0.07
CONC	0.10	-0.07	-0.23**	-0.03	0.09	0.15	0.13	-0.09	0.19	0.10	0.16	0.07	1

CONC: Sperm concentration; *P < 0.05; **P < 0.01; ***P < 0.001

3.6. *The effect of male on sperm traits*

The R^2 values associated with each of the models fitted for the different SFC's, that considered all other fixed effects of concentration, season and dilution, demonstrated that male effect is the largest contributor to the variation in the SFC's.

Table 7: The source of variation given by the fixed effect of male with its variation contribution (FE, %) and degrees of freedom (DF) on sperm viability (LIVE, %), membrane integrity (HOS, %), progressive motility (PMOT, %), motility (MOT, %).

Variation source	DF	LIVE	DF	HOS	DF	PMOT	MOT
Male	7	14.14**	7	4.17***	9	13.95***	19.37***
TCSS	108	3418.55	96	3389.41	291	33646.05	20358.37
Error mean square	98	2677.30	86	1587.59	278	16996.19	14583.10
R²		0.22		0.53		0.49	0.28
CV (%)		7.84		6.76		17.83	10.93

TCSS: Total corrected sum of squares; CV: Coefficient of variation; *P < 0.05; **P < 0.01; ***P < 0.001

Table 8: The source of variation given by the fixed effect of male with its variation contribution (FE, %) and degrees of freedom (DF) on sperm kinematic traits, namely curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat-cross frequency (BCF, Hz).

Variation source	DF	VCL	VSL	VAP	ALH	LIN	STR	WOB	BCF
Male	9	11.36***	9.77***	10.67***	7.02**	7.26***	15.18***	10.62***	11.80***
TCSS	291	51526.27	52884.94	66181.98	45.41	12945.99	13825.66	11079.01	549.94
Error mean square	278	30416.79	25087.29	35356.91	37.50	7300.09	9176.65	6263.93	461.03
R²		0.41	0.53	0.47	0.17	0.44	0.34	0.43	0.16
CV (%)		15.45	23.33	20.68	15.06	10.15	9.63	7.47	7.50

TCSS: Total corrected sum of squares; CV: Coefficient of variation; *P < 0.05; **P < 0.01; ***P < 0.001

Table 7 and Table 8 indicate that the male effect contributed 4.17 to 19.3 % of the variation associated with all sperm traits with the largest contribution to the percentage MOT and smallest to HOS. Males differed ($P < 0.01$) from one another for most of the SFC's except for BCF ($P > 0.05$). Variations among males for MOT, STR, VCL, LIN, VAP, PMOT and VSL are displayed in Figure 4.

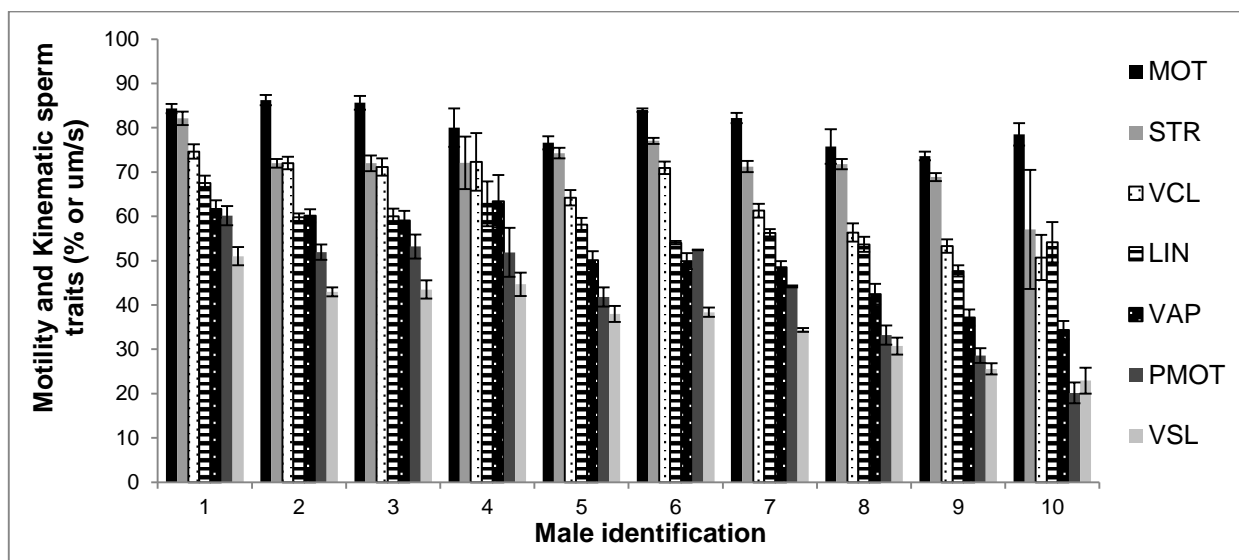


Figure 4: Ostrich male variation for sperm traits, namely motility (MOT, %), straightness (STR, %), curve-linear velocity (VCL, $\mu\text{m/s}$), linearity (LIN, %), average path velocity (VAP, $\mu\text{m/s}$), progressive motility (PMOT, %), straight-line velocity (VSL, $\mu\text{m/s}$). Standard errors are indicated by vertical bars at the means.

Male semen was categorized as good, average and poor, summarized in Table 9. Categorization depended on the distribution of closely related sperm traits (PMOT, MOT, VCL, VSL, VAP, LIN), based on Pearson correlation coefficients among males. Although the sample sizes of males were very small in this study ($n = 10$), males could be subjectively categorized on average values for the different SFC's according to the variation displayed between males.

Table 9: Categorization of ostrich males in relation to the quality of their sperm traits, namely progressive motility (PMOT, %), motility (MOT, %), curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$) and linearity (LIN, %).

Sperm trait	Poor	Average	Good
PMOT	< 40 %	40 – 50 %	> 50 %
MOT	< 70 %	70 – 80 %	> 80 %
VCL	< 60 $\mu\text{m/s}$	60 – 70 $\mu\text{m/s}$	> 70 $\mu\text{m/s}$
VSL	< 30 $\mu\text{m/s}$	30 – 40 $\mu\text{m/s}$	> 40 $\mu\text{m/s}$
VAP	< 40 $\mu\text{m/s}$	40 – 50 $\mu\text{m/s}$	> 50 $\mu\text{m/s}$
LIN	< 50 %	50 – 60 %	> 60 %

3.7. *The effect of season, dilution, male and year on sperm concentration*

Sperm concentration means were found to differ ($P < 0.001$) when fitted as the response variable in a GLM model, with the fixed effect of season, dilution, male and year. The latter FE contributed 28 % to the total variation associated with sperm concentration. Sperm concentration was influenced by the fixed effects of season ($P < 0.001$) and male ($P < 0.001$), but not by dilution rate ($P > 0.05$) or year ($P > 0.05$). Season contributed 6.17 % towards sperm concentration. Sperm concentration was different ($P < 0.001$) between seasons, with the highest ($P < 0.001$) concentration in summer (mean \pm SE = $3.42 \times 10^9 \pm 7.33 \times 10^7$ sperm cells / mL) and the lowest in winter (mean \pm SE = $3.17 \times 10^9 \pm 6.30 \times 10^7$ sperm cells / mL) and spring (mean \pm SE = $2.97 \times 10^9 \pm 8.13 \times 10^7$ sperm cells / mL), with no difference between the latter two. The relationship between seasonality and sperm concentration was confirmed by a highly significant positive Pearson's relationship ($r = 0.3$) and a linear relationship of $Y = 2.9 \times 10^9 + 1.8 \times 10^8 X$ ($R^2 = 0.08$; $P < 0.001$).

Male made the largest contribution to the variation associated with sperm concentration (FE = 16.04 %), compared to season, dilution rate and year. However, only some males differed ($P < 0.05$) from one another for sperm concentration with variation presented in

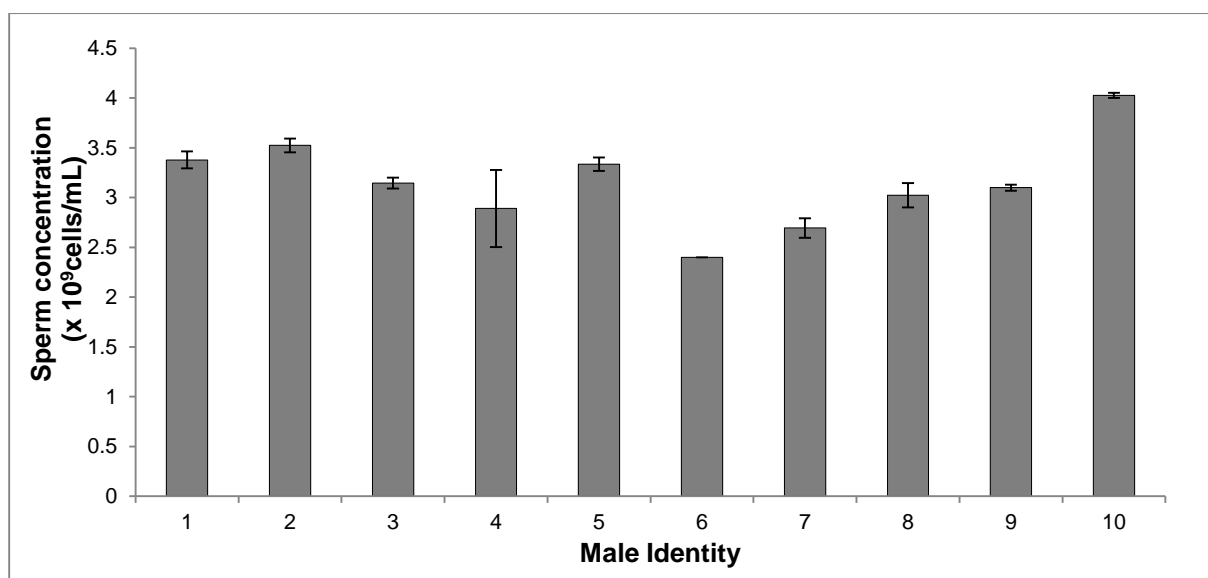


Figure 5.

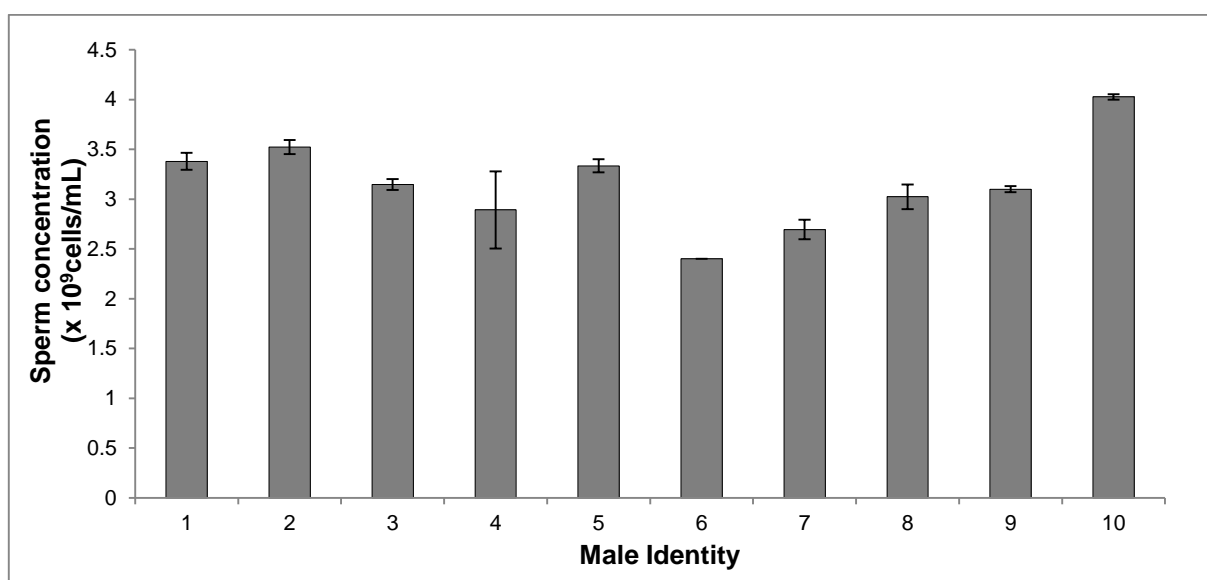


Figure 5: Between-male variation in sperm concentration. Standard errors are indicated by vertical bars at the means.

4. Discussion

4.1. The effect of season, dilution and year on sperm traits

Results indicated that season is the most influential effect on sperm traits, compared to semen dilution, year and sperm concentration, if male is considered as a random effect. Seasonality is a common phenomenon in most species, including the Ostrich and Emu (a close relative of the ostrich). Seasonality may limit reproduction to specific times of the year for the greater likelihood of offspring survival (Jarvis *et al.*, 1985; Williams *et al.*, 1995; Malecki *et al.*, 1997; Blache *et al.*, 2001). Extensive husbandry systems, like that of the ostrich, emu or free-range chickens, are more prone to the effects of seasonality, compared to indoor housing systems. An important effect, like seasonality, may increase

survivability traits through natural selection but may also be detrimental for production efficiency in terms of a steady flow of chicks throughout the year. Seasonality may be induced by a change in photoperiod, temperature, rainfall, social interactions and resource availability (Blache *et al.*, 2001; Hemberger *et al.*, 2001; Lambrechts, 2004). Different seasons may affect fresh semen function traits, resulting in impacts upon the fertilization success of the male (natural or artificial breeding system) as well as the percentage of sperm surviving processing for short- and long-term storage. The first account of ostrich seasonality on ejaculate quality in terms of volume, concentration, motility score, morphology and libido was reported by Bonato *et al.* (2014). However detailed functional sperm traits had not been considered for the ostrich, until this study.

Sperm viability and membrane integrity, (in terms of percentage live sperm -LIVE- and percentage hypo-osmotic resistant sperm -HOS- respectively) showed less dependence on season compared to motility and kinematic sperm traits although the same seasonal trend was displayed among all traits. LIVE and HOS levels reported in this study were significantly different between seasons with spring yielding the highest percentage of LIVE and HOS, compared to winter. These results are consistent with those of Bonato *et al.* (2014), who found a higher percentage of live-normal sperm derived from Nigrosin/Eosin stained slides for ostrich males in the spring to early summer, compared to winter. The high level of sperm viability and membrane integrity can possibly be associated with the ostrich's higher reproductive activity during the warmer spring months, compared to the colder winter months (Degen *et al.*, 1994; Soley and Groenewald, 1999; Rybnik *et al.*, 2012). However, it was inconsistent with other avian studies that reported low HOS due to higher temperatures, causing heat stress and testicular function disturbance (Saeid and Al-Soudi, 1975; Datta *et al.*, 1980; Santiago-Moreno *et al.*, 2009). The difference between the ostrich and other domestic avian species, like the chicken can possibly be explained by the intra-abdominal location of the ostrich testes, as well as other physiological adaptations that make them more resistant to heat stress (Maclean, 1996; Soley and Groenewald, 1999; Hemberger *et al.*, 2001).

Highest sperm motility in terms of the percentage progressive sperm (PMOT) and total percentage of motile sperm (MOT) were obtained in summer months, while the lowest were observed during winter months, with distinct differences between each of the seasons. Kinematic sperm traits, specifically sperm velocity (VCL, VSL and VAP) and swim quality (LIN and WOB) displayed the same trend as that of the PMOT and MOT.

The swim straightness (STR) of sperm seemed to be less sensitive towards seasonal change, as there was no difference between summer and spring although STR was still higher in these seasons compared to winter. The latter results are not in agreement with literature obtained for the ostrich, finding sperm motility to be fairly consistent over different seasons. The latter result can possibly be explained by the method of motility evaluation (Bonato *et al.*, 2014). However results were consistent with free-range chicken species, where sperm motility decreased with decreased photoperiod and temperature, as experienced during winter (Santiago-Moreno *et al.*, 2009).

Variations in sperm concentration due to seasonal changes were observed with higher sperm concentrations in summer, compared to spring and winter with no difference between the latter two. The effect of seasonal changes in sperm traits for the ostrich has also been reported by Rybnik *et al.* (2012) and Bonato *et al.* (2014). Furthermore, Degen *et al.* (1994) found that the increase of day light length observed in spring and early summer was associated with elevated androgen levels, and more specifically testosterone, a hormone responsible for sperm production and maturation, which could potentially increase sperm concentration (Degen *et al.*, 1994). It is clear from the results reported in this study that sperm function traits follow the same pattern as that of the ostrich breeding season with highest reproduction activity and sperm function traits in spring and early summer. Knowledge regarding ejaculate quality and quantity in the different seasons will allow managerial manipulation through assisted reproduction techniques to increase reproduction efficiency for instance, good quality ejaculates cryopreserved during spring and early summer may be inseminated during winter, when females are in production, but when ejaculates are of a poorer quality.

Dilution of the neat ostrich ejaculate caused an initial loss (~ 3 %) of sperm viability (LIVE) and membrane integrity (HOS), but has been shown to be an important attribute to maintain sperm function for further processing. Dilution is important to prolong sperm life for evaluation and storage purposes, specifically for ejaculates of low volume and high sperm cell concentration to avoid substrate depletion, toxin accumulation, pH change and increased metabolic activity (Clarke *et al.*, 1982; Bilgili *et al.*, 1987). A significant increase in the percentage of progressive motile sperm (PMOT) was observed upon dilution, as well as for important sperm velocity traits. The improvement of VCL, VSL and VAP associated with dilution can possibly be explained by the ability of diluted semen samples to maintain sperm function, compared to undiluted samples. Undiluted samples

specifically deteriorate quickly after collection, due to agglutination and hence are difficult to evaluate compared to diluted samples (Malecki *et al.*, 2008). Ciereszko *et al.* (2010) similarly observed these effects in a recent study done on ostrich semen that was evaluated undiluted and diluted (1:3) with a non-specific ostrich extender.

BCF was the only sperm trait influenced ($P < 0.05$) by year that showed significant variation between 2013 and 2014. BCF is an indication of cell oscillation and is based on specific sperm cell paths expressed in Hz (number of video frames per second). Since BCF was the only SFC influenced by year it is reasonable to suggest that the upgrade in CASA system during this time- frame could explain the variation in BCF values since Boryshpolets *et al.* (2013) reported variation in BCF values associated with a change in CASA system although the same system settings were applied.

4.2. The effect of male and sperm concentration on sperm traits and the relationship among them

The effect of male contributed substantially to the variation encountered with all the sperm function traits evaluated in this study. PMOT, MOT, VCL, VSL, VAP and LIN were observed to vary the most among males. These motility and kinematic traits could be used to categorize male and ejaculates. Sperm function characteristic and reproductive performance variation among males have been reported in other avian reproductive studies and also specifically for the ostrich (Kamar and Badreldin, 1959; Bonato *et al.*, 2010, 2011, 2014). Genetic differences among males may possibly explain the male effect observed in this study for a trait like MOT, where variation was the highest among males. Documented genetic differences in terms of seminal plasma protein concentration, amidase activity and fatty acid composition could contribute to the variation associated with sperm traits, as reported by Surai *et al.* (1998) and Ciereszko *et al.* (2010). For instance plasma proteins unique to a specific male have been found to affect sperm motility (Yoshida *et al.*, 2008; Rodrigues *et al.*, 2012) either negatively (Schoneck *et al.*, 1996) or positively (Somlev *et al.*, 1996). The percentage motile sperm has been found to be highly heritable in both mammal (cattle: 0.79; Pepper-Yowell, 2011), and avian species (Beijing-You: 0.85; Hu *et al.*, 2013). It is thus possible that ostrich males may have the same genetic variation in MOT, which would allow genetic selection and improvement of sperm motility and associated sperm traits. This is very convenient since MOT, that also indicates structural and functional integrity, may be used to identify males with good reproductive capacity because of its high correlation with fertilization potential

(Wishart and Palmer, 1986; Froman *et al.*, 1999; Blesbois *et al.*, 2008; Pepper-Yowell, 2011).

Variation in sperm concentration among ostrich males has been observed in this study. Previous studies on ostrich sperm concentration are contradictory, since some report between male variations for sperm concentration (Rybnik *et al.*, 2012) while others did not (Bonato *et al.*, 2010). However, outcomes may possibly be highly dependent on the number of males included in the study (Bonato *et al.*, 2010). The variation in sperm concentration among males can involve several factors like feed intake, body size, androgen levels, age, collection frequency and the individual's genetic make-up (Malik *et al.*, 2013). For instance, large cockerels with higher body weights are associated with increased testicular size and produce more sperm cells during spermatogenesis resulting in a higher sperm concentration (Adeyemo *et al.*, 2007; Mosenene, 2009). Furthermore, it was found that sperm concentration influenced sperm function in terms of the percentage hypo-osmotic resistant sperm: very low and very high sperm concentrations were detrimental to cells' membrane integrity as they were associated with lower percentage HOS, indicating fewer cells with functional membrane integrity. High and low sperm concentrations are associated with high levels of oxidative stress and a mediator to low fertility levels (Murphy *et al.*, 2013; Agarwal *et al.*, 2014a, b). Oxidative stress causes peroxidative damage to the cell membrane that is mostly constructed out of unsaturated fatty acids which lack the necessary cytoplasmic component containing anti-oxidants (Lenzi *et al.*, 2002; Murphy *et al.*, 2013). The loss of fatty acids, up to 60 % in severe oxidative stress conditions, renders membrane function by decreasing its fluidity, increasing non-specific permeability to ions, and inactivating membrane bound receptors and enzymes. This ultimately contributes to the poor membrane integrity of the cell, which could have possibly resulted in the lower numbers of hypo- osmotic resistant sperm observed.

Most of the correlations obtained for ostrich sperm traits observed in this study are in agreement with that reported in other avian and mammalian studies that offer the opportunity for indirect selection of associated traits and ejaculate evaluation by means of single traits assessment. Motility traits, PMOT and MOT, showed a strong positive affinity for each other and for kinematic sperm traits of VCL, VSL, VAP, LIN and WOB. VCL, VSL and VAP were highly positively correlated with each other and with STR, LIN and WOB. Although no correlation was obtained between PMOT and sperm viability

(LIVE), LIVE was positively correlated with MOT, VSL, VAP, LIN and WOB. The latter is consistent with other avian studies and suggests that any of the two characters will fairly well represent the other (Kamar and Badreldin, 1959). No correlation was obtained between HOS and LIVE, which is inconsistent with Santiago- Moreno *et al.* (2009) who reported a very high correlation of $r = 0.86$ ($P < 0.001$) between HOS and LIVE for free-range chickens.

The close affiliation between most sperm motility and kinematic traits enabled male identification on the basis of higher values for some of the most important motility and kinematic traits. High sperm velocity traits and decreased deviation from linearity were important determinants of fertilization success since it may influence the ability of the sperm to traverse the female reproductive tract to reach the site of sperm storage and fertilization (Froman *et al.*, 1999; King *et al.*, 2000). King *et al.* (2000) reported categorization of Turkey males according to a sperm mobility index and found VSL, VCL, VAP, LIN and BCF to be significantly higher in the high-mobility group with strong positive correlations between the mobility group and certain kinematic traits. Sperm velocity traits have also been used as indirect indicator of mitochondrial function of sperm (Graham *et al.*, 1984). The latter can be used for a speedy evaluation to determine sample quality for AI or suitability for further processing that may include short- or long-term storage purposes.

5. Conclusion

The variation among and within males for ejaculate sperm functional traits, including motility, kinematic traits, viability and membrane integrity, indicate the importance of evaluation prior to breeding of birds or semen processing for storage. A trait like sperm concentration, which has been used as the indicator of semen quality in most commercial ejaculate evaluation systems, together with ejaculate volume and motility scoring, is shown to be inadequate since some SFC's decline when the sperm concentration increases above a certain level. Moreover, variation among males is difficult to identify with a subjective motility scoring system. The identification of males with high SFC values *in vitro* may potentially improve fertilization ability *in vivo*, since these SFC's have been well correlated with fertilization success in other species. Favourable relationships between sperm function traits simplify evaluation of males since it is only necessary to consider two or three of these SFC's. The effects of season and dilution rate should be considered when using AI for breeding, or when semen processing for storage or

evaluation is scheduled to take place since both factors affect the SFC's appreciably. Late spring and early summer ejaculate evaluation should be sufficient to give a good representation of the male's SFC status and ejaculate suitability for further storage processing. Winter collections would be suitable when aggressive males are considered for evaluation, since testosterone levels are linked with photoperiod length and are lower during this season (Degen *et al.*, 1994), however SFC values should be corrected for the loss associated with seasonality. Dilution of ejaculates are necessary to maintain sperm function for further evaluation and processing purposes to maintain progressive motility and sperm velocity, traits directly correlated with fertilizing ability, when compared to neat ejaculates. It is however important that an optimal dilution rate in the most appropriate medium at a suitable temperature be established, specifically for the ostrich, to guarantee maximal sperm function maintenance for evaluation purposes and further processing. The latter would provide maximum utilization of good males during artificial insemination purposes since several inseminations for multiple females would be possible.

6. Acknowledgements

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CHAPTER III

DEVELOPMENT OF AN OSTRICH SPECIFIC STORAGE EXTENDER

The objectives of this study were: 1) to develop an ostrich specific diluent by means of a complete seminal plasma mineral evaluation (atomic absorption spectrophotometry), and 2) to test the storage ability of the two diluents (OS1 and OS2) formulated to maintain ostrich sperm survival over a 48 hour period at 5 °C. Formulation of OS1 was based on macro minerals (Na, K, P, Ca, Mg) and OS2 on micro minerals (Se and Zn), measured in the ostrich seminal plasma. Storage ability was evaluated by measuring sperm motility traits that included CASA parameters using the Sperm Class Analyzer® (SCA). A decrease of ~ 20 % in the percentage motile (MOT) and progressive motile (PMOT) sperm was recorded over a 24 hour storage period, when diluted in either of the OS diluents. Diluent type had no effect on sperm traits associated with curve-linear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP) or motility (MOT or PMOT). Deterioration of motile quality of sperm was associated with decreased linearity, straightness and wobbling of sperm extended without micro elements in OS1 most possibly due to oxidative damage during semen processing and storage. Male variation specifically in terms of PMOT, MOT, VCL, VSL and VAP was observed and must be considered for storage of high quality ejaculates suitable for artificial insemination purposes.

1. Introduction

Artificial insemination (AI) was advocated as a viable method to address the low and variable reproductive performance of ostriches (Malecki *et al.*, 2008), while it was also suggested as a potential solution for problems with the recording and analysis of reproduction data in a structured breeding programme (Cloete *et al.*, 2008). AI can overcome the primary reason for low fertility due to male-female incompatibility or poor sperm production (Ya-jie *et al.*, 2001; Malecki and Martin, 2003). The extension or dilution of semen has been shown to be a critical first step for semen processing, storage and preservation, as well as for maintaining sperm function *in vitro* (Blesbois *et al.*, 2005; Blesbois and Brillard, 2007) or for AI (Lake, 1960; Sexton, 1977; Sexton and Fewlass, 1978; Sexton and Giesen, 1982; Bootwalla and Miles, 1992; Tselutin *et al.*, 1995, 1999; Gerzilov *et al.*, 2011). Dilution and storage of semen offer the advantage of increased semen volume that allows for several females to be inseminated with a single ejaculate from a genetically superior male (Bootwalla and Miles, 1992) thus allowing for an increased selection intensity on the male side. In the case of low semen production, dilution can be used to extend semen to improve the efficiency of sperm usage. Dilution protects semen during storage for the maintenance of fertilizing ability during several hours of storage or transportation to distant on-farm insemination (Lake, 1960; Bootwalla and Miles, 1992).

With respect to storing ostrich semen, several diluents have been tested that include EK (Lukaszewicz, 2002), Lake's (Lake, 1960), sodium chloride (NaCl) diluents (Malecki *et al.*, 2008), Ovodyl (IMV, l'Aigle, France), minimum essential medium (MEM, GIBCO BRL Company), RPMI-1640 medium (GIBCO BRL Company) and SL (0.85 % NaCl), ZS (planning diluent), NG (modified NAUPE diluent), NF (modified NAUPE diluent), SJ₁ (modified lactalbumin hydrosylate diluent), SJ₂ (lactalbumin hydrosylate diluent)). However, these extenders were found to offer limited capacity to the maintenance of sperm viability (Ya-jie *et al.*, 2001; Malecki and Kadokawa, 2011). Ya-jie *et al.* (2001) found that MEM, RPMI-1640 and SJ₁ prolonged semen storage until complete sperm cell death for up to 80 hours, with a mean overall live sperm percentage of 26.1 ± 10.1 %. Malecki and Kadokawa (2011) found the EK extender to yield more (11 ± 1 %) live sperm, as well as higher post-thaw sperm motility compared to the HS-1 extender (Hanzawa *et al.*, 2010).

The limitation of damage exerted on the sperm cell during dilution is dependent on key elements that include a species specific extender or diluent. Specific requirements are due to unique metabolic requirements of the specie's sperm with its composition mostly based on that of the specie's seminal plasma (SP) (Siudzinska and Lukaszewicz, 2008). SP is the fluid medium mediating the chemical functions of the ejaculate, and facilitating survival and transport of spermatozoa (Mann, 1964; Juyena and Stelletta, 2012). Macro minerals (Na, K, P, Ca, Mg, Cl) and trace minerals (Mn, Zn, Cu, Fe, Se), energy substrate (glucose, fructose, sorbitol, glycerylphosphocholine) and organic components (citric acid, amino acids, peptides, proteins, lipids, hormones, cytokines) are some of the main constituents of SP with differences within and between species (Lake and Ravie, 1979; Atanasov *et al.*, 2007; Blesbois and Brillard, 2007; Long and Bakst, 2008; Siudzinska and Lukaszewicz, 2008; Barbas and Mascarenhas, 2009). Minerals are also important basic components of semen diluents as they contribute to maintaining osmotic balance, form part of important enzymatic systems and have been correlated directly with progressive motility in other avian species like the chicken (Aghaei *et al.*, 2010; Juyena and Stelletta, 2012). Notable differences in SP's major mineral components do exist between avian species (emu, a close relative to the ostrich: Malecki *et al.*, 2000; turkey: Cherms, 1967; and chicken: El Jack and Lake, 1969). The specific effects of these elements on sperm are not well covered in species-specific literature. These elements have been identified as key constituents of sperm function, maintenance and fertilization which are all vital

elements for the preservation of sperm function and eventually female fertility (Asadpour, 2012; Juyena and Stelletta, 2012). Hence, the first aim of this study was to develop an ostrich specific diluent by means of a complete ostrich seminal plasma mineral evaluation (atomic absorption spectrophotometry) that will maintain sperm viability at a high level and allow semen storage for AI in Experiment 1. The second aim was to test the storage ability of the two ostrich specific diluents (OS1 and OS2) on maintaining sperm survival over a 48 hour period at 5 °C in Experiment 2.

2. Material and Methods

2.1. Animal population and semen preparation

Males for both trials were chosen for their reliability in terms of ejaculate quality, as well as their willingness to cooperate during semen collection using the dummy female method (Rybnik *et al.*, 2007).

In Experiment 1, ejaculates were collected from seven South African Black males, aged between 3 and 7 years, at different daily intervals (day 1, 3, 7, 11, 15, 19, 21, 23, 25, 26, 27, 28) for a period of 28 days during spring (August to September). Birds were allocated to the trial to obtain initial seminal plasma mineral composition and developing two forms of diluent (OS1 and OS2) for ostrich semen.

In Experiment 2, (replicated three times), ejaculates were collected from three *South African Black* males during summer (December). The ejaculates were tested with the ostrich specific diluent adapted for macro (OS1) and micro minerals (OS2) obtained from mean concentrations of elements found in the seminal plasma during Experiment 1. Semen from each ejaculate was diluted with either OS1 or OS2. The diluted samples were then stored for 48 hours at 5 °C. Sperm motility evaluation of the neat sample and initial diluted sample took place 30 minutes after collection, while motility of the diluted stored samples was evaluated over a 48 hour storage period at time intervals of 5, 15, 24 and 48 hours.

2.2. Semen preparation

Sperm concentration was recorded in both experiments with the aid of a spectrophotometer (Spectrawave, WPA, S800, Biochrom). The transmittance values were used to calculate the sperm concentration, using a regression equation previously

developed using the actual sperm counts from a haemocytometer. Mineral composition of the seminal plasma (SP) was determined only in Experiment 1 in a sample obtained by separating the SP from the sperm by double centrifugation at 1250 revolutions per minute (RPM) for 10 min at 20 °C. Seminal plasma aliquots were stored at -20 °C until atomic absorption spectrophotometry was used to determine the concentration of each element.

2.3. *In vitro* sperm function evaluation

2.3.1 *Motility evaluation*

Sperm motility traits were evaluated only in Experiment 2 by means of the Sperm Class Analyzer® (SCA) version 5.3 (Microptic S.L., Barcelona, Spain) at different storage intervals of 0, 5, 15, 24 and 48 hours. All sperm motility recordings were made after re-suspension of the neat sperm, as well as treated sperm, in a standard motility buffer using Sodium Chloride (150mM) and TES (20mM) with male specific seminal plasma (2 %), to a final sperm concentration of 20×10^6 sperm cells / mL. After re-suspension the tube was placed in a 38 °C water bath for 1 minute. For motility recording 2 µl of diluted semen was placed onto a pre-warmed slide covered gently with a cover glass (22 x 22 mm), and allowed to settle for 20 seconds prior to recording. Seven to nine different fields were captured until at least 500 motile sperm were evaluated. At least nine fields were captured randomly to eliminate bias towards higher sperm concentrations or motility. Sperm motility traits recorded included motility (MOT, %), progressive motility (PMOT, %), curve-linear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), amplitude of lateral head displacement (ALH, µm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat-cross frequency (BCF, Hz).

2.4 *Statistical analyses*

Sperm traits as percentages, and with skewed distribution (as determined by the Shapiro-Wilk test: $P < 0.05$) were transformed using the arc sine of the percentage mean square-root ($\text{degree} \cdot \arcsin \sqrt{\%}$), while the sperm concentration was transformed to natural logarithms. Analyses included a distribution analysis and summary statistics to obtain variance parameters and graphs, describing the sperm traits. The total number of records, means, standard deviation, minimum, maximum and coefficient of variation (CV) were determined for each sperm trait. The contribution of each fixed effect (FE) to a particular sperm trait was evaluated by expressing the sum of squares for such an effect, as a

percentage of the total corrected sum of squares (TCSS) (Leighton *et al.*, 1982; Smith, 2010). Least squares means, standard errors (S.E.) and coefficients of variation (CV) were calculated and subjected to Tukey's multiple range tests to investigate differences between least squares means. Correlations (Pearson) and regressions (linear and non-linear) were used to determine significant ($P < 0.05$) relationships between traits.

Separate general mixed models (GLMM) were performed to evaluate the effect of collection day (D), semen volume (S, as a linear covariate), plasma volume (P, as a linear covariate), sperm concentration (C, as a linear covariate) and male (M, as the random effect to account for the repeated sampling of the same males) on the different seminal plasma mineral components. GLMM for sperm traits in terms of motility and kinematics included fixed effects of semen processing stage (R, neat, diluted or stored), storage time (T), diluent type (O), sperm concentration (C, as a linear covariate) and male (M) as a random effect. The general linear model (GLM) procedure was applied to assess the specific effect of variation between males in terms of sperm motility traits. All analyses were done using the Statistical Analysis System (SAS, version 9.3).

Seminal plasma minerals for Experiment 1 included sodium (Na), potassium (K), magnesium (Mg), calcium (Ca), phosphorus (P), boron (B), aluminium (Al), vanadium (V), chrome (Cr), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), selenium (Se), molybdenum (Mo), barium (Ba) and lead (Pb).

Example of the GLMM fitted, with Y being the dependent seminal plasma mineral component, in Experiment 1:

$$Y_{ij} = \mu + M_i + D_j + b_0 (S)_{ij} + b_1 (P)_{ij} + b_2 (C)_{ij} + e_{ij}$$

Where:

- Y_{ij} = Seminal plasma mineral
- μ = population mean
- M_i = random effect of the i^{th} male ($i = 1, 2, 3, 4, 5, 6, 7$)
- D_j = fixed effect of the j^{th} collection day ($j = 1, 3, 7, 11, 15, 19, 21, 23, 25, 26, 27, 28$)
- S_{ij} = semen volume fitted as a linear covariate
- P_{ij} = plasma volume fitted as a linear covariate
- C_{ij} = sperm concentration fitted as a linear covariate
- b_0 = regression coefficients of Y_{ij} on semen volume (S)
- b_1 = regression coefficients of Y_{ij} on seminal plasma volume (P)
- b_2 = regression coefficients of Y_{ij} on sperm concentration (C)
- e_{ij} = random error

Example of the GLMM fitted, with Y being the dependent sperm traits of motility, in Experiment 2:

$$Y_{ijkl} = \mu + M_i + R_j + O_k + T_l + b_0 (C)_{ijkl} + e_{ijkl}$$

Where:

- Y_{ijkl} = Sperm trait under assessment
- μ = population mean
- M_i = random effect of the i^{th} male ($i = 1, 2, 3$)
- R_j = fixed effect of the j^{th} processing stage ($j = \text{neat, diluted, stored}$)
- O_k = fixed effect of the k^{th} extender ($k = \text{Os1, Os2}$)
- T_l = fixed effect of the l^{th} storage time ($l = 0, 5, 15, 24, 48, 72$ hours)
- C_{ijkl} = sperm concentration fitted as a linear covariate
- b_0 = regression coefficients of Y_{ijk} on sperm concentration (C)
- e_{ijkl} = random error

3. Results

Experiment 1: Seminal plasma mineral composition determination and ostrich specific diluent development

3.1. Descriptive statistics

The mean (\pm SE) sperm concentration amounted to $3.77 \times 10^9 \pm 1.49 \times 10^8$ cells / mL, semen volume was 1.96 ± 0.14 mL and seminal plasma volume was 0.81 ± 0.07 mL with CV's of respectively 33.5, 60.8 and 72.1 % over 72 records. The summary of the statistics that included the number of records, mean, standard deviation, minimum, maximum and coefficient of variation for the different seminal plasma minerals components are set out in Table 10.

Table 10: Summary statistics of seminal plasma mineral concentrations determined during experiment 1.

SP mineral	Records	Mean (mM/L)	SD	Minimum (mM/L)	Maximum (mM/L)	CV (%)
Na	62	91.85942	17.30403	47.07304	139.88696	18.84
K	62	32.41265	8.41430	12.44276	48.98692	25.96
Mg	62	0.92417	0.34839	0.46183	2.25082	37.70
Ca	62	2.90148	0.63420	1.71012	4.79223	21.86
P	62	3.78829	2.53886	1.54331	13.69112	67.02
B	62	0.11930	0.04641	0.03484	0.22966	38.90
Al	62	0.00400	0.00340	0.00034	0.02253	84.90
V	62	0.00005	0.00005	0.00001	0.00027	97.07
Cr	31	0.00007	0.00012	0.00000	0.00062	167.03
Mn	62	0.00136	0.00095	0.00042	0.00467	70.01
Fe	62	0.00520	0.00237	0.00200	0.01353	45.51
Co	62	0.00034	0.00013	0.00013	0.00081	39.83
Ni	56	0.00043	0.00057	0.00004	0.00283	131.91
Cu	51	0.00236	0.00482	0.00006	0.02447	204.27
Zn	62	0.02654	0.02732	0.00421	0.15396	102.96
As	62	0.00005	0.00002	0.00001	0.00010	35.04
Se	62	0.00395	0.00133	0.00064	0.00883	33.64
Mo	62	0.00018	0.00013	0.00003	0.00057	72.36
Ba	62	0.00035	0.00016	0.00013	0.00090	45.32
Pb	61	0.00011	0.00027	0.00000	0.000003	198.68

SP: Seminal plasma; SD: Standard deviation; CV: Coefficient of variation; Na: Sodium; K: Potassium; Mg: Magnesium; Ca: Calcium; P: Phosphorus; B: Boron; Al: Aluminium; V: Vanadium; Cr: Chrome; Mn: Manganese; Fe: Iron; Co: Cobalt; Ni: Nickel; Cu: Copper; Zn: Zinc; As: Arsenic; Se: Selenium; Mo: Molybdenum; Ba: Barium; Pb: Lead

The ostrich diluent (OS1 and OS2) formulations were based on ostrich seminal plasma mineral composition as well as components of known importance in the literature for avian diluent configuration after Experiment 1 was concluded. The constituents of OS1 and OS2 can be seen in Table 11.

Table 11: Compounds contributing to the two ostrich diluents adapted for macro minerals (OS1) and micro minerals (OS2) according to the seminal plasma of the ostrich.

Component	Empirical formula	Molecular Weight	OS1 (g/L)	OS2 (g/L)
D-Glucose	$C_6H_{12}O_6$	180.16	10.00	10.00
Magnesium acetate (4H₂O)	$(CH_3COO)_2Mg \times 4H_2O$	214.46	0.18	0.18
Tripotassium citrate (H₂O)	$K_3C_6H_5O_7$	324.40	4.30	4.30
Sodium glutamate (H₂O)	$C_5H_8NNaO_4H_2O$	187.13	16.00	16.00
Sodium Phosphate dibasic (DiSodium anhydrous)	Na_2HPO_4	142.01	0.60	0.60
TES	$C_6H_{15}NO_6S$	229.25	4.59	4.59
Calcium chloride	$CaCl_2$	110.98	0.33	0.33
Sodium Selenite	Na_2O_3Se	172.94	-	0.001
Zinc Chloride	$ZnCl_2$	136.30	-	0.0055

TES: OS1: Ostrich diluent based on macro minerals OS2: Ostrich diluent based on micro minerals

The contribution of each FE (%) with its degrees of freedom (DF), R-square value and CV (%) are presented in Table 12 and Table 13 for the different seminal plasma mineral components.

Table 12: Source of variation given by the fixed effect of sperm concentration, semen volume, seminal plasma volume and day of collection with their variation contribution (FE, %), with its degrees of freedom (DF) on seminal plasma macro minerals namely Sodium (Na), Potassium (K), Phosphorus (P), Calcium (Ca) and Magnesium (Mg).

Source of variation	DF	Na	K	P	Ca	Mg
Sperm concentration	1	2.35	1.02	10.19**	1.21	10.96**
Semen volume	1	6.90**	21.12***	2.20	2.36	0.04
Seminal plasma volume	1	8.05*	3.35	1.19	3.93	3.03
Day	11	13.11	4.03	12.18	10.52	10.97
TCSS	61	18265.20	4318.83	393.19	24.53	7.40
Error mean square	47	12760.70	2970.45	306.17	20.13	5.79
R ²		0.30	0.31	0.22	0.18	0.22
CV (%)		17.94	24.53	67.37	22.56	37.99

TCSS: Total corrected sum of squares; CV: Coefficient of variation; *P < 0.05; **P < 0.01; ***P < 0.001

Table 13: Source of variation given by the fixed effect of sperm concentration, semen volume, seminal plasma volume and day of collection with their variation contribution (FE, %) with its degrees of freedom (DF) on seminal plasma micro minerals, Boron (B), Zinc (Zn), Iron (Fe), Aluminium (Al), Selenium (Se), Copper (Cu), Manganese (Mn), Cobalt (Co), Chrome (Cr), Nickel (Ni), Lead (Pb), Barium (Ba), Molybdenum (Mo), Arsenic (As), Vanadium (V).

Source of variation	DF	B	Zn	Fe	Al	Se	Cu	Mn	Co	Cr	Ni	Pb	Ba	Mo	As	V
Sperm concentration	1	3.59*	8.21*	11.47*	11.41**	8.26*	1.80	8.12*	1.29	14.6	0.04	8.20*	1.29	1.63	0.66	0.76
Semen volume	1	21.37**	2.17	2.02	0.13	2.34	4.64	5.15	2.11	1.90	0.03	12.48**	5.99*	1.00	1.69	4.71
SP volume	1	1.21	0.97	3.39	0.00	3.66	0.01	2.39	3.68	1.45	0.03	0.19	0.23	1.46	6.08	0.52
Day	11	21.62*	16.03	12.60	23.02	11.12	14.18	12.34	14.58	34.87	35.15**	9.52	26.96	76.54***	16.51	38.73**
TCSS	61	0.13	0.05	0.0003	0.0007	0.0001	0.0012	8.12	1.1*10 ⁻⁶	14.76	1.8*10 ⁻⁵	2.8*10 ⁻⁶	1.5*10 ⁻⁶	1.0*10 ⁻⁶	1.9*10 ⁻⁸	1.7*10 ⁻⁷
Error mean square	47	0.06	0.03	0.0003	0.0005	8.4*10 ⁻⁵	0.0008	5.15	8.4*10 ⁻⁷	1.90	7.6*10 ⁻⁶	1.9*10 ⁻⁶	9.1*10 ⁻⁷	2.3*10 ⁻⁷	1.5*10 ⁻⁸	9.4*10 ⁻⁷
R²		0.56	0.25	0.23	0.28	0.22	0.28	0.26	0.24	0.39	0.58	0.29	0.41	0.77	0.24	0.44
CV (%)		29.30	101.72	45.55	81.85	33.79	204.17	68.66	39.50	179.33	99.32	191.44	39.82	39.27	34.78	82.41

TCSS: Total corrected sum of squares; EMS: error mean square; CV: Coefficient of variation; *P < 0.05; **P < 0.01; ***P < 0.001

3.2. The effect of sperm concentration on seminal plasma minerals

Sperm concentration (mean \pm SEM = $3.77 \times 10^9 \pm 1.49 \times 10^8$ cells/mL) contributed ($P < 0.05$) to the variation associated with the concentration of the macro minerals of P (FE = 10.19 %; $P = 0.017$) and Mg (FE = 10.96 %; $P = 0.014$). The medium negative correlation coefficient ($r = -0.31$; $P = 0.016$) indicated an inverse linear relationship between sperm concentration and Mg with no correlation ($P > 0.05$) between sperm concentration and P. The latter was confirmed by the linear regression of $Y = 1.26 - 0.0088x$ ($P = 0.016$; $R^2 = 0.09$), implying that for every 1×10^9 / mL sperm concentration increase, a decrease of 0.0088 mM/L in Mg was observed (Figure 6).

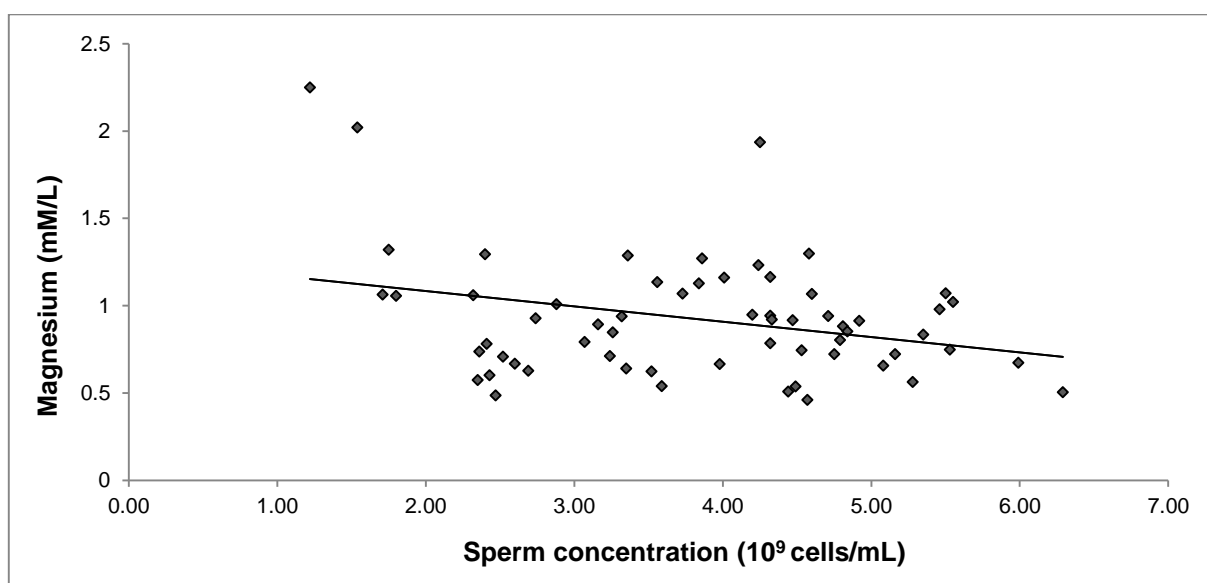


Figure 6: The linear relationship between magnesium (Mg, mM/L) and sperm concentration (10^9 sperm cells/mL).

The concentrations of the micro minerals B (FE = 3.05 %; $P = 0.053$), Zn (FE = 8.21 %; $P = 0.028$) Fe (FE = 11.47 %; $P = 0.011$), Al (FE = 11.41 %; $P = 0.009$), Se (FE = 8.26 %; $P = 0.030$), Mn (FE = 8.12 %; $P = 0.028$) and Pb (FE = 8.20 %; $P = 0.026$) varied with sperm concentration. Only Se had a medium positive correlation ($r = 0.266$; $P = 0.037$) with sperm concentration. This relationship was best explained by a linear regression ($Y = \alpha + \beta_1X$) of $Y = 0.00283 + 0.00029X$; $R^2 = 0.07$; $P = 0.037$; Figure 7).

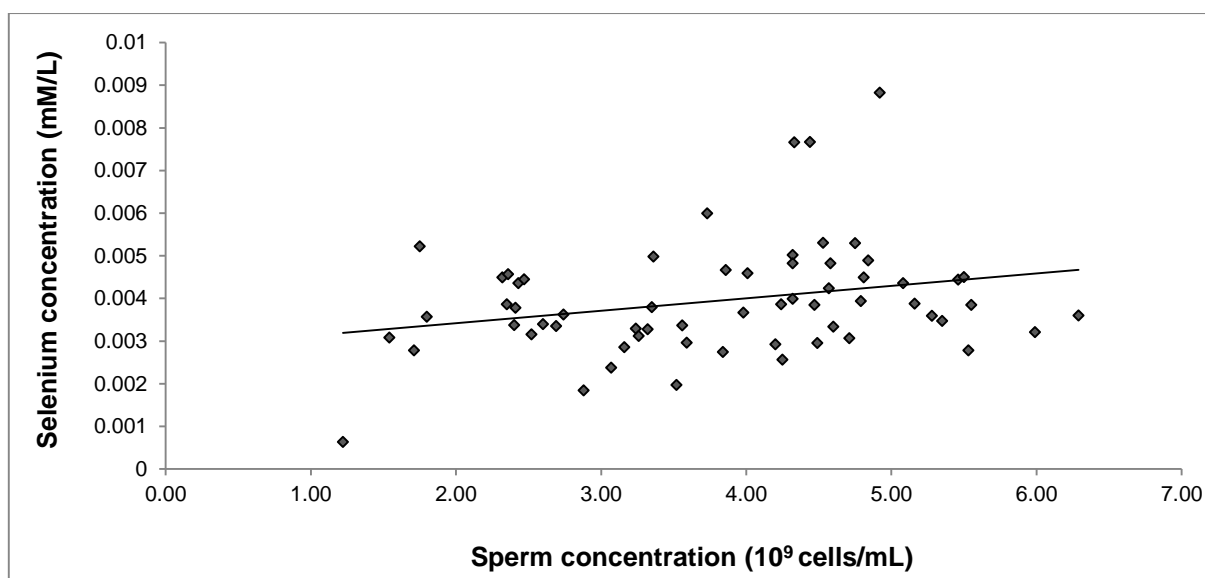


Figure 7: The linear relationship between Selenium (Se, mM/L) and sperm concentration (10⁹ sperm cells/mL).

Medium negative correlations were obtained for the concentrations of Mn ($r = -0.318$; $P = 0.012$) and Fe ($r = -0.262$; $P = 0.040$) with sperm concentration. No correlations ($P > 0.05$) were recorded between sperm concentration and B, Zn, Al and Pb, although the FE contribution of 8.21 % for sperm concentration on Zn is noteworthy. The negative correlations obtained between Mn and Fe with sperm concentration is indicative of an inverse linear relationship. The linear regression of Mn concentration on sperm numbers ($Y = 0.00232 - 2.5 \times 10^{-4}X$; $R^2 = 0.10$; $P = 0.012$), as well as Fe ($Y = 0.00716 - 5.1 \times 10^{-4}X$; $R^2 = 0.069$; $P = 0.040$) displayed in Figure 8. Mn and Fe showed the same linear relationship pattern, but at different element concentrations, Mn being lower than that of Fe over the sperm concentration range.

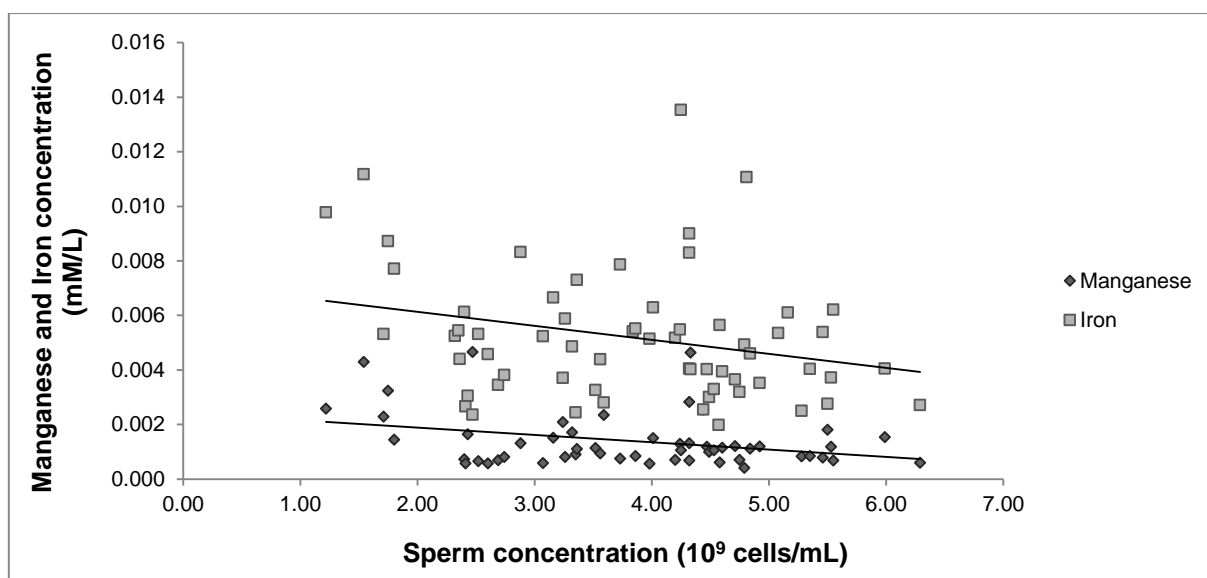


Figure 8: The linear relationship between Manganese (Mn, mM/L), as well as Iron (Fe, mM/L) with sperm concentration.

The relationship between Mn and Fe was investigated with a strong positive correlation ($r = 0.70$; $P < 0.0001$) being obtained between these two minerals. The relationship was elucidated by a linear regression of $Y = 0.0028 + 1.74X$; $R^2 = 0.49$; $P < 0.0001$; (Figure 9). It may be expected that the Fe concentration will increase with 1.74 mM/L for every 1 mM/L increase in Mn.

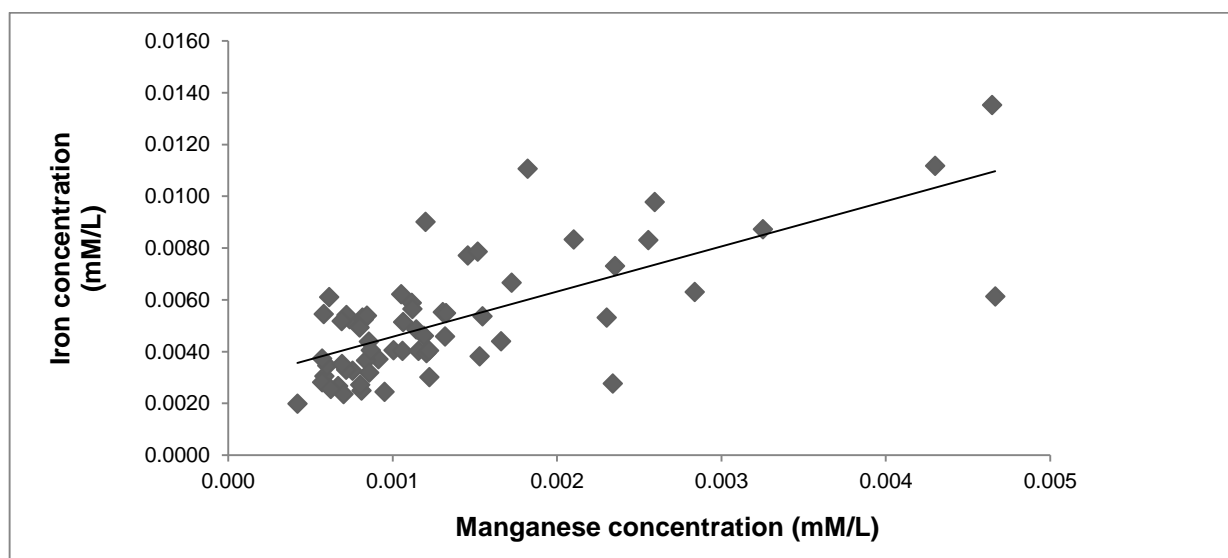


Figure 9: The linear relationship between Manganese (Mn, mM/L) and Iron (Fe, mM/L) concentration.

3.3. The effect of semen volume on seminal plasma minerals

Semen volume (mean \pm SEM = 2.13 ± 0.14 mL) contributed ($P < 0.01$) to the variation associated with the macro minerals of Na (FE = 6.90 %; $P = 0.0083$) and K (FE = 21.12 %; $P = 0.0004$). A negative correlation ($r = -0.29$; $P = 0.02$) was obtained between Na and

semen volume, with a medium positive correlation ($r = 0.44$; $P = 0.0003$) between semen volume and K. The micro minerals boron (FE = 21.37 %; $P < 0.0001$), lead (FE = 12.48 %; $P = 0.0067$) and Ba (FE = 5.99 %; $P = 0.0347$) were also influenced by semen volume. B and K showed large variation in relation to semen volume. A medium negative correlation ($r = -0.56$; $P < 0.0001$) was obtained between B and semen volume. An inverse linear relationship ($Y = 0.167 - 0.023X$; $R^2 = 0.31$; $P < 0.0001$) was obtained between B and semen volume whereby B decreased as the volume of semen increased (Figure 10).

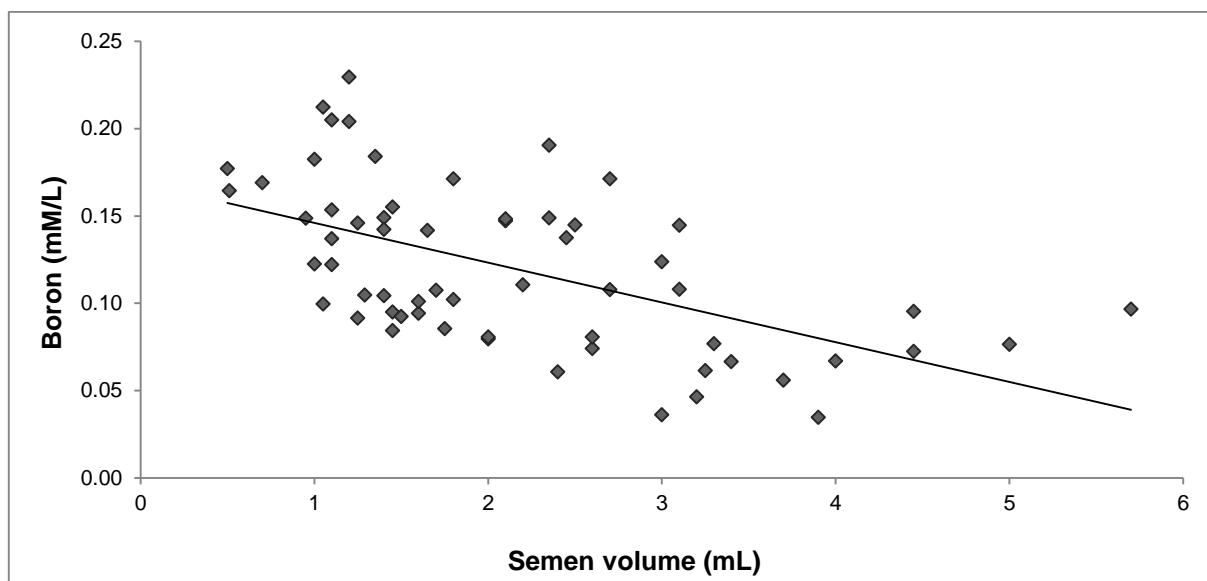


Figure 10: The linear inverse relationship between Boron (B, mM/L) and semen volume (mL).

A low positive correlation was obtained between Pb ($r = 0.39$; $P = 0.0017$) and this was confirmed by a linear relationship ($Y = -0.00005 + 0.000074X$, $R^2 = 0.16$, $P = 0.0017$; Figure 11).

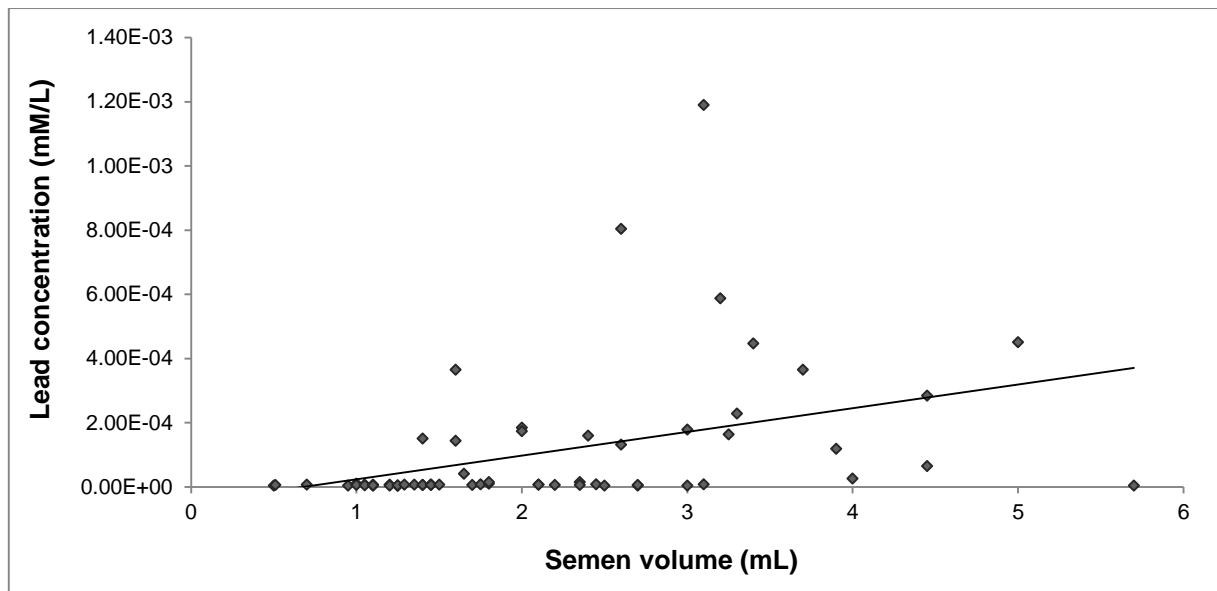


Figure 11: The linear relationship between Lead (Pb, mM/L) and semen volume (mL).

The low positive correlation ($r = 0.33$; $P = 0.0082$) between Ba and semen volume could be explained by the linear relationship $Y = 0.00025 + 0.000047X$ ($R^2 = 0.11$; $P = 0.0082$). Ba levels increased with an increase in semen volume (Figure 12).

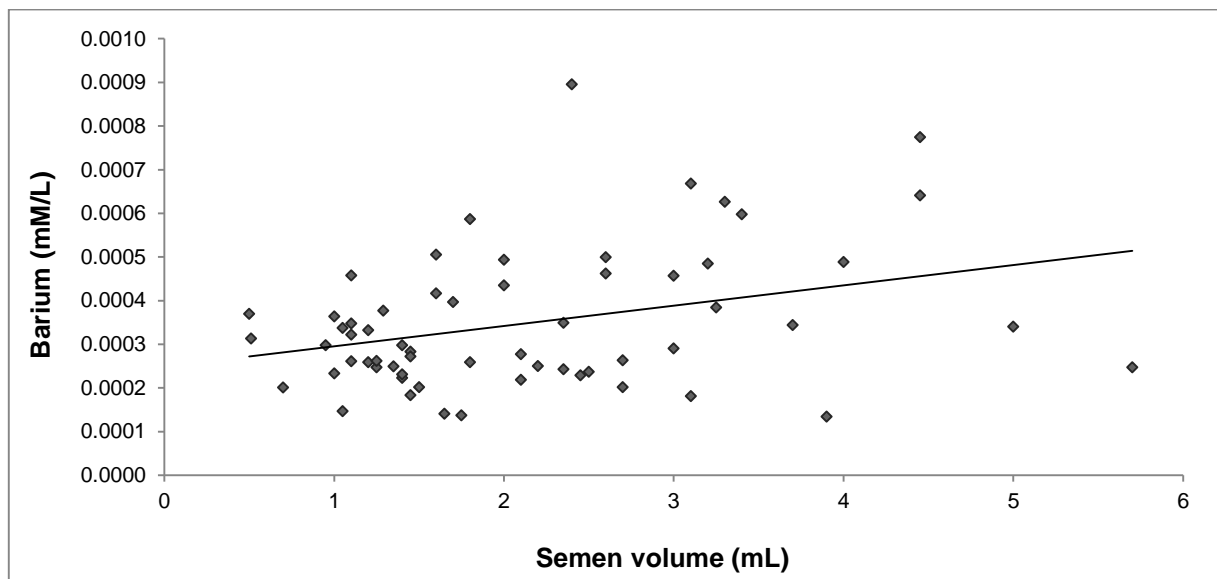


Figure 12: The linear relationship between Barium (Ba, mM/L) and semen volume (mL).

3.4. The effect of seminal plasma volume on seminal plasma minerals

Seminal plasma volume (mean \pm SEM = 1.43 ± 0.55 mL) contributed ($P < 0.05$) to the variation associated with Na (FE = 8.05 %; $P = 0.02$). The correlation obtained between Na and seminal plasma volume ($r = -0.35$; $P = 0.0056$) indicate an inverse relationship

that was confirmed by the linear regression $Y = 93.84 - 1.388X$ ($R^2 = 0.12$; $P = 0.0056$; Figure 13).

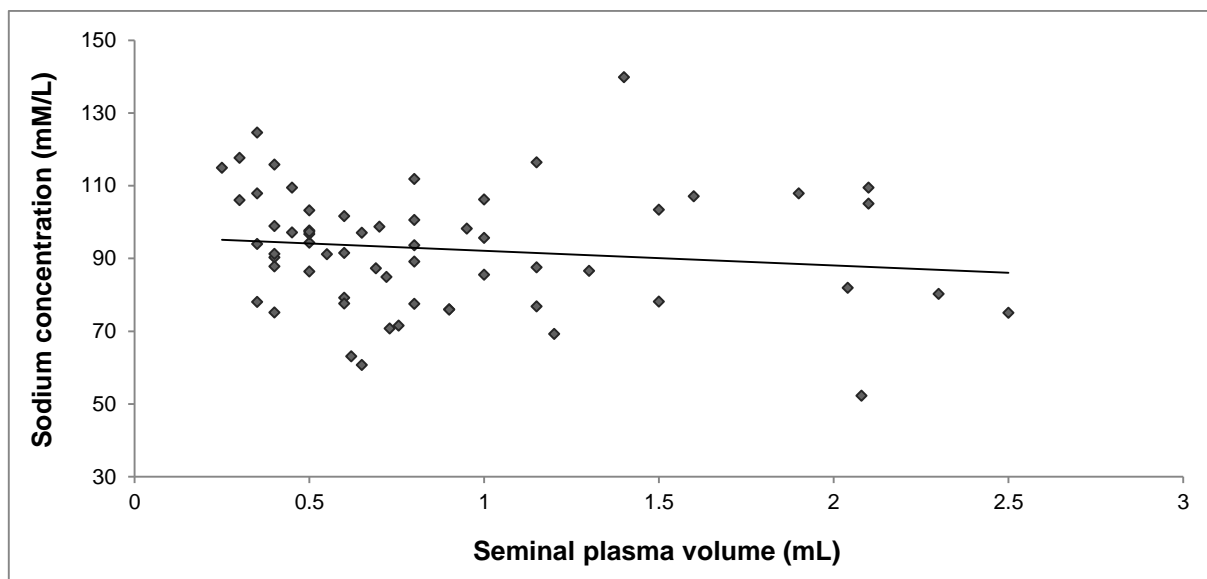


Figure 13: The inverse linear relationship between Sodium (Na, mM/L) and seminal plasma volume (mL). Standard error is indicated by vertical bars at the mean.

3.5. *The effect of collection day on seminal plasma minerals*

Day of collection contributed ($P < 0.05$) to the variation associated with the concentration of the micro minerals B (FE = 21.62 %; $P = 0.0376$), Ni (FE = 35.15 %; $P = 0.0041$), Mo (FE = 76.54 %; $P < 0.0001$) and V (FE = 38.73 %; $P = 0.044$) and not with any macro minerals. Days 7 and 19 represented the highest and lowest B concentrations and were different from each other (0.158 ± 0.019 mM/L; 0.079 ± 0.007 mM/L, respectively; $P < 0.05$), but not different ($P > 0.05$) from the rest of the days (Figure 14).

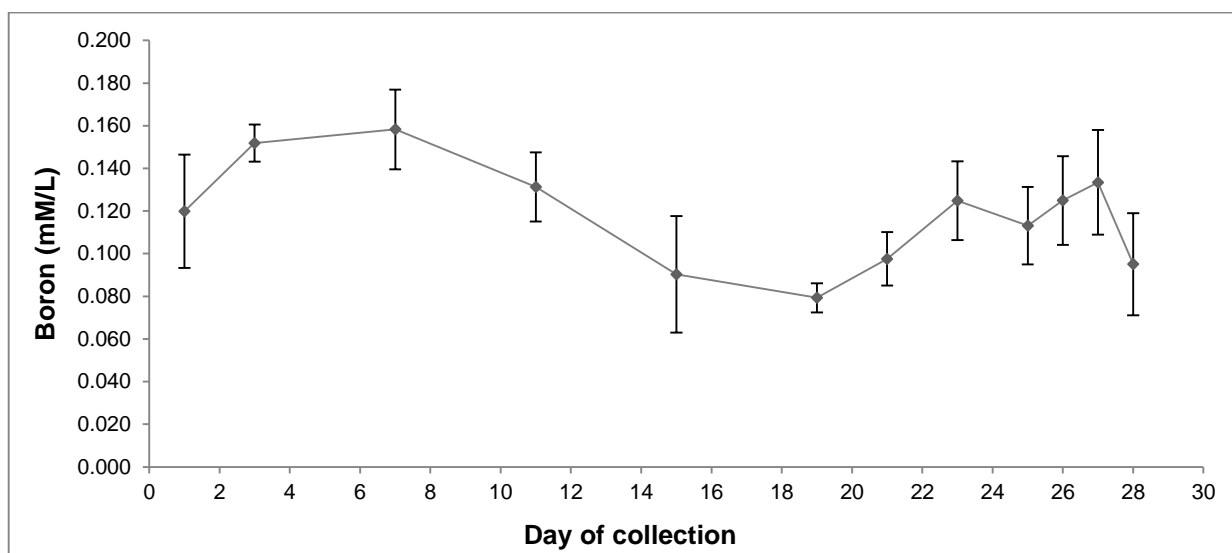


Figure 14: The influence of collection day on Boron concentration. Standard error is indicated by vertical bars at the means.

Day 1 (0.0014 ± 0.0004 mM/L) of collection was the highest in terms of Ni concentration and was different ($P < 0.05$) from all the other days except Days 3 (0.0012 ± 0.0004 mM/L) and 11 (0.0007 ± 0.0002 mM/L) as presented in Figure 15.

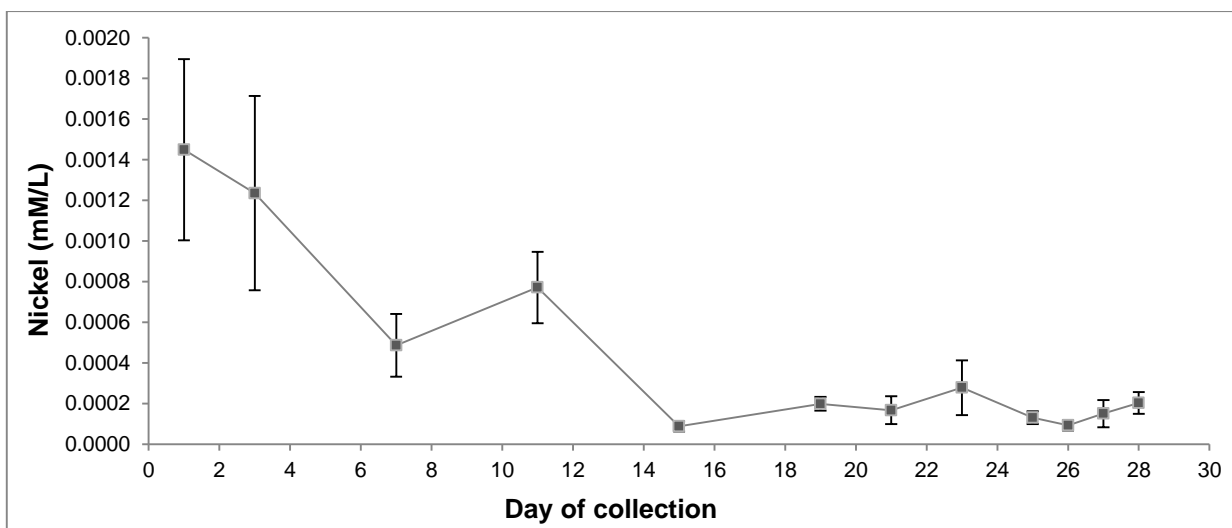


Figure 15: The influence of collection day on Nickel (Ni, mM/L). Standard error is indicated by vertical bars at the mean.

Mo concentration showed very high variation associated with the day of collection. The highest concentrations were found on Days 26 (0.00035 ± 0.00004 mM/L), 27 (0.00040 ± 0.00007 mM/L) and 28 (0.00039 ± 0.00006 mM/L), which did not differ from each other ($P > 0.05$), but from all the other days of collection. V concentration was the highest on day 15 (0.00018 ± 0.00007 mM/L) and was different ($P < 0.05$) to all other days, except

for Day 27 (0.00008 ± 0.00004 mM/L; $P > 0.05$). The influence of collection day on Mo and V is presented in Figure 16.

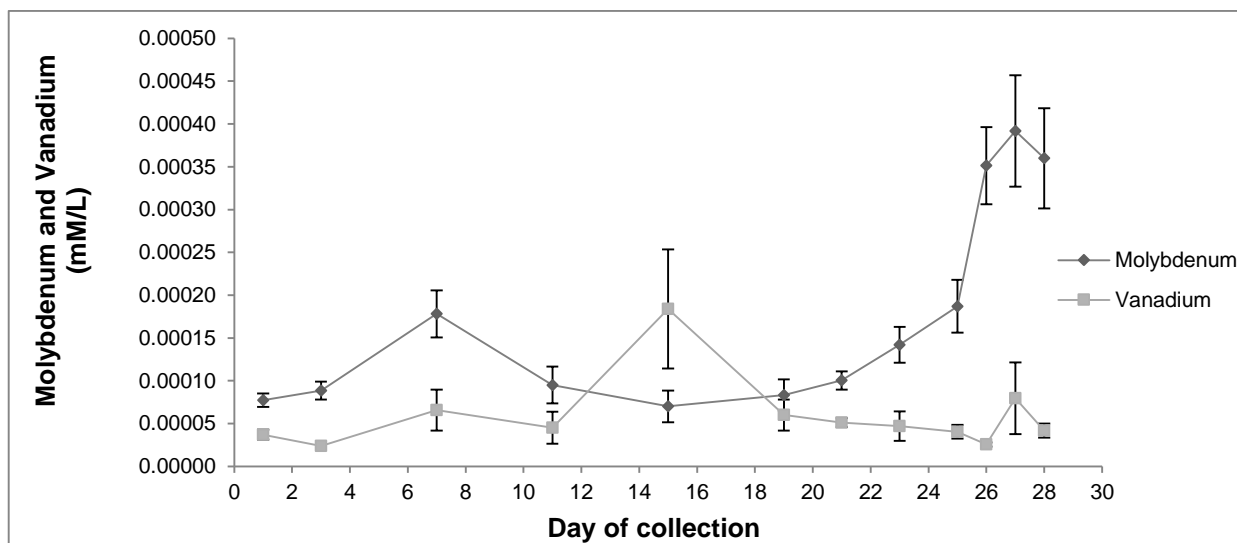


Figure 16: The influence of collection day on Molybdenum (Mo, mM/L) and Vanadium (V, mM/L) concentration. Standard error is indicated by vertical bars at the mean.

Experiment 2: The effect of the ostrich specific diluent on sperm function in vitro

3.6. Descriptive statistics

The mean (\pm SE) sperm concentration amounted to $3.37 \times 10^9 \pm 3.48 \times 10^7$ / mL with a minimum of 2.84×10^9 and a maximum of 4.01×10^9 / mL and a CV of 11.22 %. PMOT = 37.54 ± 2.36 %, MOT = 70.34 ± 1.97 %, VCL = 55.68 ± 1.94 μ m/s, VSL = 35.84 ± 1.76 μ m/s, VAP = 44.91 ± 2.03 μ m/s, ALH = 1.96 ± 0.06 μ m, LIN = 59.73 ± 1.56 %, STR = 76.26 ± 1.12 %, WOB = 76.35 ± 1.37 % and BCF = 7.80 ± 0.23 Hz was obtained across the analyses (Table 14). A total of 108 records were evaluated for sperm motility and kinematic traits with CV's ranging from 15.32 to 65.30 %.

Table 14: Description of sperm motility and kinematic traits used to assess sperm survival over 48 hours stored in the ostrich specific diluents (OS1 and OS2).

Sperm Trait	Records	Mean	SD	Minimum	Maximum	CV (%)
Progressive motile (%)	108	37.54	24.51	0.00	77.70	65.30
Motile (%)	108	70.34	20.50	9.90	94.60	29.15
Curve-linear velocity ($\mu\text{m/s}$)	108	55.68	20.14	12.60	99.50	36.18
Straight-line velocity ($\mu\text{m/s}$)	108	35.84	18.24	0.70	72.50	50.90
Average path velocity ($\mu\text{m/s}$)	108	44.91	21.13	2.70	91.00	47.05
Amplitude of lateral head displacement (μm)	108	1.96	0.58	0.00	2.80	29.58
Linearity (%)	108	59.73	16.20	5.60	81.00	27.12
Straight (%)	108	76.26	11.69	26.30	94.20	15.32
Wobble (%)	108	76.35	14.24	21.30	92.30	18.65
Beat - cross frequency (Hz)	108	8.00	2.42	0.00	11.40	30.31

SD: Standard deviation; CV: Coefficient of variation

The contribution of each FE (%), with its degrees of freedom (DF), R-square value and CV (%) are set out in Table 15 and Table 8 for sperm motility traits. The least squares means for FE's of these traits are presented in Table 7 and Table 18. The latter is indicative of the influence each fixed effect (sperm concentration, storage time, diluent type, processing stage and male) exerted on the sperm traits.

Table 15: The source of variation for the fixed effect of sperm concentration, storage time, diluent type and processing stage with their variation contribution (FE, %) and degrees of freedom (DF) on sperm kinematic traits, progressive motility (PMOT, %), motility (MOT, %), curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat- cross frequency (BCF, Hz).

Variation source	DF	PMOT	MOT	VCL	VSL	VAP	ALH	LIN	STR	WOB	BCF
Sperm concentration	1	2.42	2.96	1.04	1.20	0.82	1.29	0.88	1.09	0.40	0.10
Storage time	1	51.90***	45.53***	43.53***	48.90***	44.37***	37.73***	51.44***	42.04***	48.60***	29.06***
Diluent type	1	0.00	0.06	0.02	0.28	0.13	0.01	2.57**	3.47**	1.81*	0.32
Stage	2	5.54***	1.54**	11.82***	10.89***	11.46***	1.91*	3.71**	4.63**	3.48**	2.26
TCSS	114	33509.75	19821.45	44438.73	36301.32	48841.01	331.13	10791.35	6046.76	9315.59	1661.16
Error mean square	109	6331.75	3848.59	11550.80	8892.28	12348.17	182.39	3899.48	3145.29	2993.97	1105.39
R ²		0.811	0.80	0.74	0.76	0.75	0.45	0.64	0.48	0.68	0.33
CV (%)		21.18	10.14	18.49	25.22	23.70	16.40	11.78	8.75	8.49	19.78

Stage: processing stage (neat, diluted, soled); TCSS: Total corrected sum of squares; CV: Coefficient of variation; *P < 0.05, **P < 0.01, ***P < 0.001

Table 16: The least square means (\pm S.E) of sperm concentration, storage time, diluent type and processing stage on the sperm kinematic traits, progressive motility (PMOT, %), motility (MOT, %), curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat- cross frequency (BCF, Hz).

VS	PMOT	MOT	VCL	VSL	VAP	ALH	LIN	STR	WOB	BCF
SC	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
ST	***	***	***	***	***	***	***	***	***	***
0 hours	67.54 \pm 1.61 ^a	88.43 \pm 1.20 ^a	80.81 \pm 2.27 ^a	57.05 \pm 1.40 ^a	71.14 \pm 2.26 ^a	2.30 \pm 0.07 ^a	70.80 \pm 0.87 ^a	80.54 \pm 1.01 ^a	87.93 \pm 0.58 ^a	8.21 \pm 0.17 ^a
5 hours	53.62 \pm 1.94 ^b	79.42 \pm 1.22 ^b	69.34 \pm 2.21 ^b	49.53 \pm 1.95 ^b	59.38 \pm 2.23 ^b	2.29 \pm 0.05 ^{ba}	71.04 \pm 0.85 ^{ab}	83.28 \pm 0.80 ^b	85.17 \pm 0.62 ^b	8.84 \pm 0.13 ^{ba}
15 hours	29.39 \pm 5.25 ^{cd}	71.26 \pm 3.34 ^{cd}	47.71 \pm 3.07 ^{cd}	29.84 \pm 2.85 ^{cd}	37.32 \pm 3.19 ^{cd}	2.02 \pm 0.05 ^{cd}	61.67 \pm 2.69 ^{cd}	79.39 \pm 1.43 ^{cd}	77.39 \pm 2.29 ^{cd}	8.95 \pm 0.22 ^{cba}
24 hours	24.47 \pm 2.94 ^{dc}	67.92 \pm 2.00 ^{dc}	45.96 \pm 1.75 ^{dc}	26.84 \pm 2.20 ^{dc}	34.08 \pm 2.12 ^{dc}	1.99 \pm 0.04 ^{dc}	56.97 \pm 2.71 ^{dc}	77.19 \pm 1.66 ^{dc}	73.08 \pm 2.01 ^{dc}	9.33 \pm 0.18 ^{dc}
48 hours	3.78 \pm 0.90 ^e	37.63 \pm 3.38 ^e	28.66 \pm 1.71 ^e	10.66 \pm 1.37 ^e	16.39 \pm 1.55 ^e	1.14 \pm 0.16 ^e	34.61 \pm 3.59 ^e	59.38 \pm 3.66 ^e	54.48 \pm 3.32 ^e	4.57 \pm 0.81 ^e
OS	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	**	**	*	P > 0.05
OS1	39.68 \pm 4.82 ^a	73.12 \pm 2.78 ^a	56.54 \pm 5.24 ^a	35.04 \pm 3.86 ^a	45.29 \pm 5.03 ^a	1.91 \pm 0.07 ^a	55.97 \pm 1.62 ^a	72.66 \pm 1.30 ^a	74.31 \pm 2.15 ^a	7.61 \pm 0.35 ^a
OS2	39.23 \pm 4.84 ^a	72.21 \pm 2.80 ^a	56.99 \pm 5.26 ^a	36.89 \pm 3.87 ^a	46.73 \pm 5.05 ^a	1.91 \pm 0.07 ^a	60.87 \pm 1.67 ^b	76.86 \pm 1.35 ^b	78.17 \pm 2.18 ^b	7.91 \pm 0.3 ^a
Stage	***	*	***	***	***	*	**	**	**	P > 0.05
Neat	28.75 \pm 5.20 ^a	72.77 \pm 3.19 ^a	45.02 \pm 5.24 ^a	25.17 \pm 4.15 ^a	33.90 \pm 5.33 ^a	1.75 \pm 0.10 ^a	52.89 \pm 2.38 ^a	71.84 \pm 2.04 ^a	71.86 \pm 2.62 ^a	7.89 \pm 0.50 ^a
Diluted	52.55 \pm 5.31 ^b	75.17 \pm 3.30 ^{ba}	69.32 \pm 5.57 ^b	45.99 \pm 4.22 ^b	58.96 \pm 3.54 ^b	1.95 \pm 0.11 ^{ba}	60.54 \pm 2.55 ^{bc}	74.14 \pm 2.20 ^{bc}	79.40 \pm 2.73 ^{bc}	6.98 \pm 0.53 ^a
Stored	37.06 \pm 4.72 ^c	70.06 \pm 2.65 ^{ca}	55.94 \pm 5.17 ^c	36.72 \pm 3.77 ^c	45.17 \pm 4.95 ^c	2.03 \pm 0.06 ^{cb}	61.84 \pm 1.36 ^{cb}	78.30 \pm 1.03 ^{cb}	77.47 \pm 2.02 ^{cb}	8.41 \pm 0.30 ^a

VS: Variation source; SC: Sperm concentration; ST: Storage time; OS: Ostrich specific diluent; Stage: processing stage (neat, diluted, stored); *P < 0.05, **P < 0.01, ***P < 0.001

3.7. The effect of sperm concentration on sperm motility traits

Sperm concentration did not affect any of the sperm traits measured ($P > 0.05$) and accounted for between 0.10 % of the variation in BCF to 2.96 % of the variation in MOT. Further analyses to explore the relationship between these traits and sperm concentration were thus discarded.

3.8. The effect of diluent type on sperm motility traits

Ostrich diluent type (OS1 and OS2) affected ($P < 0.05$) only certain sperm kinematics, namely LIN ($P = 0.0061$), STR ($P = 0.0082$) and WOB ($P = 0.0120$) with no effect ($P > 0.05$) on any of the other motility or kinematic sperm traits. The diluent's contribution towards variation was highest for STR (FE = 3.47 %), then LIN (FE = 2.57 %) and the lowest towards WOB (FE = 1.81 %). LIN (OS1 = 55.97 ± 1.62 %; OS2 = 60.87 ± 1.67 %), STR (OS1 = 72.66 ± 1.30 %; OS2 = 76.86 ± 1.35 %) and WOB (OS1 = 74.31 ± 2.15 %; OS2 = 78.17 ± 2.18 %) was highest ($P < 0.05$) in OS2, compared to OS1 (Figure 12). All three kinematic traits (STR, LIN, WOB) influenced by diluent type, were strongly positively correlated ($r = \geq 0.9$; $P < 0.0001$).

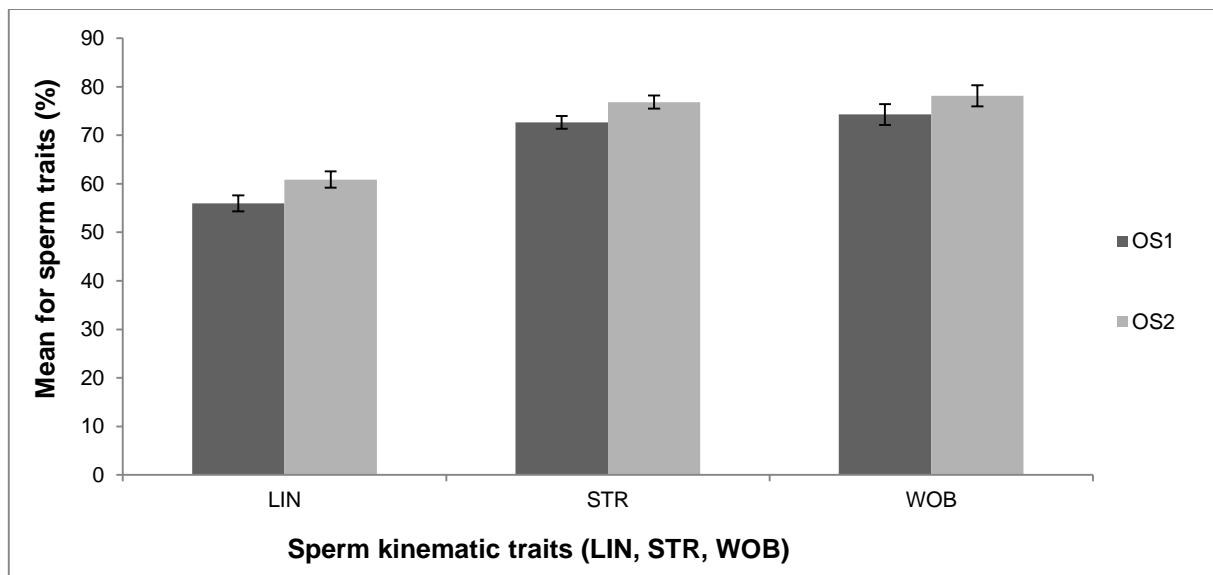


Figure 17: Kinematic sperm traits of wobble (WOB, %), straightness of swim (STR, %) and linearity (LIN, %) influenced by the Ostrich specific diluent type (OS1 and OS2). Standard error is indicated by vertical bars at the mean.

3.9. The effect of stage on sperm motility traits

Stage of processing, including neat, diluted and stored samples contributed between 1.54 (MOT) and 11.82 % (VCL) of the variation associated with all sperm traits ($P < 0.05$; Table

6). PMOT (52.55 ± 5.31 %), VCL (69.32 ± 5.57 $\mu\text{m/s}$), VSL (45.99 ± 4.22 $\mu\text{m/s}$) and VAP (58.96 ± 3.54 $\mu\text{m/s}$) was highest ($P < 0.05$) in diluted samples compared to neat and stored samples (Table 7). MOT was not significantly higher after dilution of the semen (MOT = 75.17 ± 3.30 %), compared to neat sperm motility, but significantly deteriorated with the storage (70.06 ± 2.65 %). ALH of sperm was significantly higher after semen storage (2.03 ± 0.06 μm) compared to the unprocessed neat semen (1.75 ± 0.10 μm), however not different ($P > 0.05$) from diluted semen samples. LIN, STR and WOB were significantly higher after dilution (LIN = 60.54 ± 2.55 %; STR = 74.14 ± 2.20 %; WOB = 79.40 ± 2.73 %) compared to neat semen (LIN = 52.89 ± 2.38 %; STR = 71.84 ± 2.04 %; WOB = 71.86 ± 2.62 %), but remained at the same ($P > 0.05$) levels, after storage.

3.10. The effect of storage time on sperm motility traits

Storage time influenced ($P < 0.0001$) all sperm traits and was by far the largest contributor towards the observed variation. FE ranged from 29.06 % for BCF to 51.90 % for PMOT, compared to smaller values for the other FE, namely sperm concentration, processing stage and diluent type (Table 6). PMOT, MOT, VCL, VSL, VAP, LIN, STR and WOB all deteriorated ($P < 0.05$) from 0 to 15 hours of storage, with no significant further deterioration between 15 and 24 hours. Thereafter another significant decrease occurred from 24 hours to 48 hours for all traits (Figure 13). ALH did not decrease ($P > 0.05$) from 0 to 5 hours, but decreased ($P < 0.05$) from 5 to 15 hours, with no difference between 15 and 24 hours. Another significant decrease followed from 24 to 48 hours, same as for all the other traits measured. BCF was stable between 0 to 24 hours of storage with no significant decrease ($P > 0.05$) while from 24 to 48 hours a significant decrease was observed ($P < 0.05$). The regression of most sperm traits namely PMOT, MOT, VCL, VSL, VAP, ALH and BCF on storage time were best ($P < 0.0001$), explained by the following linear regressions: PMOT ($Y = 54.40 - 1.04X$; $R^2 = 0.66$), MOT ($Y = 86.43 - 0.95X$; $R^2 = 0.79$), VCL ($Y = 68.36 - 0.80X$; $R^2 = 0.58$), VSL ($Y = 47.50 - 0.74X$; $R^2 = 0.61$), VAP ($Y = 58.38 - 0.85X$; $R^2 = 0.60$), ALH ($Y = 2.30 - 0.02X$; $R^2 = 0.51$) and BCF ($Y = 9.26 - 0.07X$; $R^2 = 0.34$). The linear equations obtained for PMOT, MOT, VCL, VSL, VAP, ALH and BCF indicated declines of 1.04 %, 0.95 %, 0.80 $\mu\text{m/s}$, 0.74 $\mu\text{m/s}$, 0.85 $\mu\text{m/s}$, 0.02 μm and 0.07 Hz respectively, for every hour of storage.

The regressions of LIN, STR and WOB on storage time were best ($P < 0.0001$) explained by the quadratic functions of $Y = \alpha + \beta_1X + \beta_2X^2$ and given as LIN ($Y = 70.35 - 0.70X +$

0.0013X²; R² = 0.59), STR (Y = 82.17 – 0.32X – 0.001X²; R² = 0.39) and WOB (Y = 86.26 – 0.71X + 0.003X²; R² = 0.62). The latter indicated that LIN, STR and WOB would be maintained at maxima of 67.09, 81.31 and 84.6 % after 4.7, 2.7 and 2.3 hours of storage respectively, before deterioration of these traits will take place with a further increase in storage time. The influence of storage time on each sperm trait, except for ALH and BCF is illustrated in Figure 18.

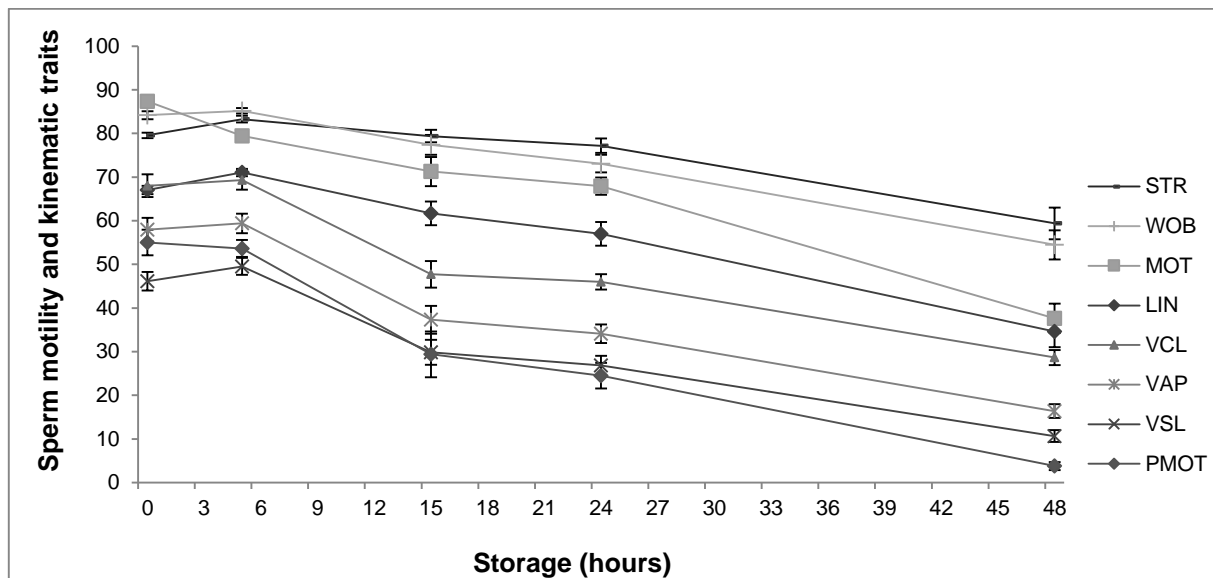


Figure 18: The influence of storage time over 48 hours on the motility and kinematic sperm traits, progressive motile (PMOT, %), motility (MOT, %), curve-linear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linear (LIN, %), straight (STR, %) and wobble (WOB, %). Standard error is indicated by vertical bars at the mean.

3.11. The effect of the diluent x storage time interaction on sperm motility traits

LIN, STR and WOB were the only sperm kinematic traits influenced ($P < 0.0001$) by an interaction of semen diluent and storage time, with no difference ($P > 0.05$) between the sperm traits at initial dilution (equivalent to 0 hours of storage). LIN (OS1 = 53.86 ± 3.05 %; OS2 = 59.99 ± 3.05 %), STR (OS1 = 75.12 ± 2.93 %; OS2 = 79.26 ± 2.93 %) and WOB (OS1 = 70.93 ± 2.72 %; OS2 = 75.28 ± 2.72 %) were maintained by OS1 and OS2 until 24 hours of storage ($P > 0.05$). OS2 maintained a higher ($P < 0.001$) percentage LIN (OS1 = 27.14 ± 2.90 %; OS2 = 42.13 ± 2.90 %), STR (OS1 = 51.39 ± 2.78 %; OS2 = 67.36 ± 2.78 %) and WOB (OS1 = 48.04 ± 2.59 %; OS2 = 61.06 ± 2.59 %), with increased storage time of up to 48 hours. The effect of diluent type with an increase in storage time for STR and LIN is shown in Figure 19.

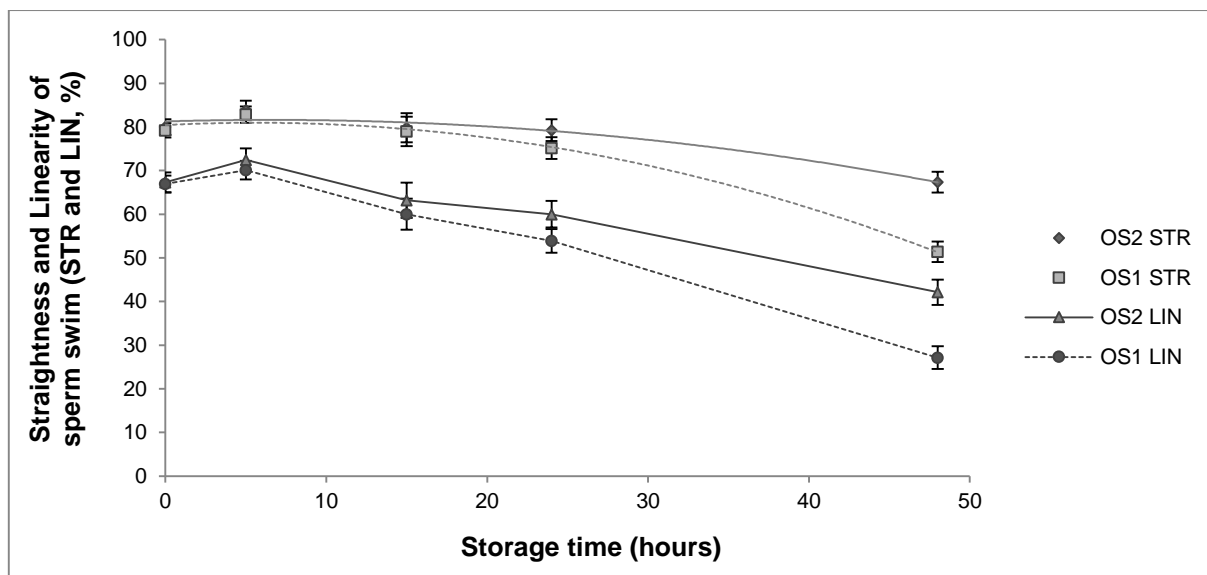


Figure 19: The influence of diluent type (OS1 and OS2) over a storage time of 48 hours on straightness (STR, %) and linearity (LIN, %) of sperm swim. Standard error is indicated by vertical bars at the mean.

3.12. The effect of male and the interaction of male and storage time on sperm motility traits

Males fitted as an FE contributed to the variation associated with PMOT ($P < 0.001$), MOT ($P < 0.01$), VCL ($P < 0.001$), VSL ($P < 0.001$), VAP ($P < 0.001$) and WOB ($P < 0.05$) and accounted for 2.03 % (WOB) to 6.72 % (VCL) of the observed variation, as presented Table 8. Furthermore, VCL, VSL and VAP showed the most variation (> 4 %) between males. Overall, means for two of the males (Male 1 and 2) were mostly not different from each other ($P > 0.05$) while Male 3 was significantly superior compared to the other two males for PMOT, MOT, VCL, VSL, VAP and WOB (Table 9).

MOT was the only sperm trait influenced ($P < 0.0001$) by the interaction effect of male with storage time, demonstrated in Figure 20. Although MOT declined for all males from 0 hours to 48 hours of storage, Male 3 and, to a lesser extent Male 2, maintained MOT better than Male 1. MOT for Male 3 only declined with 39.44 % from 0 hours to 48 hours of storage, compared to Male 1 (47.93 %) and Male 2 (67.42 %).

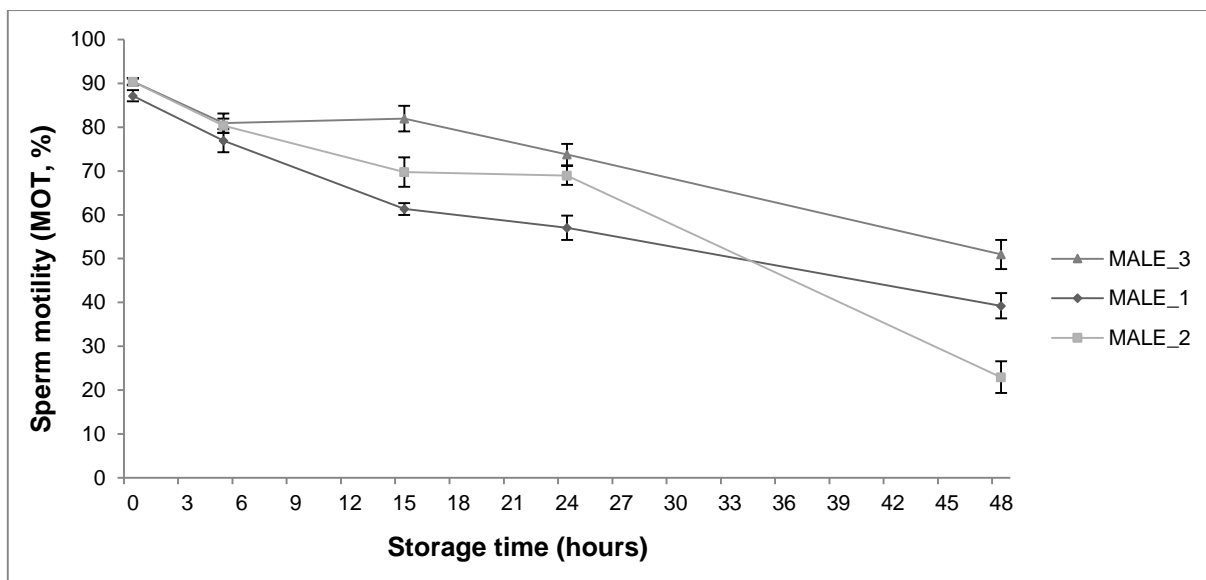


Figure 20: The interaction of male and storage time on sperm motility (MOT, %) over 48 hours of storage. Standard error is indicated by vertical bars at the mean.

Table 17: Source of variation given by the fixed effect of male with its variation contribution (FE, %) and degrees of freedom (DF) on sperm motility traits of PMOT, MOT VCL, VSL, VAP, ALH, LIN, STR, WOB and BCF.

Variation source	DF	PMOT	MOT	VCL	VSL	VAP	ALH	LIN	STR	WOB	BCF
Male	2	4.03***	1.81**	6.72***	4.44***	5.65***	1.39	0.94	0.45	2.03*	1.75
TCSS	114	66972.64	46914.39	44438.73	36301.32	48841.01	36.31	28374.67	14768.04	21962.21	645.36
Error mean square	104	10729.4	6974.19	7344.80	6067.58	8094.91	14.71	8083.71	6541.16	5988.61	293.87
R ²		0.84	0.85	0.83	0.83	0.83	0.59	0.72	0.56	0.72	0.54
CV (%)		26.83	11.48	15.09	21.32	19.65	19.11	14.71	10.38	9.92	20.79

TCSS: Total corrected sum of squares; CV: Coefficient of variation; *P < 0.05, **P < 0.01, ***P < 0.001

Table 18: Influence of male as a fixed effect on PMOT, MOT VCL, VSL, VAP, ALH, LIN, STR, WOB and BCF with least square means \pm standard errors (S.E).

Variation source	PMOT	MOT	VCL	VSL	VAP	ALH	LIN	STR	WOB	BCF
Male	***	**	***	***	***	P > 0.05	P > 0.05	P > 0.05	*	P > 0.05
Male 1	32.64 \pm 2.15 ^a	68.57 \pm 1.74 ^a	51.74 \pm 1.82 ^a	32.05 \pm 1.68 ^a	41.15 \pm 1.93 ^a	1.88 \pm 0.08 ^a	56.52 \pm 1.91 ^a	73.67 \pm 1.73 ^a	74.47 \pm 1.63 ^a	7.59 \pm 0.39 ^a
Male 2	36.99 \pm 1.91 ^a	71.51 \pm 1.55 ^a	51.33 \pm 1.62 ^a	32.20 \pm 1.50 ^a	40.84 \pm 1.72 ^a	1.82 \pm 0.07 ^a	57.01 \pm 1.70 ^a	73.99 \pm 1.54 ^a	73.79 \pm 1.45 ^a	7.31 \pm 0.34 ^a
Male 3	48.77 \pm 2.51 ^b	77.95 \pm 2.03 ^b	67.27 \pm 2.13 ^b	43.70 \pm 1.95 ^b	56.11 \pm 2.25 ^b	2.04 \pm 0.09 ^a	61.95 \pm 2.22 ^a	76.76 \pm 2.02 ^a	80.60 \pm 1.90 ^b	8.45 \pm 0.45 ^a

*P < 0.05, **P < 0.01, ***P < 0.001; Means with different letters differ (P < 0.05)

4. Discussion

4.1. *The effect of sperm concentration, semen volume, seminal plasma volume and day of collection on seminal plasma minerals*

Variation in seminal plasma minerals of ostrich males was observed in the study and associated with sperm concentration, seminal plasma volume and day of collection. Sperm concentration contributed to the variation associated with Mg. The negative correlation and inverse linear regression of Mg concentration on sperm concentration is consistent with results reported by Gur and Demirci (2000). The latter can possibly be explained by the need to establish homeostasis to maintain a proper balance of Mg concentration in combination with Na, K and Ca. It has been found that Mg as well as K levels in seminal plasma is lower, compared to that within the sperm cell compared to Na and in some cases Ca which is present at higher concentrations in the seminal plasma, compared to the sperm cell. The relative large amount of Mg in the cell is associated with mitochondria aggregation in the midpiece (El Jack and Lake, 1969). Thus the more sperm cells per mL (higher sperm concentration) the smaller the quantity of Mg in the seminal plasma. High levels of Mg in seminal plasma have been associated with superior quality ejaculates in humans and are closely correlated with Zn and Ca (Homonnai *et al.*, 1978). This can possibly be attributed to the role of Mg in numerous intracellular processes that involves mostly enzyme systems responsible for transport, storage and the use of energy. Mg has been found essential for enzymes involved in anaerobic glycolysis and the release of energy in the form of adenosine triphosphate (Mg-ATP) that directly affects sperm motility (Mann, 1964; Viski *et al.*, 1997). The stabilising effect of the cation Mg relates to polyphosphate compounds that include nucleic acid duplexes (RNA and DNA), in the production of proteins and antioxidants, such as glutathione. Mg also plays an important modulating role in the regulation of K (Na-K pump) and Ca (Mann, 1964; Viski *et al.*, 1997; Owczary *et al.*, 2008).

Variations in Se, Mn and Fe concentration were associated with a change in sperm concentration. Se is directly involved in spermatogenesis and higher concentrations are associated with an increased sperm count (Shalini and Bansal, 2006). Se has been recognized in several species as an essential mediator limiting oxidative stress, a common restraint of sperm function when oxidants outnumber antioxidants (Vaisberg *et al.*, 2005; Bansal and Bilaspuri, 2011). Ejaculates or insemination doses with high sperm concentration are specifically prone to oxidative stress, representing a necessity for a

higher Se concentration (Murphy *et al.*, 2013). The exact anti-oxidative mechanism of Se is not known, but it could possibly be involved as an enzymatic anti-oxidant co-factor, such as glutathione peroxidase (GPx) (Barber *et al.*, 2005). GPx is one of the most important anti-oxidant systems in seminal plasma, with a function to reduce reactive oxygen species like hydrogen peroxides to water and alcohol (Surai *et al.*, 1998; Barber *et al.*, 2005; Dimitrova *et al.*, 2007).

The reduction of the Mn concentration in relation to an increase in sperm concentration could potentially be explained by the strenuous demands on Mn by the sperm cell at higher concentrations. Mn forms an integral part of the enzymatic anti-oxidants (like Se), limiting oxidative stress only as part of superoxide dismutase (SOD) that spontaneously dismutates superoxide anions (O_2^-) to form O_2 and H_2O_2 that is less damaging (Sikka, 1996). SOD activity is the first line of defence against oxidative stress in seminal plasma (Matos *et al.*, 2009) and has been positively correlated with sperm concentration (Marzec-Wróblewska *et al.*, 2011). Mn has been found to enhance sperm motility, viability, capacitation and acrosome reaction through a cascade of actions. Mn has been found to stimulate adenylate-cyclase activity in sperm which enhances cyclic adenosine monophosphate (cAMP) (Bilaspuri and Bansal, 2008). The latter may lead to protein phosphorylation and increased intracellular Ca levels that may result in increased hyperactivation, as well as the maintenance of membrane integrity and the viability of sperm (Lapointe *et al.*, 1996; Guraya, 1999; Bansal and Bilaspuri, 2008; Bilaspuri and Bansal, 2008).

The inverse relationship between Fe and sperm concentration, as well as the strong positive correlation between Fe and Mn in this study can possibly be explained by the positive relationship between SOD activity and seminal plasma Fe concentration noted in previous studies. Ferrous ions (Fe_{2+}) have been found to induce or increase the rate of lipid peroxidation (oxidative stress), but also opposing the actions of Mn by decreasing sperm motility, viability and increased mid-piece defects that impair capacitation and acrosome reaction (de Lamirande and Gagnon, 1993, 1995; Huang *et al.*, 2001; Bansal and Bilaspuri, 2008). It is worth mentioning that although Fe-SOD forms have been isolated in prokaryotes and some eukaryotes, little is known about its presence in semen.

Semen volume contributed to the variation associated with Na and K (and even more so for K), while seminal plasma volume predominantly influenced the Na. Na and K cations

in seminal plasma regulate the osmotic balance to support the sperm cells and form part of many important enzymes (Cevik *et al.*, 2007). Na concentration decreased with an increase in semen and seminal plasma volume, and has been considered as the principal cation in seminal plasma with higher concentrations in the seminal plasma, compared to sperm cells (Cragle *et al.*, 1958; Lake, 1971). It has been reported in several avian species that K is more concentrated within sperm cells than in the seminal plasma (Cragle *et al.*, 1958; Lake, 1971). Hence, the increase in K in relation to semen volume observed in this study is potentially due to K's natural metabolic inhibiting activity. In that sense, a decrease in sperm motility when higher K concentrations are present in SP has been noted by Massányi *et al.* (2003).

B is believed to be an essential trace element for sperm function, but its specific role has not directly been proven in avian species (Nielsen, 1996). B concentration decreased with an increase in semen volume and the lowest B concentration was recorded closest to the middle of the trial. High B seminal plasma levels in mammals have been shown to contribute to the sperm quality traits of concentration, total sperm output, sperm motility, live sperm and normal sperm (Elkomy *et al.*, 2014). B has been shown to affect macro minerals (Ca, Cu, Mg) and cellular metabolism of other substances directly involved in life processes, such as glucose, triglyceride and reactive oxygen (Nielsen, 1994), although no correlations have been established between the suggested minerals in this study. The close interaction between B and Ca found in other studies has been suggested to affect and maintain cell membrane characteristics, as well as cellular signal transduction (Nielsen, 1994). B has also been postulated to reduce oxidative stress and improve the lipid profile with animals having a natural ability to establish homeostasis of B in the body without allowing it to reach toxic levels (Hunt, 1998; Basoglu *et al.*, 2011).

Pb is known as a toxic seminal plasma metal that demonstrated an increase in concentration, with increased semen volume. Pb has been associated with impaired motility, vitality, higher DNA fragmentation % and elevated seminal plasma ROS concentrations at high levels (Taha *et al.*, 2013). Xu *et al.* (1993) reported Pb concentration in seminal plasma to have no association with sperm traits related to motility and morphology.

Ba, a dense alkaline metal and an essential nutrient, has been found to block cell membrane K channels with Ca agonist properties. It has been suggested that the effects

of K and Ca metabolism mediate the toxic effects of Ba since toxicosis may occur (Anonymous, 2005; Piasta *et al.*, 2011). The concentrations of the trace elements Ni, Mo, and V showed fluctuation according to the day of collection. High initial concentrations of Ni were recorded with possible depletion towards the end of the trial. In contrast, Mo concentrations showed a build up towards the last three days of collection. V showed a mid-collection spike and again on the second last day of collections. It is not easy to explain these trends, and no comparable results have been found for ostriches or other ratites.

4.2. The effect of extender type, stage, storage time, sperm concentration and male on sperm motility traits

The ostrich specific diluents, OS1 and OS2, have shown to maintain sperm motility traits in terms of PMOT and MOT, velocity traits in terms of VCL, VSL, VAP as well as ALH and BCF equally. A difference in diluent capability in terms of the percentage STR, LIN and WOB was recorded for the two diluents. OS2, adapted for the micro-minerals Se and Zn, improved swim quality of sperm as reflected by a higher % STR, LIN and WOB. LIN has been associated with *in vivo* fertility in mammals (Budworth *et al.*, 1988; Farrell *et al.*, 1998) and high mobility phenotypic males in Turkeys (King *et al.*, 2000). STR has been shown to be best maintained over longer storage periods (48 hours), when diluted in OS2. Se and Zn are both micro minerals with strong anti-oxidative properties (Vaisberg *et al.*, 2005; Bansal and Bilaspuri, 2011; Murphy *et al.*, 2013) and the improvement in swimming quality by supplementing the diluent with these elements may be indicative of the limitation of oxidative stress during standard semen processing and storage.

All sperm traits, except BCF was affected by standard processing stages that include, neat, diluted and stored. Neat semen samples decreased in quality, most probably due to agglutination while the dilution of semen better maintained sperm traits (Ciereszko *et al.*, 2010). All sperm traits deteriorated with prolonged storage for up to 48 hours, but dilution with OS2 appeared to perform better towards the end of the storage time.

The possible explanation for the non-significant effect of sperm concentration on sperm motility traits can be the uniform distribution of sperm concentration between and within the limited number of triplicated males (Bonato *et al.*, 2011). The small coefficient of variation (CV = 11.22 %) confirms that the latter may stem from the selection of specific

males prior to the trial based on previous sperm trait knowledge. All sperm motility traits decreased with an increase in storage time over a 48 hour period. Although a linear relationship best described the deterioration in sperm traits (PMOT, MOT, VCL, VSL, VAP, ALH, BCF) over the 48 hours of storage LIN, STR and WOB displayed a slight deviation from this trend and was better described by a quadratic relationship. The difference in relationships of LIN, STR and WOB with storage time might be explained by the ability of particularly the OS2 extender to maintain these three swim quality traits better compared to other traits as indicated by the interaction of diluent with storage time or being less sensitive to the hazardous effects of prolonged storage. Males differed from each other in terms of measured sperm traits (PMOT, MOT, VCL, VSL, VAP and WOB), but only MOT varied among males, in combination with storage time. Males differed in their ability to maintain MOT over the 48 hours storage period even though the initial quality in terms of MOT was no different among males at 0 hours of storage. The latter suggested the possibility of a repeatable male effect for certain sperm traits as discussed in Chapter II and by Cloete *et al.* (2014).

5. Conclusion

The ostrich specific diluent has shown to be capable of improving sperm function maintenance over longer storage periods. Sperm swim quality traits (LIN, STR and WOB) can be better maintained over a 48 hours storage period when the OS diluent is adapted for the micro minerals of Se and Zn. Male selection is equally important to identify males with good quality ejaculates. Initial high motility traits would guarantee good motility at the end of processing and stored semen acceptable for artificial insemination. Improvement of sperm motility traits and maintenance over longer storage periods can potentially be achieved by further modification of the diluent and by the further study of other putative key contributors to the eventual quality of stored semen, like dilution rate, temperature and the rate of cooling.

6. Acknowledgements

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CHAPTER IV

PROLONGED STORAGE OF OSTRICH SEMEN AT 5 °C THROUGH INTERMEDIATE DILUTION

Optimal dilution rate, dilution temperature and storage time have been recognized as vital steps in the processing of semen for storage at above zero temperatures that have not yet been determined in the ostrich. Semen of four South African Black ostrich males was individually diluted with an ostrich specific diluent (OS1), immediately after collection at a 1:1, 1:2, 1:4 and 1:8 semen/diluent ratio with a diluent set at 5, 21 and 38 °C. Semen was then transferred to an iced water bath at 5 °C within 15 min and stored for 48 hours. Semen was evaluated at different storage intervals of 1, 5, 24 and 48 hours. A panel of sperm function tests was conducted to evaluate semen, including sperm motility and kinematic parameters, measured by the Sperm Class Analyzer®, percentage live sperm measured by fluorescence SYBR14®/PI (LIVE/DEAD®), percentage of sperm able to resist the hypo-osmotic swelling (HOS) stress test and sperm morphology determined by Nigrosin-Eosin staining. The sperm functions reflected by progressive motility (PMOT), motility (MOT), sperm kinematics, viability and membrane integrity were best maintained at higher dilution rates of 1:4 to 1:8 with an interpolated dilution rate 1:6 being ideal for the ostrich, compared to lower rates of 1:1 to 1:2 at all storage intervals measured. Sperm morphology was unaffected by dilution rate. A decrease in MOT and PMOT was associated with the use of pre-cooled extender at 5 °C compared to higher dilution temperatures of 21 and 38 °C. The beneficial effect of a higher dilution temperature (21 °C) becomes more prominent in terms of PMOT at higher dilution rates (1:4 to 1:8) and extended storage periods of 24 hours (and longer). All sperm functions can be maintained with limited deterioration for up to 24 hours in chilled semen at 5 °C. Semen from specific males was more suitable for chilled semen storage with no significant loss in sperm function. Such males can be classified as good-chillers, compared to other males that exhibited a good initial semen quality but semen deteriorating markedly over extended storage periods for the functions measured (poor-chillers).

1. Introduction

The fundamentals of a successful artificial insemination (AI) program have been set for the ostrich in terms of reliable stress free semen collection (Rybnik *et al.*, 2007), semen collection frequency, timing and output (Bonato *et al.*, 2011, 2014) and the duration of female sperm storage (Malecki *et al.*, 2004). More recently, the foundation of sperm function traits (Chapter II) and the development of an ostrich specific semen extender (Chapter III) were established, creating the basis and necessity for an efficient liquid storage protocol to further the application of AI on a commercial scale in the ostrich industry. AI has the potential to overcome poor productivity in the ratite industry that is associated with variable fertility and reproductive efficiency, depending on the proportion of fertile eggs produced by each female (Deeming, 1996; Van Schalkwyk *et al.*, 1996, 2000; Bunter and Graser, 2000; Bunter *et al.*, 2001; Cloete *et al.*, 2008; Dzoma and

Motshegwa, 2009; Dzoma, 2010). The primary reason for the latter is hypothesized to stem from poor sperm supply through male-female incompatibility or poor sperm production (Malecki and Martin, 2003; Malecki *et al.*, 2008).

Liquid storage of semen is not a new concept since Van Wambeke (1967) showed *in vitro* short-term storage to be possible by maintaining diluted domestic fowl semen for up to 24 hours at 0 °C without a major loss of fertility. Ya-jie *et al.* (2001) found that ostrich semen can stay viable for 72 hours at 5 °C in MEM (Minimum Essential Medium) extender, although the bulk of deterioration in sperm viability occurs within the first 48 hours, with no reference to the daily rate of sperm loss. However, since ostrich sperm can be stored in the female sperm storage tubules and can still fertilize eggs up to four weeks after the last copulation an alternative approach is clearly needed (Malecki *et al.*, 2004).

Semen dilution has been shown to be a crucial first step to avoid semen agglutination and to maintain sperm function for evaluation and further processing purposes, specifically in the ostrich (Ciereszko *et al.*, 2010; Chapter II and III). Undiluted sperm will lose most of their function as well as fertilizing ability within minutes after collection due to substrate depletion, increased metabolic by-products and pH change (Sexton, 1976, 1977, 1978, 1979; Sexton and Fewlass, 1978; Clarke *et al.*, 1982; Bilgili *et al.*, 1987; McDaniel *et al.*, 1998; Parker and McDaniel, 2003, 2004). An optimal dilution rate needs to be established when the duration of *in vitro* storage increases to reduce deleterious factors present in the seminal plasma, but also to complement the natural protective and nutritional elements of seminal plasma (Blesbois and de Reviers, 1992; Blesbois and Brillard, 2007). Over-dilution should however be avoided, since elevated sperm respiration, morphological changes and loss of motility can occur (Clarke *et al.*, 1982).

Together with dilution, using an appropriate extender, the semen must be pre-cooled (4 to 7 °C) to slow down catabolic and degradative processes as well as bacteriospermia build up (Wishart, 1982, 1989; Giesen and Sexton, 1983; Blesbois and Brillard, 2007; Sood *et al.*, 2012). Abrupt cooling of semen from physiological or room temperature to temperatures ≥ 0 °C may be stressful to sperm, especially when reaching the critical temperature zone of 15 to 20°C, where the physical properties of the cell membrane is changed appreciably (Hammerstedt *et al.*, 1990). The temperature at which dilution takes place is very important as dilution with a pre-cooled extender is already the onset of cooling. Improper cooling of neat semen can cause irreversible damage to the sperm cell

through the phenomenon known as chilling injury or cold shock (Watson, 1981; Watson and Morris, 1987; Drobnis *et al.*, 1993; Petrunkina, 2007). Injury immediately upon chilling or inflicted over an extended storage interval has been described as irreversible damage that occurs when sperm is cooled too low (≥ 0 °C), but still non-freezing temperatures (Watson and Morris, 1987; Drobnis *et al.*, 1993; Arav *et al.*, 1996; Saragusty *et al.*, 2005). Sensitivity of sperm to cooling and cellular response may differ between species most probably due to species specific membrane compositions (Watson and Morris, 1987; Blesbois *et al.*, 2005). For instance the emu, a close relative of the ostrich, has been found to be more tolerant towards low collection temperatures of 5 °C, with increased resistance of sperm to chilling injury attributed to the high percentage of unsaturated fatty acids present (heneicosanoic, arachidonic and docosatetraenoic acids) (Sood *et al.*, 2012).

The aim of this study was to develop an effective short-term storage protocol that would enable ostrich breeders to collect, dilute, cool and store semen for hours or days at temperatures ≥ 0 °C for either on site artificial insemination purposes or transportation to a distant farm or for retail purposes. The latter is especially of great benefit to countries like South Africa where ostrich farms are of substantial size (922 to 3130 hectares on average) and are serviced mostly by rural roads that may prolong semen transportation (Cupido, 2005). Furthermore, inseminations to multiple females per ejaculate from a single male with a high workload would also be possible with diluted stored semen, allowing the maximum utilization of superior males (Bootwalla and Miles, 1992). Semen extension during periods of low semen production such as winter would be made possible through short-term storage (Bootwalla and Miles, 1992). The assisted reproduction techniques of short-term storage and artificial insemination thereof would allow for a structured breeding program in the ostrich industry as advocated by Cloete *et al.* (2008).

2. Material and methods

2.1. Animal population

Ejaculates were collected from four South African Black (SAB) ostrich males, aged between 3 and 7 years using the dummy female method (Rybnik *et al.*, 2007). They were allocated to the trial evaluating dilution rate (1:1, 1:2, 1:4; 1:8), dilution temperature (5, 21, 38 °C) and storage time (1, 5, 24, 48 hours) in a factorial experimental design. Males were chosen for their reliability in terms of ejaculate quality and willingness to mount the

dummy female. Males in the resource population were screened from the commercial ostrich breeding flock maintained at the Oudtshoorn Research Farm on the basis of behavioural attributes rendering them suitable for AI (referred as desirable behaviour repertoires and described in Bonato and Cloete, 2013). The origin of the ostrich flock and the general management procedures implemented therein were described previously (Van Schalkwyk *et al.*, 1996; Bunter and Cloete, 2004; Cloete *et al.*, 2008).

2.2. Semen preparation

Ejaculates from individual ostrich males were diluted immediately after collection at a 1:1, 1:2, 1:4 and 1:8 semen:diluent ratio, with the ostrich specific diluent (OS1 adapted to pH7, Chapter III set at different temperatures of 5, 21 and 38 °C. After dilution, samples were transferred to a pre-cooled water bath at 5 °C and transported to the on-site lab. Semen was evaluated for sperm specific functions that included motility, viability, membrane integrity and sperm morphology after 1, 5, 24 and 48 h of storage at 5 °C.

Sperm concentration was obtained with the aid of a spectrophotometer (Spectrawave, WPA, S800, Biochrom) with transmittance values that were previously calibrated against actual cell counts using a haemocytometer. Aliquots of 20 µl semen diluted 1:400 (v/v) with phosphate buffered saline solution containing 10 % formalin were used for the latter.

2.3. In vitro sperm function evaluation

2.3.1. Motility evaluation

Sperm motility traits were evaluated by means of the Sperm Class Analyzer® (SCA) version 5.3 (Microptic S.L., Barcelona, Spain). All sperm motility recordings were made after re-suspension of the neat sperm, as well as treated sperm in a standard motility buffer using Sodium Chloride (150mM) and TES (20mM) with male specific seminal plasma (2 %), to a final sperm concentration of 20×10^6 sperm cells/mL. After re-suspension the tube was placed in a 38 °C water bath for 1 minute. For sperm motility recording 2 µl of diluted semen was placed onto a pre-warmed slide covered gently with a cover glass (22 x 22 mm) and allowed to settle for 20 seconds, prior to recording. Seven to nine different fields were captured until at least 500 motile sperm were evaluated. At least nine fields were captured randomly to eliminate bias towards higher sperm concentration or motility. Sperm motility traits included motility (MOT, %), progressive motility (PMOT, %), curve-linear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s),

average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat- cross frequency (BCF, Hz).

2.3.2. Viability evaluation

Sperm viability (LIVE, %) was measured using the LIVE/DEAD® Sperm Viability Kit from Life technologies that contains the SYBR® 14 and Propidium Iodide (PI) fluorescent stains. All sperm viability recordings were made after re-suspension of neat sperm, as well as treated sperm in the standard Ostrich diluent at pH7 to a final sperm concentration of 20×10^6 sperm cells/mL. The SYBR® 14 working solution was prepared in a HEPES/NaCl medium to a 1:49 concentration (v/v) SYBR® 14 to HEPES/NaCl solution. Sperm suspension aliquots of 250 μl were re-suspended with 1.5 μl membrane-permeant SYBR® 14 working solution and incubated for 10 minutes at a temperature controlled environment of 38 °C. After incubation, 2 μl of the next fluorescent stain, Propidium Iodide (PI), was added and incubated for 10 minutes, whereafter cells were evaluated. For evaluation of viable (green) and non-viable (red/or green with red) sperm a 2 μl droplet was placed on a glass slide and covered with a cover glass (22 x 22 mm) and allowed to settle for 20 seconds, prior to recording. The fluorescent sperm was observed and photographed under 10x microscopy with an Olympus BX41 epifluorescent microscope (Olympus Optical Co., Tokyo, Japan), equipped with a filter, camera (ColorView IIIu Soft Imaging System) and software package (analysis FIVE, Olympus Soft Imaging Solutions GmbH, Münster) to count viable and non-viable sperm. Nine to ten different fields were randomly captured until at least 500 spermatozoa were evaluated. Distorted fields as well as fields that included drift or debris or clumps of sperm were avoided. The SYBR® 14 nucleic acid stain labels live sperm with green fluorescence, and membrane-impermeable PI labels the nucleic acids of membrane-compromised sperm with red fluorescence.

2.3.3. Membrane integrity evaluation

Sperm membrane integrity was measured using the Hypo-osmotic swelling test (Jeyendran *et al.*, 1984) adapted specifically for the ostrich by means of preliminary experimental exploration. The neat sperm samples for hypo-osmotic swelling resistant sperm (HOS, %) were prepared at the same time as the motility evaluation. All sperm membrane integrity recordings were made after re-suspension of neat sperm and treated sperm in a standard salt (NaCl/H₂O) solution adapted to 25 mOsm to a final sperm concentration of 20×10^6 sperm cells/mL. For HOS recording 2 μl of diluted semen was

placed onto a pre-warmed slide, using a heated stage set at 38 °C, covered gently with a cover glass (22 x 22 mm) and allowed to settle for 20 seconds prior to recording. Sperm was captured using the Sperm Class Analyzer® (SCA) version 5.3 (Microptic S.L., Barcelona, Spain) with a Basler A312fc digital camera (Basler AG, Ahrensburg, Germany) mounted on an Olympus BX41 microscope (Olympus Optical Co., Tokyo, Japan) equipped with phase contrast optics. Seven to nine different fields were captured randomly until good representation (500 sperm) was reached and bias towards higher sperm concentration eliminated. Distorted fields as well as fields that included drift or debris or clumps of sperm were excluded.

2.3.4. Morphological evaluation

The average proportion of live normal (NORM, %), live abnormal (ABNRM, %) and dead sperm was recorded for each diluted aliquot after counting 300 stained sperm on each replicated slide stained with a Nigrosine-Eosin stain. Live, viable sperm with intact membranes, excludes Eosin stain and stays ivory white, while dead (non-viable) sperm possess permeable cell membranes that permit the Eosin to stain the sperm pinkish or magenta. Nigrosin enhances contrast between background (stains blue) and stained/unstained sperm to be evaluated (Lake and Stewart, 1978).

2.4. Statistical analyses

Sperm traits as percentages, and with skewed distribution (as determined by the Shapiro-Wilk test: $P < 0.05$) were transformed using the arc sine of the percentage mean square-root ($\text{degree} \cdot \arcsin \sqrt{\%}$), while sperm concentration was transformed to natural logarithms. Analyses included distribution analysis and summary statistics to obtain variance parameters and graphs describing the sperm traits. The total number of records, mean, standard deviation, minimum, maximum and coefficient of variation (CV) was obtained for each sperm trait. The contribution of each fixed effect (FE) to a particular SFC was evaluated by expressing the sum of squares for such an effect as a percentage of the total corrected sum of squares (TCSS) (Leighton *et al.*, 1982).

General Mixed Models (GLMM) were performed to evaluate the influence of dilution rate (D), dilution temperature (T), storage time (S) and sperm concentration (C; as a linear covariate) influencing the different sperm traits with the inclusion of male (M) as random effect to account for the repeated sampling of the same males. General linear model

(GLM) was applied to assess the specific effect of variation between males and its interactions with other fixed effects (D, T, S, and C).

Example of the GLMM fitted with Y being the dependent sperm traits of motility, viability, membrane integrity and sperm morphology:

$$Y_{ijkl} = \mu + M_i + D_j + T_k + S_l + b_0 (C)_{ijkl} + e_{ijkl}$$

Where:

- Y_{ijkl} = Sperm trait under assessment
- μ = population mean
- M_i = random effect of the i^{th} male ($i = 1, 2, 3, 4$)
- D_j = fixed effect of the j^{th} dilution rate ($j = 1:1, 1:2, 1:4, 1:8$ semen/diluent ratio)
- T_k = fixed effect of the k^{th} dilution temperature ($k = 5, 21, 38$ °C)
- S_l = fixed effect of the l^{th} storage time ($l = 1, 5, 24, 48$ hours)
- C_{ijkl} = sperm concentration fitted as a linear covariate
- b_0 = regression coefficient of Y_{ijkl} on sperm concentration (C)
- e_{ijkl} = random error

Sperm function traits included motility traits derived from CASA, viability, membrane integrity (HOS) and the number of morphological normal and abnormal sperm that was fitted individually in each model as the dependent variables. Motility traits included motility (MOT, %), progressive motility (PMOT, %), curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat-cross frequency (BCF, Hz). Viability included the percentage live (LIVE) and dead sperm, while membrane integrity included the number of hypo-osmotic resistant (HOS) and non-resistant sperm. Morphology included the number live normal (NORM) and abnormal live (ABNRM) sperm. Least squares means, standard errors (S.E) and variation coefficients (CV) were calculated and subjected to Tukey's multiple range tests to investigate differences between least squares means. Correlations (Pearson) and regressions (linear and non-linear) were applied to investigate significant ($P < 0.05$) relationships between traits. Statistical Analysis System (SAS, version 9.3) was used for all the analyses performed.

3. Results

3.1. Descriptive statistics

Across the whole analyses sperm trait (mean \pm SE) values were recorded as LIVE = 72.94 ± 0.95 %, HOS = 63.73 ± 1.65 %, PMOT = 22.56 ± 0.96 %, MOT = 51.04 ± 1.28 %, VCL = 47.27 ± 0.92 $\mu\text{m/s}$, VSL = 22.31 ± 0.83 $\mu\text{m/s}$, VAP = 30.63 ± 0.8 $\mu\text{m/s}$, ALH = 2.25 ± 0.06 μm , LIN = 53.02 ± 0.87 %, STR = 54.91 ± 1.46 %, WOB = 66.06 ± 0.83 % and BCF = 7.04 ± 3.0 Hz. The total number of records, mean, standard deviation, minimum, maximum and coefficient of variation (CV) was obtained for each sperm trait and are set out in Table 19.

Table 19: The description of sperm traits that included sperm viability (LIVE, %), membrane integrity (HOS, %), normal morphology (NORM, %), abnormal morphology (ABNRM, %), progressive motility (PMOT, %) and motility (MOT, %), curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat- cross frequency (BCF, Hz) for 4 (repeated) ostrich males used in Experiment 1: investigating the effect of dilution rate (1:1, 1:2, 1:4, 1:8) and dilution temperature (5, 21, 38 °C) over 48 hours storage at 5 °C.

Sperm trait	Records	Mean	S.D	Minimum	Maximum	CV (%)
Live (%)	307	72.94	16.76	18.04	98.15	22.98
Hypo osmotic swelling resistant (%)	210	63.73	23.95	1.46	94.85	37.58
Live normal (%)	184	48.76	26.31	2.14	90.06	53.96
Live abnormal (%)	184	45.56	25.43	9.32	90.83	55.82
Progressive motile (%)	322	22.58	16.74	0.50	70.61	74.13
Motile (%)	322	51.04	22.94	2.67	96.00	44.94
Curve-linear velocity ($\mu\text{m/s}$)	322	47.27	16.56	3.56	83.68	35.03
Straight-line velocity ($\mu\text{m/s}$)	322	22.31	14.91	0.00	67.40	66.82
Average path velocity ($\mu\text{m/s}$)	322	30.63	15.68	1.11	73.90	51.19
Amplitude of lateral head displacement (μm)	322	2.25	1.07	0.00	4.50	47.55
Linearity (%)	322	53.02	15.61	0.00	86.68	29.45
Straight (%)	322	54.91	26.23	0.00	96.36	47.77
Wobble (%)	322	66.06	14.91	30.00	92.47	22.56
Beat- cross frequency (Hz)	322	7.04	2.99	0.00	13.01	42.46

S.D: Standard deviation; CV: Coefficient of variation

The contribution of each fixed effect (FE, %) with its degrees of freedom (DF), R-square value and coefficient of variation (CV, %) is shown in

Table 20 for LIVE, HOS, NORM, ABNRM, PMOT and MOT. The kinematic sperm traits (VCL, VSL, VCL, VAP, ALH, LIN, STR, WOB and BCF) are set out in Table 21. The least squares means for the different sperm traits are showed in Table 22 and Table 23.

Table 20: The source of variation induced by the fixed effect of sperm concentration, dilution rate, dilution temperature and storage time with their variation contribution (FE, %) with its degrees of freedom (DF) on sperm viability (LIVE, %), membrane integrity (HOS, %), normal morphology (NORM, %), abnormal morphology (ABNRM, %), progressive motility (PMOT, %) and motility (MOT, %).

Source of variation	DF	LIVE	DF	HOS	DF	NORM	ABNRM	DF	PMOT	MOT
Dilution Rate	3	9.06***	3	22.85***	3	2.07	1.65	3	7.78***	3.76***
Dilution Temp (°C)	2	0.74	2	0.26	2	0.02	0.06	2	1.15*	0.90*
Storage Time (h)	3	34.03***	3	21.77***	3	21.95***	18.25***	3	45.13***	44.51***
TCSS	307	41172.9	209	51149.8	183	51073.0	45469.5	302	46063.0	68280.3
Error mean square	299	23264.8	201	27123.1	175	38736.3	36402.9	294	22336.2	35454.9
R square		0.43		0.47		0.24	0.20		0.52	0.48
CV, %		14.85		21.69		33.91	34.10		32.81	23.31

TCSS: Total corrected sum of squares; CV: Coefficient of variation; *P < 0.05; **P < 0.01; ***P < 0.001.

Table 21: The source of variation as induced by the fixed effect of sperm concentration, dilution rate, dilution temperature and storage time with their variation contribution (FE, %) with its degrees of freedom (DF) on sperm kinematic traits, namely curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat- cross frequency (BCF, Hz).

Source of variation	DF	VCL	VSL	VAP	ALH	LIN	STR	WOB	BCF
Dilution Rate	3	8.20***	8.09***	8.52***	7.44***	8.71***	4.51**	9.40***	10.84***
Dilution Temp ($^{\circ}\text{C}$)	2	0.44	0.28	0.34	0.28	0.56	0.19	0.12	0.34
Storage Time (h)	3	35.23***	22.91***	34.33***	22.50***	12.97***	3.69**	21.78***	22.71***
TCSS	321	88026.81	71327.41	78933.02	2304.30	30156.08	88904.47	28311.60	7164.32
Error mean square	313	50603.85	49809.22	45893.23	1634.813	23698.31	81766.71	19780.20	4840.17
R square		0.43	0.30	0.42	0.29	0.21	0.10	0.30	0.32
CV, %		26.90	56.55	39.53	27.83	18.62	33.45	14.48	26.78

TCSS: Total corrected sum of squares; CV: Coefficient of variation; *P < 0.05; **P < 0.01; ***P < 0.001

Table 22: The least square means (\pm S.E) of dilution rate, dilution temperature and storage time on sperm viability (LIVE, %), membrane integrity (HOS, %), normal morphology (NORM, %), abnormal morphology (ABNRM, %), progressive motility (PMOT, %) and motility (MOT, %) with least square means \pm standard errors (S.E).

Variation source	LIVE	HOS	NORM	ABNRM	PMOT	MOT
Dilution Rate	***	***	P > 0.05	P > 0.05	***	***
Dilution 1:1	65.86 \pm 2.42 ^a	52.88 \pm 17.18 ^a	42.49 \pm 6.90	50.77 \pm 6.20	16.91 \pm 4.35 ^a	45.94 \pm 5.53 ^a
Dilution 1:2	73.41 \pm 2.43 ^{bc}	62.49 \pm 17.17 ^b	46.51 \pm 6.90	47.84 \pm 6.20	20.83 \pm 4.32 ^b	51.19 \pm 5.52 ^b
Dilution 1:4	76.61 \pm 2.42 ^{cbd}	78.35 \pm 17.18 ^c	49.23 \pm 6.90	45.01 \pm 6.20	26.36 \pm 4.31 ^{cd}	55.44 \pm 5.51 ^{cdb}
Dilution 1:8	78.61 \pm 2.46 ^{dc}	82.21 \pm 17.20 ^{dc}	52.12 \pm 6.90	42.29 \pm 6.20	27.16 \pm 4.32 ^{dc}	56.36 \pm 5.53 ^{dc}
Dilution Temp	P > 0.05	P > 0.05	P > 0.05	P > 0.05	*	*
5 °C	74.08 \pm 2.33	67.47 \pm 17.15	47.00 \pm 6.83	46.13 \pm 6.15	20.36 \pm 4.27 ^a	49.60 \pm 5.45 ^a
21 °C	75.61 \pm 2.34	68.33 \pm 17.16	47.50 \pm 6.84	47.12 \pm 6.15	23.36 \pm 4.28 ^b	52.15 \pm 5.46 ^b
38 °C	71.18 \pm 2.34	71.14 \pm 17.18	48.27 \pm 6.84	46.18 \pm 6.15	24.73 \pm 4.28 ^b	54.94 \pm 5.46 ^b
Storage Time	***	***	***	***	***	***
1 hour	85.05 \pm 2.52 ^a	82.38 \pm 17.16 ^a	52.68 \pm 6.89 ^a	42.26 \pm 6.19 ^a	37.42 \pm 4.31 ^a	70.24 \pm 5.52 ^a
5 hours	78.17 \pm 2.40 ^{bc}	76.10 \pm 17.18 ^b	55.97 \pm 6.89 ^{ba}	38.88 \pm 6.19 ^{ba}	27.82 \pm 4.31 ^b	62.36 \pm 5.52 ^b
24 hours	74.50 \pm 2.41 ^{cb}	65.18 \pm 17.18 ^c	55.62 \pm 6.89 ^{cba}	39.65 \pm 6.19 ^{cba}	18.21 \pm 4.32 ^c	46.59 \pm 5.52 ^c
48 hours	56.76 \pm 2.42 ^d	52.27 \pm 17.19 ^d	26.09 \pm 6.94 ^d	65.13 \pm 6.19 ^d	7.81 \pm 4.39 ^d	29.74 \pm 5.55 ^d

Dilution Temp: Dilution temperature; *P < 0.05; **P < 0.01; ***P < 0.001; Means with different letters differ (P < 0.05)

Table 23: The least square means (\pm S.E) of dilution rate, dilution temperature and storage time on the curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat- cross frequency (BCF, Hz).

Variation source	VCL	VSL	VAP	ALH	LIN	STR	WOB	BCF
Dilution Rate	***	***	***	***	***	**	***	***
Dilution 1:1	43.13 \pm 8.38 ^a	20.69 \pm 9.75 ^a	28.08 \pm 9.49 ^a	1.81 \pm 0.17 ^a	50.33 \pm 9.34 ^a	52.12 \pm 10.59 ^a	62.29 \pm 6.66 ^a	6.29 \pm 1.21 ^a
Dilution 1:2	48.56 \pm 8.38 ^b	24.03 \pm 9.75 ^b	32.40 \pm 9.49 ^b	2.17 \pm 0.17 ^{bcd}	55.01 \pm 9.33 ^b	56.91 \pm 10.58 ^{ba}	66.29 \pm 6.66 ^b	6.84 \pm 1.21 ^{ba}
Dilution 1:4	53.16 \pm 8.38 ^{cd}	28.26 \pm 9.75 ^c	36.61 \pm 9.49 ^{cd}	2.44 \pm 0.17 ^{cbd}	58.86 \pm 9.33 ^{cd}	62.04 \pm 10.57 ^{cb}	71.57 \pm 6.65 ^c	8.14 \pm 1.21 ^c
Dilution 1:8	54.45 \pm 8.38 ^{dc}	30.66 \pm 9.75 ^d	39.09 \pm 9.49 ^{dc}	2.44 \pm 0.17 ^{dcb}	61.21 \pm 9.33 ^{dc}	65.08 \pm 10.58 ^{dbc}	73.64 \pm 6.63 ^{dc}	8.50 \pm 1.21 ^{dc}
Dilution Temp	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
5 °C	48.49 \pm 8.36	25.09 \pm 9.74	33.05 \pm 9.49	2.27 \pm 0.16	56.54 \pm 9.32	59.49 \pm 10.50	68.78 \pm 6.64	7.63 \pm 1.20
21 °C	51.05 \pm 8.36	26.97 \pm 9.74	35.21 \pm 9.48	2.20 \pm 0.16	57.73 \pm 9.32	60.27 \pm 10.51	68.99 \pm 6.63	7.42 \pm 1.20
38 °C	49.94 \pm 8.36	25.68 \pm 9.74	33.88 \pm 9.48	2.17 \pm 0.16	54.78 \pm 9.32	57.35 \pm 10.51	67.56 \pm 6.65	7.28 \pm 1.20
Storage Time	***	***	***	***	***	**	***	***
1 hour	61.07 \pm 8.38 ^a	33.72 \pm 9.74 ^a	44.85 \pm 9.49 ^a	2.65 \pm 0.17 ^{ab}	62.29 \pm 9.33 ^{ab}	62.79 \pm 10.57 ^a	76.28 \pm 6.65 ^a	8.37 \pm 1.21 ^a
5 hours	56.99 \pm 8.38 ^b	31.25 \pm 9.75 ^b	40.39 \pm 9.49 ^b	2.60 \pm 0.17 ^{ba}	60.90 \pm 9.33 ^{ba}	63.73 \pm 10.57 ^{ba}	73.74 \pm 6.65 ^{ba}	8.65 \pm 1.21 ^{ba}
24 hours	44.10 \pm 8.38 ^c	21.48 \pm 9.75 ^c	28.03 \pm 9.49 ^c	2.14 \pm 0.17 ^c	52.90 \pm 9.33 ^{cd}	58.15 \pm 10.57 ^{cba}	64.80 \pm 6.65 ^c	7.40 \pm 1.21 ^c
48 hours	37.15 \pm 8.39 ^d	17.19 \pm 9.75 ^d	22.91 \pm 9.49 ^d	1.47 \pm 0.17 ^d	49.31 \pm 9.34 ^{dc}	51.48 \pm 10.60 ^{dc}	58.97 \pm 6.66 ^d	5.36 \pm 1.21 ^d

Dilution Temp: Dilution temperature; *P < 0.05; **P < 0.01; ***P < 0.001; Means with different letters differ (P < 0.05)

Table 24: Pearson's correlations among ostrich sperm traits, namely sperm viability (LIVE, %), membrane integrity (HOS, %), normal morphology (NORM, %), abnormal morphology (ABNRM, %), progressive motility (PMOT, %) and motility (MOT, %), curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat- cross frequency (BCF, Hz) and sperm concentration, dilution rate (DR: 1:1 to 1:8), dilution temperature (DT: 5, 21, 38 °C) and storage time (ST: 1, 5, 24, 48 hours).

Sperm Trait	LIVE	HOS	NORM	ABNRM	PMOT	MOT	VCL	VSL	VAP	ALH	LIN	STR	WOB	BCF
DR	0.24***	0.42***	0.12	-0.12	0.17**	0.13*	0.22***	0.24 ***	0.24***	0.17**	0.25***	0.15**	0.18***	0.27***
DT	-0.06	0.07	0.04	-0.01	0.12*	0.09	0.03	0.02	0.02	-0.04	-0.04	-0.02	-0.03	-0.03
ST	-0.57***	-0.48***	-0.38***	0.35***	-0.59***	-0.66***	-0.57***	-0.46***	-0.55***	-0.42***	-0.35***	-0.16***	-0.19***	-0.46***

DR: Dilution rate; DT: Dilution temperature; ST; Storage time; *P < 0.05; **P < 0.01; ***P < 0.001

3.2. *The effect of dilution rate on sperm traits*

From the data obtained in this study, most sperm traits were influenced ($P < 0.05$) by dilution rate, except for sperm morphology (NORM, ABNRM) ($P > 0.05$). The contribution of dilution rate to the overall variation ranged from 3.76 to 23.46 % across sperm traits. The highest variation (FE = 23.46 %) was associated with HOS, while MOT and STR showed the smallest variation (FE < 5 %), associated with dilution rates.

Higher dilution rates of 1:4 and 1:8 yielded the highest ($P < 0.05$) LIVE (78.61 ± 2.46 %), HOS (82.21 ± 17.20 %), PMOT (27.16 ± 4.32 %), MOT (56.36 ± 5.53 %), VCL (54.45 ± 8.38 $\mu\text{m/s}$), VAP (39.09 ± 9.49 $\mu\text{m/s}$), ALH (2.44 ± 0.17 μm), LIN (61.21 ± 9.33 %), STR (62.04 ± 10.58 %), WOB (73.64 ± 6.63 %) and BCF (8.50 ± 1.21 Hz) compared to 1:1 and 1:2 dilutions. No significant differences were recorded between the 1:4 and 1:8 dilution rates for these traits. A 1:8 dilution produced the highest VSL (30.66 ± 9.75 $\mu\text{m/s}$), compared to 1:4 (28.26 ± 9.75 $\mu\text{m/s}$), 1:2 (24.03 ± 9.75 $\mu\text{m/s}$) and 1:1 (20.69 ± 9.75 $\mu\text{m/s}$) dilution rates.

Pearson's correlation coefficients, (ranging from $r = 0.13$ to $r = 0.42$), confirmed the positive relationships ($P < 0.05$) between dilution rate and the sperm traits measured (Table 24). An increase in dilution rate was associated with an increase in all the sperm traits (PMOT, MOT, VCL, VSL, VAP, ALH, LIN, STR, WOB, BCF), except for NORM and ABNRM. HOS showed the highest correlation coefficient of $r = 0.42$ with dilution rate. A non-linear quadratic equation ($Y = \alpha + \beta_1 X + \beta_2 X^2$; $P < 0.0001$) was applied (Figure 21) to describe the relationship ($Y = 32.64 + 15.84X - 1.30X^2$) between HOS and dilution rate. The R^2 value showed that 25 % of the variation in HOS was explained by the quadratic regression. The inflection point ($X = \pm (\beta_1/2\beta_2)$), where HOS may be maximized (80.18 %) was given by $X = 6.08$, thus a dilution rate of 1:6 semen to diluent ratio, thereafter HOS would decrease with an increase in dilution rate. A comparable non-linear relationship between LIVE and dilution rate is given by $Y = 60.67 + 6.19X - 0.51X^2$ with a R^2 value of 7.82. A maximum LIVE value of 79.45 % may be possible where $X = 6.07$ and is similar to a 1:6 dilution rate.

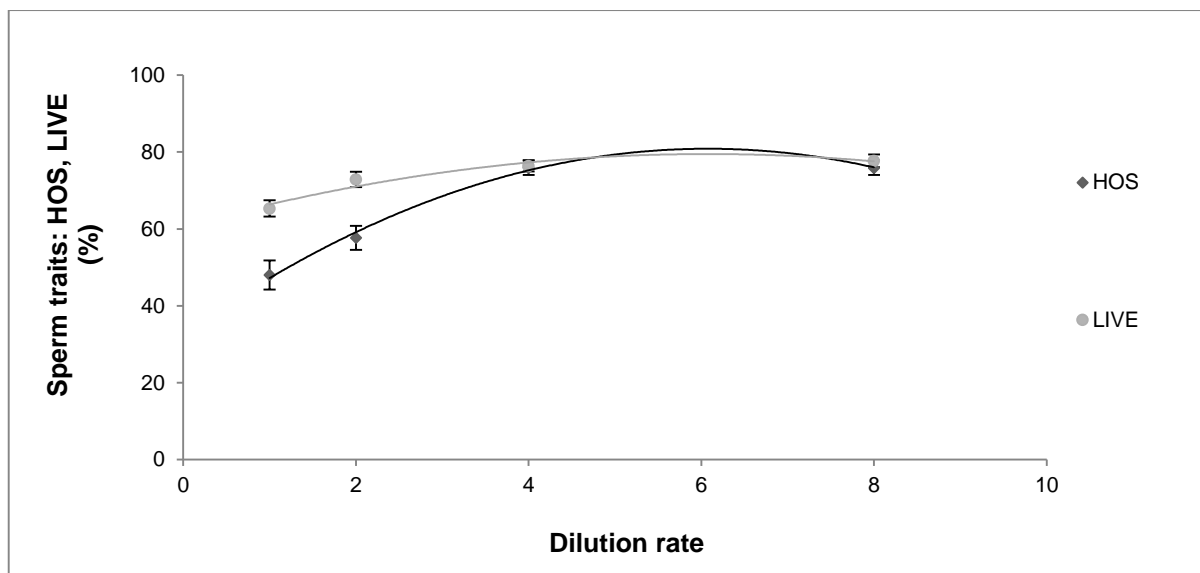


Figure 21: Polynomial relationships between the percentage hypo- osmotic swelling resistant sperm (HOS, %) cells, as well as the percentage viable sperm (LIVE, %) at different dilution rates from 1:1 to 1:8. Standard error is indicated by vertical bars at the mean.

The obtained Pearson correlation coefficients ($P < 0.05$) for motility traits namely, MOT and PMOT, with dilution rate recorded were $r = 0.13$ and $r = 0.17$ respectively. These coefficients indicated relationships, quantified by non-linear quadratic regressions (Figure 22) for the traits of MOT: $Y = 41.72 + 4.60X - 0.38X^2$; $R^2 = 0.02$ and PMOT: $Y = 13.68 + 4.27X - 0.34X^2$; $R^2 = 0.03$. The infection point ($X = \pm (\beta_1/2\beta_2)$) where MOT and PMOT would be maximized (55.62 and 27.07 %) was equal to $X = 6.28$ and $X = 6.05$, respectively. Thus a dilution rate of 1:6 semen to diluent ratio is suggested by the quadratic equation, whereafter sperm motility will decrease with an increase in dilution rate.

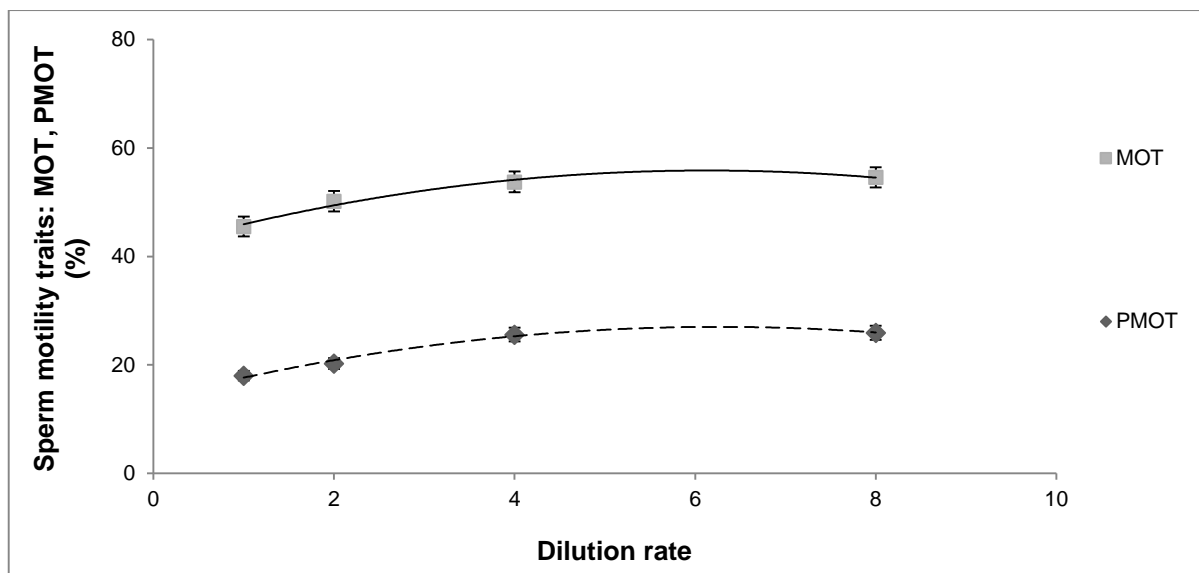


Figure 22: Polynomial relationships between the percentage motile sperm (MOT, %) as well as the percentage progressive motile sperm (PMOT, %) at different dilution rates from 1:1 to 1:8. Standard error is indicated by vertical bars at the mean.

Positive correlations ($P < 0.001$) obtained for VCL, VSL and VAP, with dilution rate ranged from 0.22 to 0.24, while those for WOB, LIN, STR, BCF, ALH ranged from 0.15 to 0.27. A non-linear quadratic equation was applied (Figure 23) to quantify the relationships between VCL ($Y = 36.09 + 5.48X - 0.44X^2$), VSL ($y = 13.18 + 4.11X - 0.30X^2$), as well as VAP ($Y = 20.71 + 4.55X - 0.34X^2$) and dilution rate. The R^2 value illustrated that 6.7; 6.9 and 7.1 % of the variation in VCL, VSL and VAP respectively was explained by the quadratic regressions. The inflection point ($X = \pm (\beta_1/2\beta_2)$ where VCL, VSL and VAP would be maximized (53.28; 27.25; 35.93 $\mu\text{m/s}$) is given by $X = 6.23; 6.85; 6.69$. Thus a dilution rate of 1:6 to 1:7 semen to diluent ratio, whereafter sperm velocity will decrease with an increase in dilution rate.

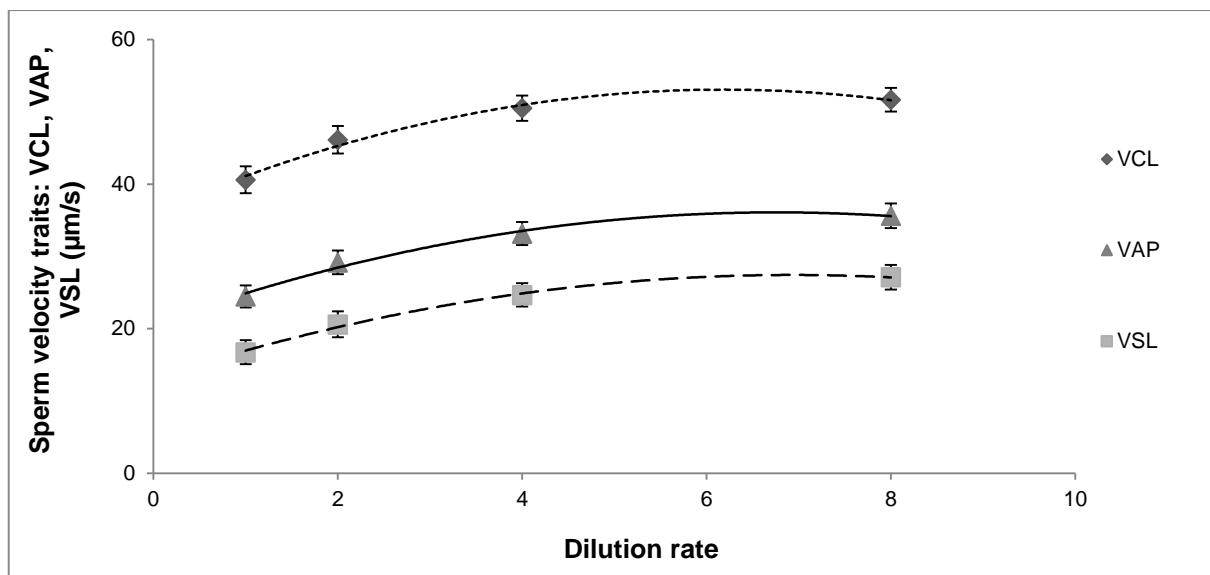


Figure 23: Polynomial relationships between sperm velocity traits namely, curve-linear velocity (VCL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$) and straight-line velocity (VSL, $\mu\text{m/s}$) at different dilution rates from 1:1 to 1:8. Standard error is indicated by vertical bars at the mean.

Non-linear quadratic equations ($Y = \alpha + \beta_1X + \beta_2X^2$; $P < 0.0001$), quantified the relationship best between WOB ($Y = 55.36 + 4.97X - 0.37X^2$), LIN ($Y = 42.76 + 4.74X - 0.35X^2$), STR ($Y = 43.03 + 5.36X - 0.39X^2$), BCF ($Y = 4.92 + 0.97X - 0.07X^2$), ALH ($Y = 1.61 + 0.33X - 0.03X^2$) and dilution rate (Figure 24 and Figure 25). The R^2 value showed that 8.8; 7.4, 3.8, 8.8, 4.6 % of the variation in WOB, LIN, STR, BCF and ALH respectively was accounted for by the quadratic regressions. The inflection point ($X = \pm (\beta_1/2\beta_2)$) where WOB, LIN and STR would be maximized (71.85, 58.59 and 61.56 %) was determined by $X = 6.64$; 6.68 and 6.92. The inflection point for BCF (8.28 Hz) was within the range of other motility and kinematic traits, with $X = 6.74$ suggesting a dilution rate of 1:6 to 1:7, whereas the inflection point for ALH (2.51 μm) was slightly lower ($X = 5.84$) and indicated a dilution rate of 1:6, similar to HOS, LIVE, MOT and PMOT, rather than 1:7 as suggested for other kinematic traits

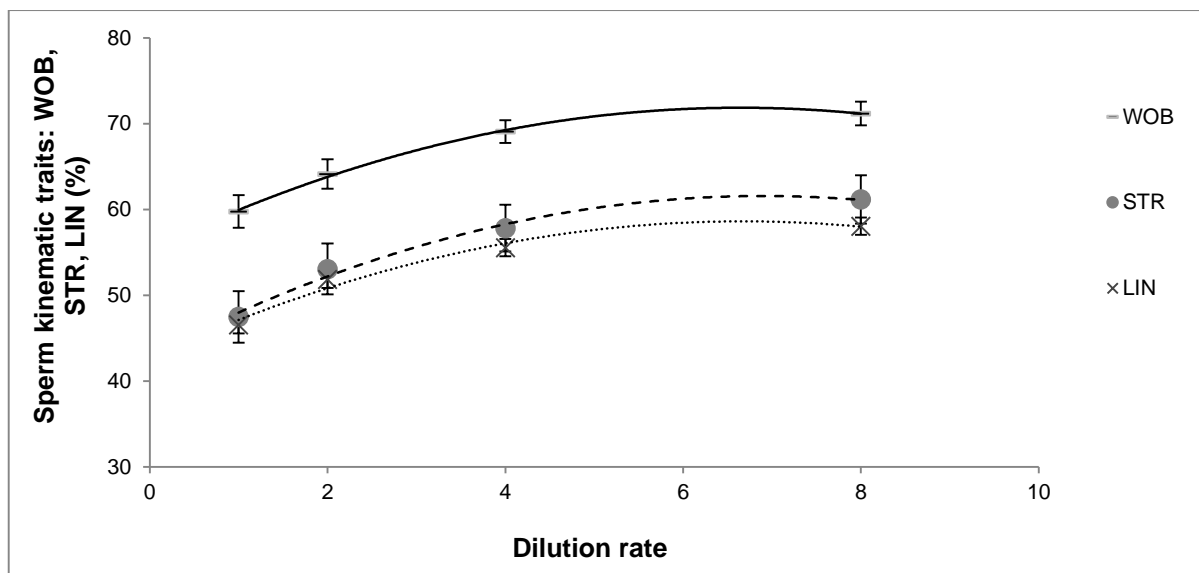


Figure 24: Polynomial relationships between sperm kinematic traits namely, wobble (WOB, %), straightness (STR, %) and linearity (LIN, %) at different dilution rates from 1:1 to 1:8. Standard error is indicated by vertical bars at the mean.

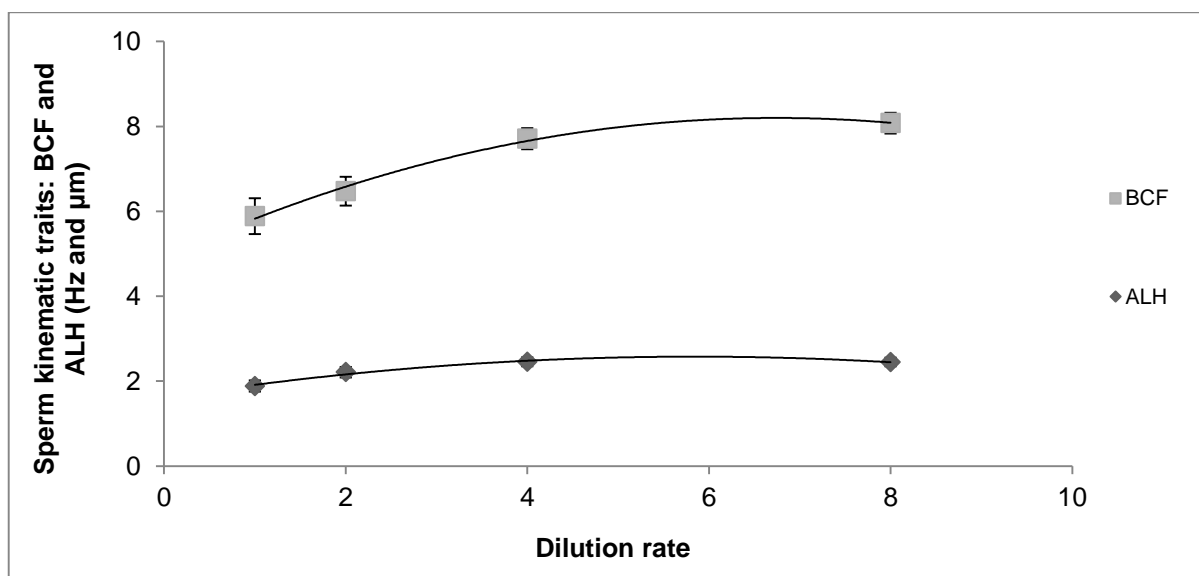


Figure 25: Polynomial relationships between sperm kinematic traits namely, beat- cross frequency (BCF, Hz) and lateral head displacement (ALH, μm) at different dilution rates from 1:1 to 1:8. Standard error is indicated by vertical bars at the mean.

3.3. The effect of dilution temperature on sperm traits

Dilution temperature contributed to a limited extent (FE = 0.90 to 1.15 %) to the variation in sperm traits compared to the other fixed effects of dilution rate and storage time. Both PMOT and MOT were highest, 24.73 ± 4.28 % and 54.94 ± 5.46 % respectively at the higher dilution temperatures of 21 and 38 °C being not different ($P > 0.05$) from each other, compared to 5 °C which was significantly different from 21 and 38 °C. A low positive Pearson correlation coefficient ($r = 0.12$; $P < 0.05$) was obtained between dilution rate

and PMOT. The relationship was quantified with a linear equation ($Y = \alpha + \beta_1 X$; $P = 0.03$; $R^2 = 0.02$), illustrated in Figure 26.

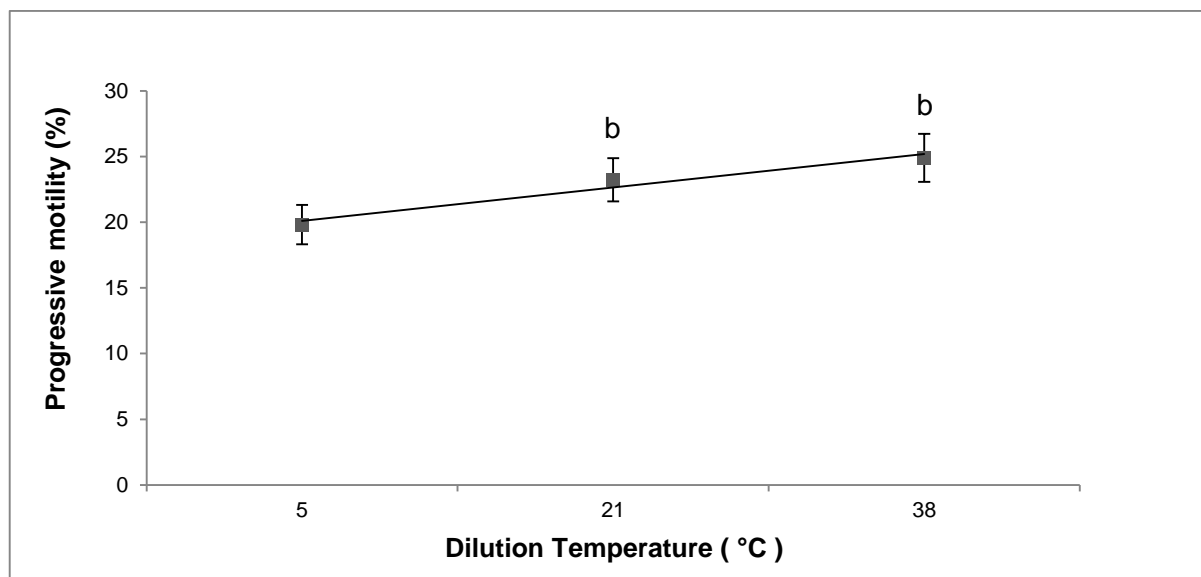


Figure 26: A linear relationship between progressive motility (PMOT, %) at different dilution temperatures (5, 21, 38 °C). Standard error is indicated by vertical bars at the mean.

The linear regression equation of $Y = 19.36 + 0.15X$ of PMOT on dilution temperature indicates a 0.15 % increase in progressive motility for every 1 °C increase from 5 to 21 °C.

3.4. The effect of storage time on sperm traits

Storage time of the semen contributed largely to the variation associated with all sperm traits with a linear decrease in all traits as time progressed from 1 to 48 hours. The variation accounted for by FE ranged from 3.62 to 45.09 % with PMOT and MOT influenced the most by storage time, while STR showed very little variation. LIVE and NORM showed limited deterioration (10.55 and 2.94 % respectively), from 1 to 24 hours, with no difference between 1, 5 and 24 hours ($P > 0.05$). LIVE and NORM decreased sharply, 17.74 to 29.53 % respectively, over the last storage period of 24 to 48 hours with 24 and 48 hours being significantly different from each other. HOS, PMOT, MOT, VCL, VSL, VAP, WOB, BCF showed a significant decrease with each storage period with all periods being different ($P < 0.05$) from each other. STR showed a limited decrease over 48 hours, with no significant differences between the storage periods. There was no decrease in LIN or ALH from 1 to 5 hours, while a significant decrease was recorded for LIN from 5 to 24 hours with 48 hours being not different ($P > 0.05$) from 24 hours. A sharp decrease ($P < 0.05$) was observed for ALH from 5 to 24 hours and from 24 to 48 hours.

Strong to low negative Pearson correlation coefficients ($P < 0.001$) for sperm traits (LIVE, HOS, NORM, ABNRM, PMOT, MOT, VCL, VSL, VAP, ALH, LIN, STR, WOB, BCF), ranging from $r = -0.16$ (STR) to -0.66 (MOT), with storage time were recorded (Table 24). Strong negative correlation coefficients, $r = -0.42$ to 0.66 , for most of the sperm traits (LIVE, HOS, PMOT, MOT, VCL, VSL, VAP, ALH, BCF) with storage time were evident, while medium strength correlation coefficients ($P < 0.001$) for NORM ($r = -0.38$), ABNRM ($r = +0.35$) and LIN ($r = -0.35$) were obtained. STR and WOB displayed weak negative correlation coefficients ($P < 0.001$) of $r = -0.16$ and $r = -0.19$ respectively, with storage time. The relationship for these sperm traits with storage time was quantified with regression analyses.

A non-linear quadratic relationship ($Y = \alpha + \beta_1X + \beta_2X^2$) was best to quantify the relationship between LIVE ($Y = 81.89 - 0.19X - 0.007X^2$; $R^2 = 0.35$; $P < 0.0001$) and NORM ($Y = 52.42 - 0.85X - 0.03X^2$; $R^2 = 0.21$; $P < 0.0001$) with storage time (Figure 27).

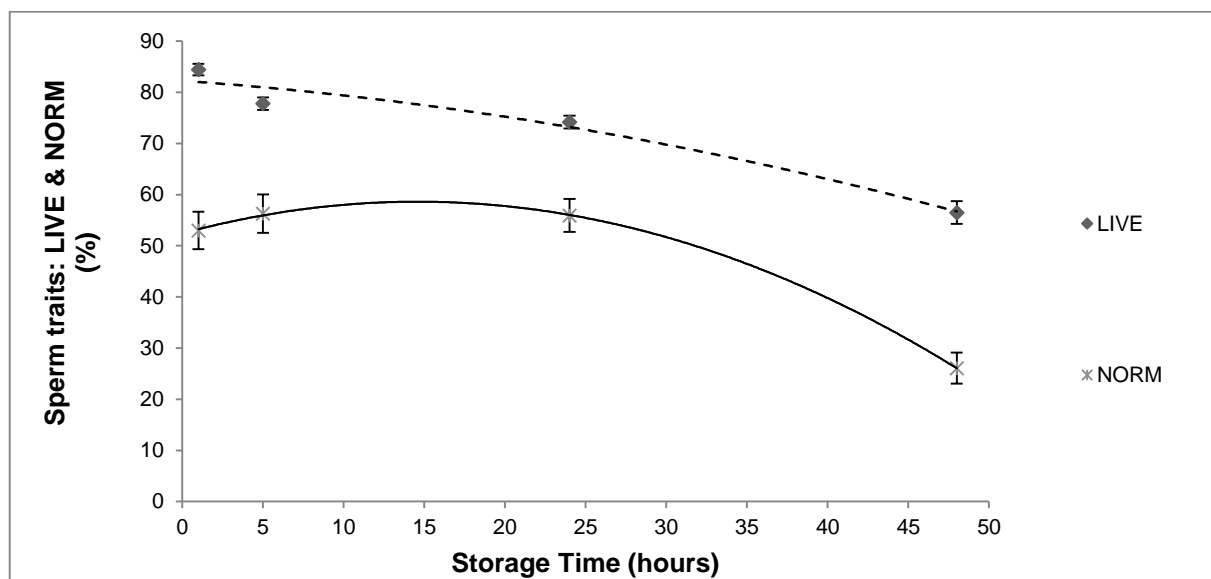


Figure 27: Non-linear relationships between sperm traits namely, sperm viability (LIVE, %) and normal sperm morphology (NORM, %) over a period of 48 hours. Standard error is indicated by vertical bars at the mean.

A linear regression ($Y = \alpha + \beta_1X$) was found best to quantify the relationship of sperm traits (WOB, HOS, PMOT, MOT, VCL, VSL, VAP, ALH and BCF) with storage time. Negative relationships for sperm traits on storage time were derived as follows: WOB ($Y = 73.12 - 0.37X$; $R^2 = 0.21$; $P < 0.0001$); HOS ($Y = 75.39 - 0.63X$; $R^2 = 0.23$; $P < 0.0001$), LIN ($Y = 58.67 - 0.30X$; $R^2 = 0.12$; $P < 0.0001$), PMOT ($Y = 34.30 - 0.45X$; $R^2 = 0.40$; $P < 0.0001$), MOT ($Y = 55.48 - 0.52X$; $R^2 = 0.43$; $P < 0.0001$) and are set out in Figure 28.

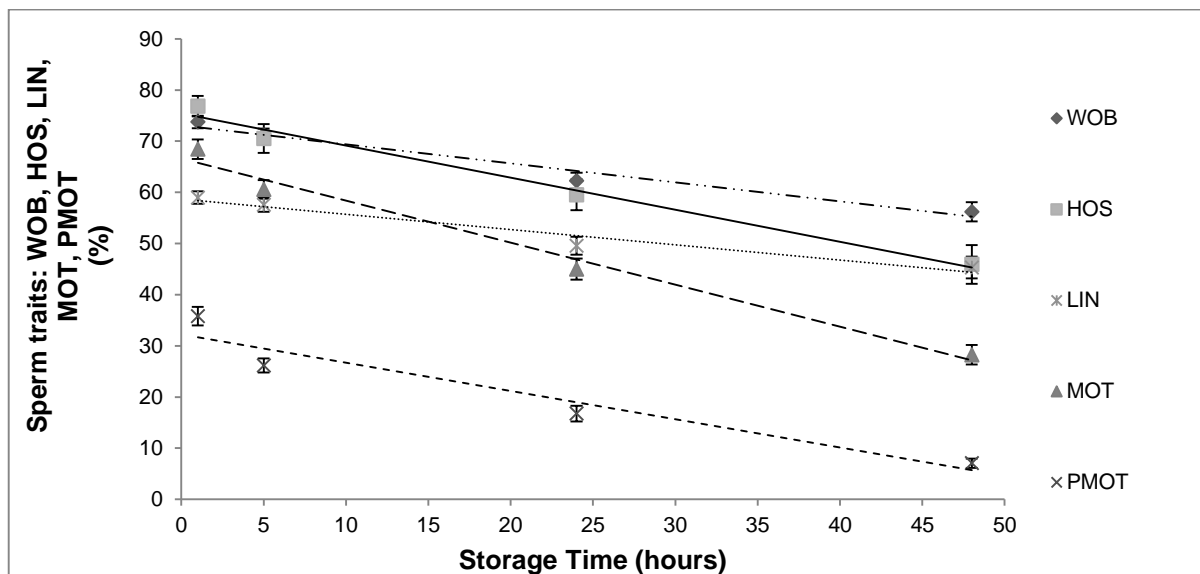


Figure 28: Linear relationships between sperm traits namely, sperm wobble (WOB, %); hypo osmotic resistant sperm (HOS, %), linearity of sperm swim (LIN, %); motility (MOT, %) and progressive motile (PMOT, %) over a period of 48 hours. Standard error is indicated by vertical bars at the mean.

Negative relationships of kinematic sperm traits with storage time were derived as follows: VCL ($Y = 56.99 - 0.51X$; $R^2 = 0.32$; $P < 0.0001$), VAP ($Y = 39.60 - 0.47X$; $R^2 = 0.31$; $P < 0.0001$), VSL ($Y = 29.31 - 0.37X$; $R^2 = 0.20$; $P < 0.0001$) (Figure 29). Corresponding regressions for ALH ($Y = 2.72 - 0.02X$; $R^2 = 0.18$; $P < 0.0001$) and BCF ($Y = 8.33 - 0.07X$; $R^2 = 0.17$; $P < 0.0001$) as displayed in Figure 30.

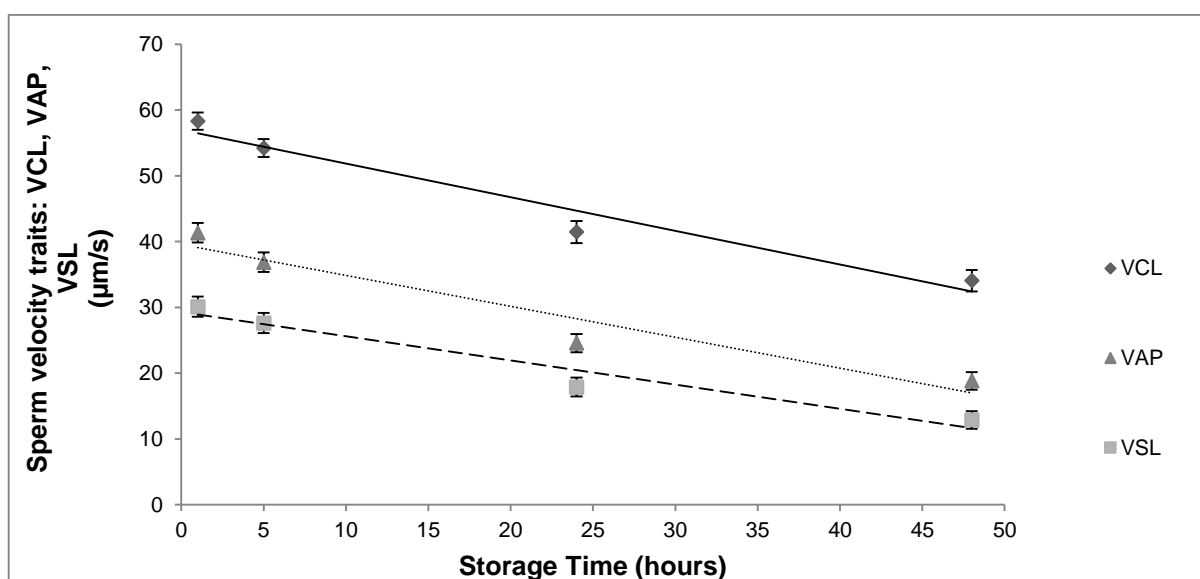


Figure 29: Linear relationships between sperm velocity traits namely, curve-linear velocity (VCL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$) and straight-line velocity (VSL, $\mu\text{m/s}$) over a period of 48 hours. Standard error is indicated by vertical bars at the mean.

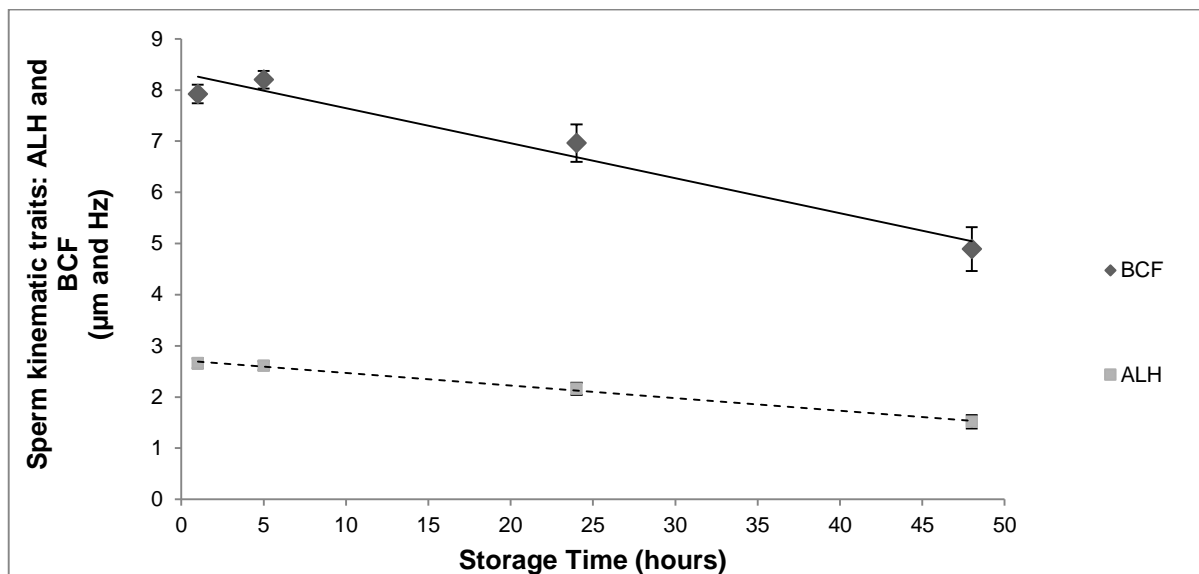


Figure 30: Linear relationships between sperm kinematic traits namely, beat- cross frequency (BCF, Hz) and lateral head displacement (ALH, µm) over a period of 48 hours. Standard error is indicated by vertical bars at the mean.

3.5. *The interaction between dilution rate and dilution temperature on sperm traits*

PMOT was the only sperm trait influenced ($P < 0.05$) by the interaction of dilution rate with dilution temperature (Figure 31). No differences ($P > 0.05$) were determined in PMOT between dilution temperatures (5, 21, 38 °C) at lower dilution rates of 1:1 and 1:2, with no difference ($P > 0.05$) between the latter two. However at higher dilution rates of 1:4 and 1:8 the effect of dilution temperature became more pronounced with higher dilution temperatures of 38 and 21 °C being significantly better in maintaining PMOT than 5 °C with no difference between dilution rates of 1:4 and 1:8.

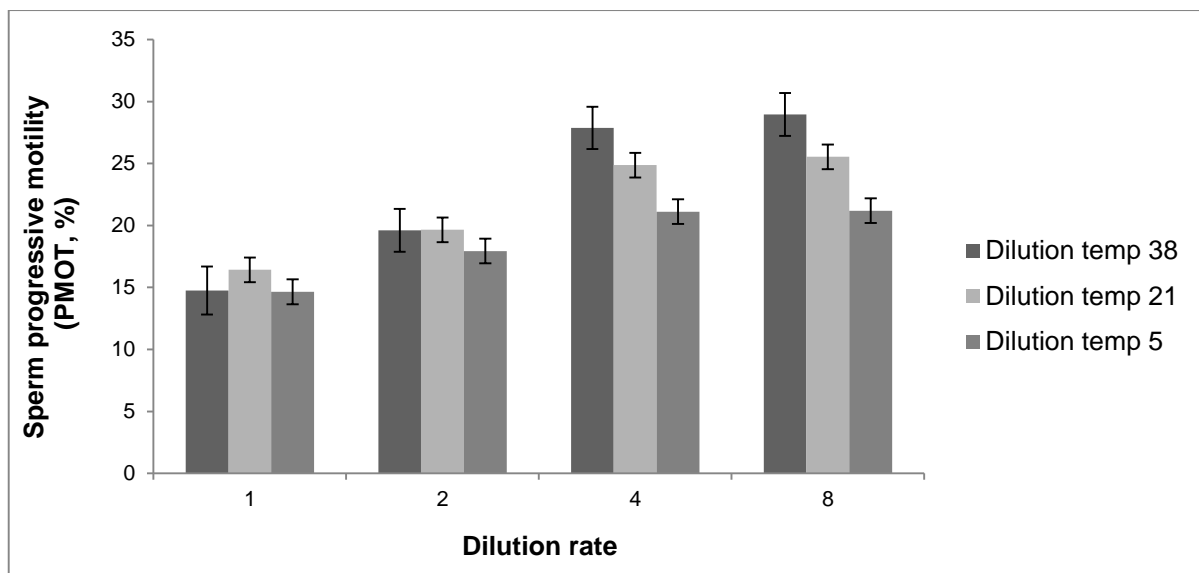


Figure 31: The interaction effect of dilution rate and dilution temperature on progressive motility (PMOT, %). Standard error is indicated by vertical bars at the mean..

3.6. The interaction between dilution rate and storage time on sperm traits

All sperm traits measured namely, LIVE, HOS, NORM, PMOT, MOT, VCL, VSL, VAP, ALH, LIN, STR, WOB and BCF showed significant variation associated with the interaction of dilution rate with storage time. For the first hour of storage no difference ($P > 0.05$) between dilution rates was observed in terms of maintaining sperm function. However, after 5 hours of storage, lower dilution rates of 1:1 to 1:2 were still not different ($P > 0.05$) from each other, but inferior compared to 1:4 and 1:8 dilution rates that were not different ($P > 0.05$) from each other. After 24 hours of storage the sperm function maintenance ability by lower dilution rates, 1:1 and 1:2 differed from each other, but were markedly below the higher dilution rates of 1:4 and 1:8 that were not different ($P < 0.05$) from each other. After 48 hours the latter trend was much more pronounced between lower and higher dilution rates with higher dilution rates of 1:4 and 1:8 being markedly superior ($P < 0.01$) in their ability to maintain sperm function. The interaction effect of dilution rate and storage time is demonstrated in Figure 32 and Figure 33 only for sperm motility (MOT, %) and sperm membrane integrity (HOS, %), although corresponding trends were observed for all other sperm traits measured.

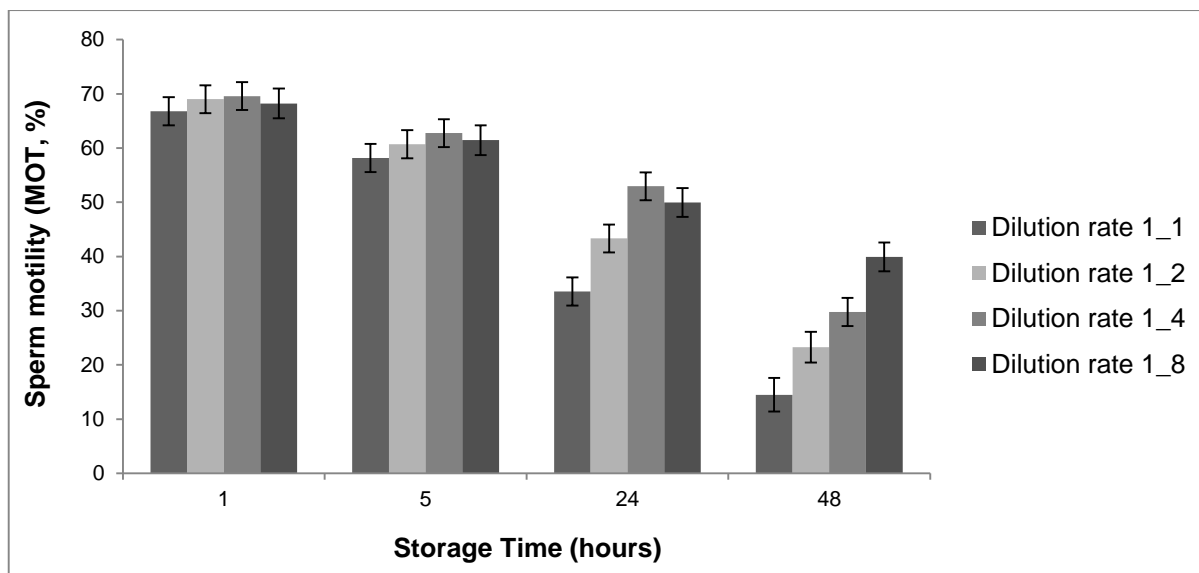


Figure 32: The interaction effect of storage time and dilution rate over a period of 48 hours for sperm motility (MOT, %). Standard error is indicated by vertical bars at the mean..

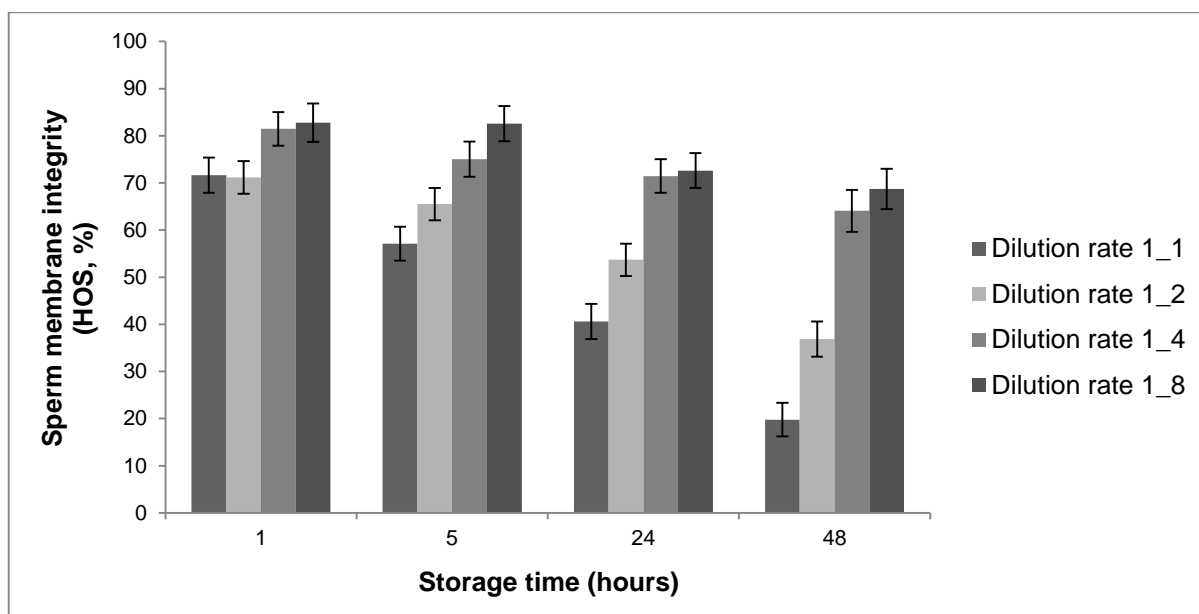


Figure 33: The interaction effect of storage time and dilution rate over a period of 48 hours for sperm membrane integrity (HOS, %). Standard error is indicated by vertical bars at the mean.

3.7. The interaction of dilution temperature with storage time for sperm traits

PMOT was the only sperm trait that was influenced ($P < 0.05$) by the interaction of dilution temperature with storage time (Figure 34). PMOT decreased gradually with an increase in storage time from 1 to 48 hours, with storage time intervals being significantly different from each other. After 1, 5 and 24 hour storage PMOT was highest ($P < 0.05$) at 38 and 21 °C, being no different from each other, compared to 5 °C. After 48 hours the effect of

dilution temperature on PMOT became insignificant, although 21 °C was most suitable in maintaining PMOT in absolute terms.

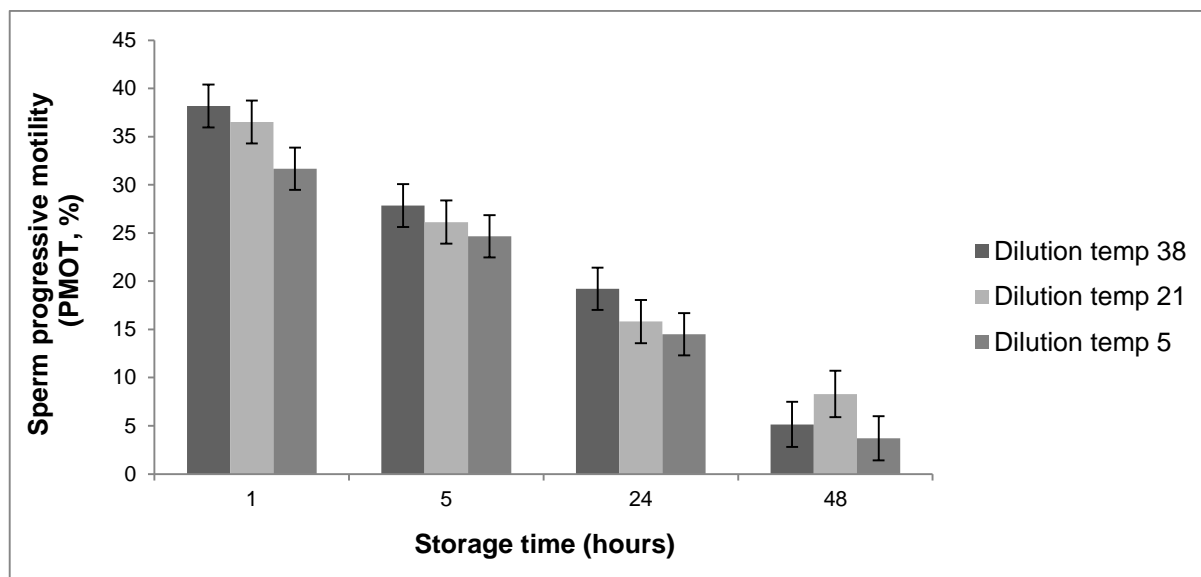


Figure 34: The interaction effect of storage time and dilution temperature (5, 21, 38 °C) over a period of 48 hours for sperm progressive motility (PMOT, %). Standard error is indicated by vertical bars at the mean.

3.8. *The interaction between male and storage time*

PMOT, MOT, BCF and NORM were highly ($P < 0.0001$) influenced by this interaction, while other traits like VCL, VSL, VAP showed less ($P < 0.05$) variation caused by the interaction effect of male with storage time. Males differed ($P < 0.05$) from each other and within each other, in their capability of maintaining measured sperm traits over a storage period up to 48 hours (Figure 35, Figure 36, Figure 37, Figure 38 and Figure 39 demonstrated these interactions for PMOT, MOT, NORM, BCF and VCL).

PMOT (Figure 35) was significantly higher for Male 3, at 1 (PMOT = 52.01 %) and 24 (PMOT = 26.76 %) hours compared to the other males that did not differ among each other after 1 to 24 hours of storage. After 48 hours of storage there was no significant difference between the males. PMOT varied significantly within males for 1, 5, 24 and 48 hours of storage. Male 2 however did not differ between 1, 5 and 24 hours of storage, but only after 48 hours of storage.

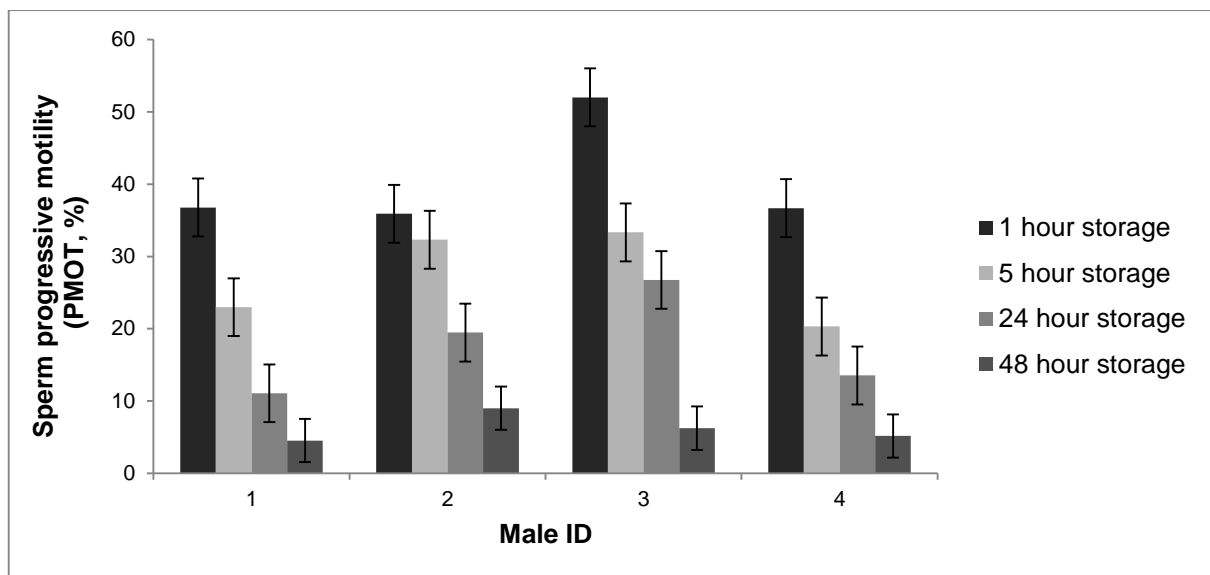


Figure 35: The interaction effect of storage time and male identity over a period of 48 hours for progressive sperm motility (PMOT, %). Standard error is indicated by vertical bars at the mean.

No differences ($P > 0.05$) were observed between males in terms of MOT (Figure 36) for storage periods of 1 to 5 hours, while there was a significant difference between males after 24 hours of storage. Males 2 and 3 were significantly better in their ability to maintain higher percentages of motile sperm (MOT = 57.57 %), with no difference ($P < 0.05$) between these males, compared to male 1 and 4 with no difference ($P > 0.05$) between the latter two (MOT = 36.13 %). After 48 hours only Male 2 differed from the other males and maintained the highest percentage of motile sperm (MOT = 38.62 %), compared to MOT = 22.63 % in other males. Within male variation in MOT was observed for all males over the 48 hour period of storage. Male 1, 2 and 4 showed no difference ($P > 0.05$) between 1 and 5 hours of storage, while Male 3 showed a sharp decrease ($P < 0.05$) from 1 to 5 hours of storage. Male 1, 3 and 4 showed a significant decrease in MOT from 5 to 24 hours and from 24 to 48 hours of storage while male 3 maintained MOT from 5 to 24 hours with no significant difference due to storage period. However, Male 3 showed a significant reduction in MOT from 24 to 48 hours of storage.

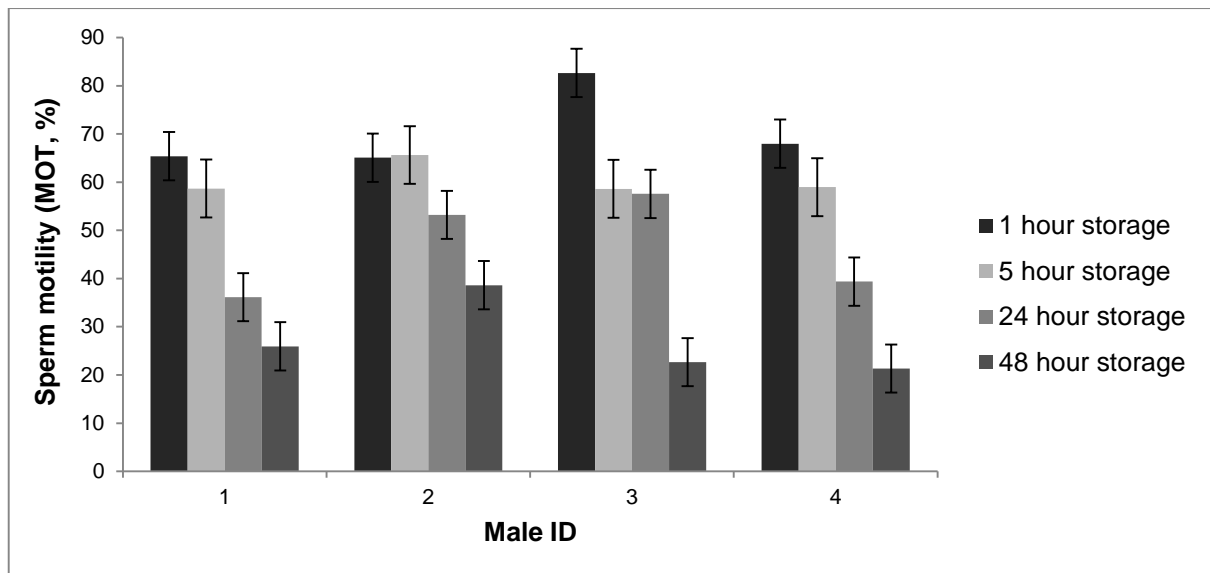


Figure 36: The interaction effect of storage time and male over a period of 48 hours for sperm motility (MOT, %). Standard error is indicated by vertical bars at the mean.

NORM (Figure 37) was significantly higher for Male 1 and 3, with no difference ($P > 0.05$) between the latter two males, compared to Male 2 and 4 at 1 and 5 hours of storage. After 24 hours of storage Male 3 maintained the highest percentage of morphologically normal sperm (76.28 %), compared to Males 1 and 2 that did not differ ($P > 0.05$) from each other. Male 4 presented the lowest ($P < 0.05$) NORM = 22.37 % at 24 hours, compared to all males. After 48 hours of storage NORM was highest for Male 2 and 3, not being significantly different from each other, compared to Male 1 and 4. Significant within male variation was obtained for NORM over the 48 hour storage period. Male 1 did not differ ($P > 0.05$) for NORM between 1 and 5 hours of storage, while there was a significant decrease from 5 to 24 hours and from 24 to 48 hours of storage. Male 2 and 3 did not differ from 1 to 24 hours of storage, but a significant decrease occurred from 24 to 48 hours. Male 4 recorded no difference ($P > 0.05$) between the different storage intervals of 1 to 48 hours of storage for NORM. However, the latter male had a markedly lower initial NORM, compared to the other males.

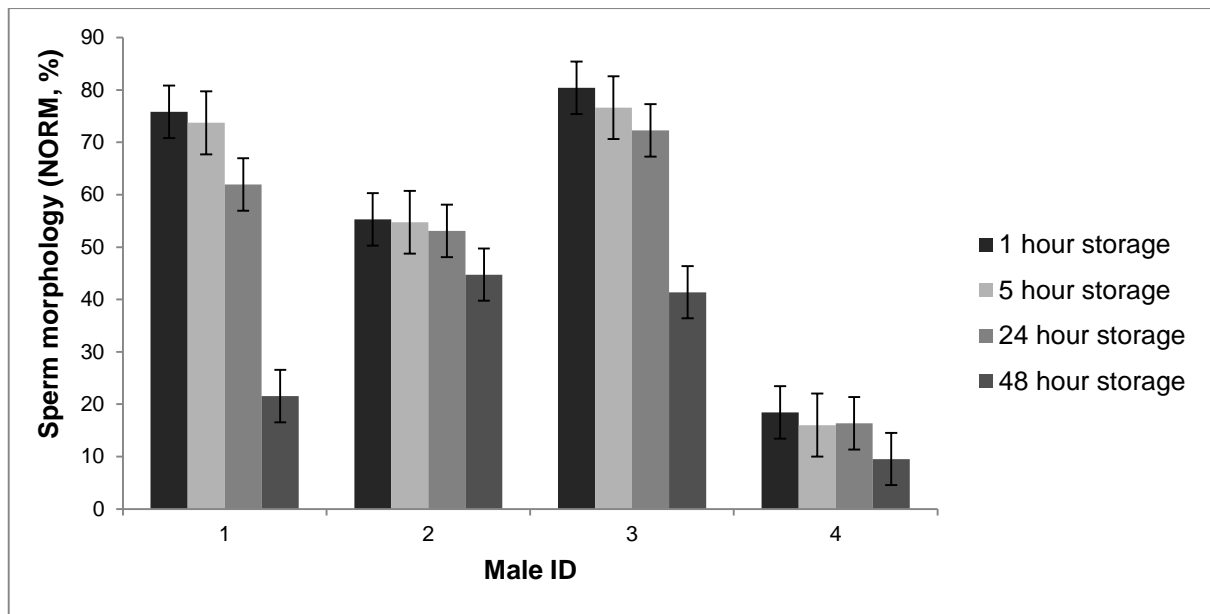


Figure 37: The interaction effect of storage time and male identity over a period of 48 hours for normal sperm morphology (NORM, %). Standard error is indicated by vertical bars at the mean.

BCF (Figure 38) was not different ($P > 0.05$) between males up to 1 to 5 hours of storage, except for Male 2 that displayed a significantly lower BCF (6.3 Hz). After 24 hours of storage, Male 3 maintained a significantly higher BCF (11.38 Hz) compared to the other males. However, no significant between male BCF variation ($P > 0.05$) was observed after 48-hour storage. Within males, BCF of Males 1 and 4 remained stable from 1 to 5 hours of storage while there was a significant decrease from 5 to 24 and from 24 to 48 hours. Male 2 showed no difference between the different storage intervals of 1, 5, 24 and 48 hours. No difference was obtained for male 3 from 1 to 24 hours while a significant reduction was evident from 24 to 48 hours of storage.

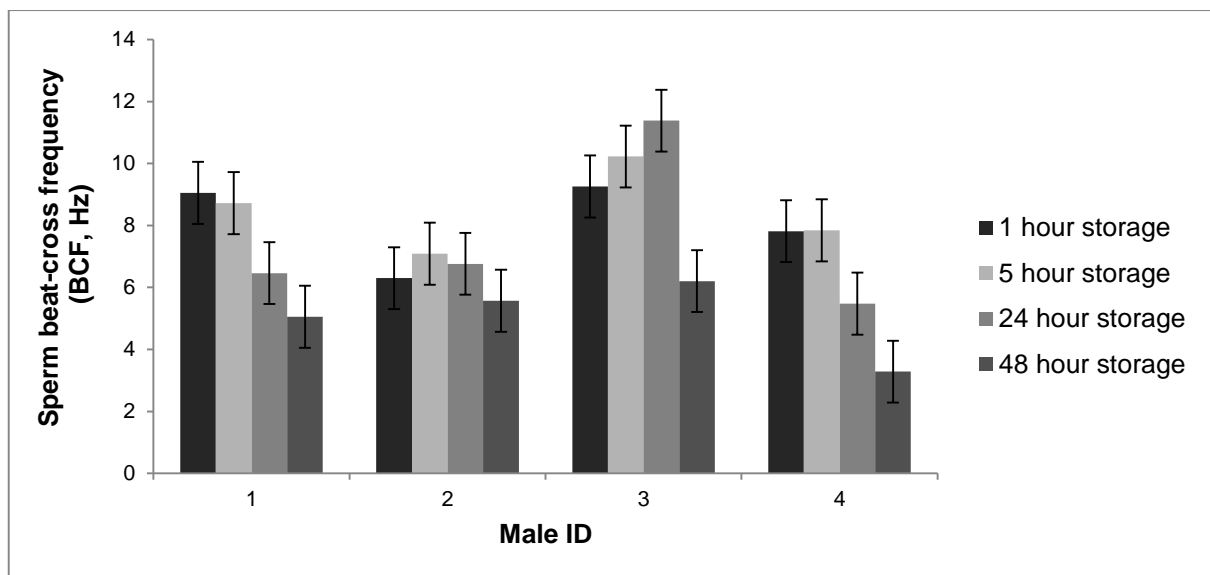


Figure 38: The interaction effect of storage time and male identity over a period of 48 hours for sperm beat -cross frequency (BCF,Hz). Standard error is indicated by vertical bars at the mean.

VCL, VSL and VAP differed ($P < 0.05$) between males at 1 hour, 5 hour, 24 hour and 48 hours of storage with the same trend evident between and within males for all three velocity traits (Figure 39). Males 2 and 4 did not differ ($P > 0.05$) after 1 hour of storage but means were significantly lower ($P < 0.05$) compared to Males 1 and 3, with no difference ($P > 0.05$) between the latter two. After 5 hours of storage only Male 4 had a significantly lower VCL than the other males with no difference ($P > 0.05$) between the latter males. After 24 hours of storage, Male 3 was significantly higher in sperm velocity compared to all the other males that showed no difference ($P > 0.05$) among each other. After 48 hours Male 4 had a significantly lower VCL than all the other males. No difference was observed between Male 1, 2 and 3 that maintained sperm velocity the best (VCL = $40.23 \mu\text{m/s}$; VSL = $28.85 \mu\text{m/s}$; VAP = $32.33 \mu\text{m/s}$).

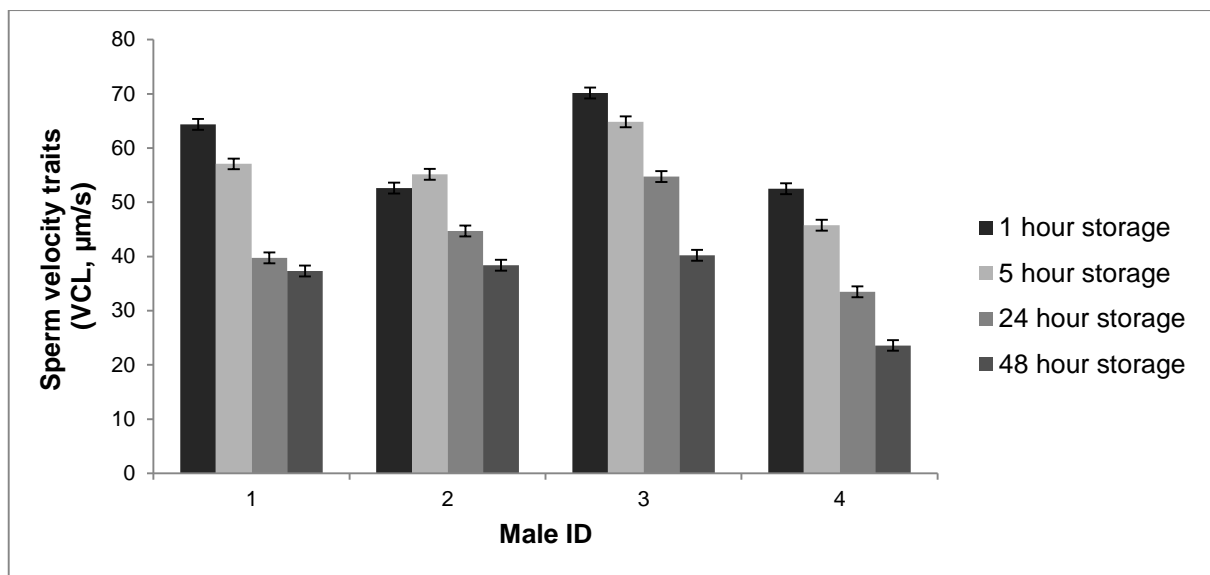


Figure 39: The interaction effect of storage time and male over a period of 48 hours for sperm velocity in terms of curve-linear velocity (VCL, $\mu\text{m/s}$). Standard error is indicated by vertical bars at the mean.

4. Discussion

4.1. *The effect of dilution rate and the interaction effect of dilution rate and storage time on sperm traits*

In this study the significant effect of dilution rate on ostrich semen has been observed in all sperm traits measured, namely LIVE, HOS, PMOT, MOT, VCL, VSL, VCL, VAP, ALH, LIN, STR, WOB, and BCF. Dilution rate had a substantial contribution, reaching 22.85 %, to the variation associated with HOS, compared to other sperm traits. The integrity of the ostrich sperm cell membrane in terms of HOS and sperm viability in terms of LIVE was best maintained by higher (1:4 to 1:8) dilution rates, compared to lower dilution rates (1:1 and 1:2). The motility traits MOT and PMOT, as well as the kinematic sperm traits VCL, VAP, VSL, ALH, LIN, STR, WOB and BCF were also improved at dilution rates (1:4 and 1:8).

The polynomial quadratic relationship between each sperm trait measured and dilution rate suggested a decrease in sperm function when under ($\leq 1:4$) or over dilution ($\geq 1:8$) is applied. Extrapolation beyond these data points, specifically $> 1:8$, is necessary to determine the extent of the impact of over-dilution. Dilution of sperm cells has a direct effect on cell homeostasis and in extreme conditions may adversely affect sperm function by compromising membrane integrity, viability, motility and ultimately fertilizing ability (Sexton, 1979; Parker and McDaniel, 2003, 2004, 2006; Chapter II). Under dilution of the semen aliquot, hence more cells per volume, has been associated with nutrient and

energy depletion, reduced O₂ and increased CO₂ concentrations, toxicity because of increased waste-product concentrations, an acidic pH, a change in electrolyte concentrations, H₂O and salt concentrations with associated volume and pressure changes (Nevo, 1965; Amann and Hammerstedt, 1980; Clarke *et al.*, 1982; Wishart, 1984; Bilgili *et al.*, 1987; Thomson and Wishart, 1991; Voet and Voet, 2004). The adverse effects associated with over-dilution is still not well understood, but it has been suggested that the reduction in seminal plasma volume, compared to the extender volume as well as the interaction between the cell and the extender components could potentially play a role (Austin and Natarajan, 1991; Blesbois and de Reviers, 1992; Garner *et al.*, 2001). Emmons and Blackshaw (1956) found that saline, a commonly used extender of semen, can hyperactivate sperm at higher dilution rates and thereby exhaust sperm. Parker and McDaniel (2006) found motility to be proportional to the increased O₂ concentration associated with an increase in dilution rate due to the metabolism of ATP, use of O₂, CO₂ generation and the exchange of Ca²⁺, Na⁺, K⁺ and Cl⁻. This increased metabolic effect of dilution on sperm might possibly be characterized by the increased enzymatic activity of the tricarboxylic acid cycle and subsequently its toxic by-products, oxygen-free radicals and malonaldehydes (Smith *et al.*, 1957; Riddle, 1968; Sexton, 1976; Wishart and Carver, 1984; Wishart, 1989).

The detrimental effects of oxidative stress have been associated with under- and over-dilution, because of the indirect effects of too high and too low sperm concentrations together with varying amounts of seminal plasma volume (Baumber and Ball, 2005; Murphy *et al.*, 2013; Agarwal *et al.*, 2014a, b). It has been shown that sperm cells exposed to large volumes of seminal plasma containing cytoplasmic droplets, a potential inducer of reactive oxygen species, are more prone to oxidative stress (Aitken and Baker, 2004; Brouwers *et al.*, 2005). The presence of cytoplasmic droplets has been observed for some individual ostrich males with droplets being more prominent in semen obtained outside of the ostrich breeding season (Du Plessis *et al.*, 2014). The cell membrane is the main target of lipid peroxidative damage, a result of oxidative stress and associated reactive oxygen species, because of its fatty acid composition and mostly the poly unsaturated fatty acid proportion which lacks the necessary cytoplasmic component containing anti-oxidants (Lenzi *et al.*, 2002; Aitken *et al.*, 2006; Khan, 2011; Murphy *et al.*, 2013). The loss of fatty acids, (up to 60% in severe oxidative stress conditions), compromises membrane function by decreasing its fluidity, increasing non-specific permeability to ions, and inactivating membrane bound receptors and enzymes ultimately also decreasing

metabolic activity (Storey, 1997). The latter may result in the impairment of sperm functional traits like membrane integrity, acrosomal integrity, morphology, viability, DNA damage, motility and ultimately fertilizing capacity of semen (Baumber *et al.*, 2000; Aitken and Baker, 2004; Kloppers *et al.*, 2008; Aitken *et al.*, 2010; Kothari *et al.*, 2010; Mahfouz *et al.*, 2010; Amaral *et al.*, 2013).

An optimal dilution rate of 1:6 for most ostrich sperm traits was predicted whereby maximum levels of HOS (80.18 %), LIVE (79.45 %), MOT (55.62 %), PMOT (27.07 %) and ALH (2.51 μm) could be maintained. Kinematic traits favored a wider range of dilution, 1:6 to 1:7, where maximum levels of VCL (53.28 $\mu\text{m/s}$), VAP (35.93 $\mu\text{m/s}$), VSL (27.25 $\mu\text{m/s}$), LIN (58.59 %), STR (61.56 %), WOB (71.85 %) and BCF (8.28 Hz) could be achieved. The 1:6 and 1:7 dilution rates are higher than the 1:1 dilution rate previously suggested for the ostrich (Malecki and Kadokawa, 2011) and for the emu (Sood *et al.*, 2011) but more aligned with a dilution rate of 1:4, suggested for the turkey (Tselutin *et al.*, 1995; Blesbois *et al.*, 2005).

The interaction effect of dilution rate with storage time on all sperm traits in this study stressed the importance of higher dilution rates (1:4 to 1:8), compared to lower dilution rates of 1:1 to 1:2, when storage of ostrich semen is considered. It was evident that sperm function after 24 and 48 hours of storage could be maintained at the same level as 5 and 24 hours of storage respectively, when higher dilution rates were used. The latter results imply that higher dilution rates at prolonged storage period diminished the harmful effects of metabolic disturbances associated with sperm storage *in vitro*. At these more suitable higher dilution rates, less metabolic by-products, more energy substrate, higher O_2 and less CO_2 concentrations as well as reactive oxygen species, per cell are maintained over longer storage periods, compared to lower dilution rates where the direct opposite conditions would likely exist (Rowell and Cooper, 1960; Nevo, 1965; Amann and Hammerstedt, 1980; Clarke *et al.*, 1982; Wishart, 1984; Bilgili *et al.*, 1987; Thomson and Wishart, 1991; Voet and Voet, 2004).

4.2. The effect of dilution temperature and the interaction effect between dilution temperature and dilution rate and between dilution temperature and storage time on sperm traits

Dilution temperature contributed to a small extent to the variation associated with only two of the sperm function traits measured namely, PMOT and MOT, suggesting a low degree of sperm cell temperature sensitivity, compared to the marked effects of dilution. However both PMOT and MOT were significantly higher (24.73 % and 54.94 % respectively) at higher dilution temperatures of 21 and 38 °C, compared to 5 °C that were associated with reductions of 4 % in MOT and 5 % in PMOT. Although this decrease in MOT and PMOT associated with a low dilution temperature can be attributed to a reduction in metabolic activity for extended preservation, and not a loss as such in MOT and PMOT, the interaction effect of dilution temperature with storage time suggests otherwise. This interaction stressed the importance of higher dilution temperatures (21 and 38 °C), compared to a lower dilution temperature of 5 °C, when storage of ostrich semen is considered. It was evident that PMOT was best maintained at higher dilution temperatures of 21 °C and 38 °C for 1 to 24 hours of storage, compared to sperm that was diluted with a pre-cooled diluent set at 5 °C. This suggests that there was in fact a loss of PMOT and MOT. PMOT could be maintained at the same level as at 5 to 24 hours of storage, when higher dilution temperatures were used for up to 48 hours of storage, compared to a dilution temperature of 5 °C. The latter suggests a degree of chilling injury experienced by sperm cells when rapidly cooled immediately after ejaculation with a pre-cooled diluent set at 5 °C that provides a fast cooling rate of ≥ 360 °C/min. The loss of motility, which is likely due to chilling injury, is probably a result of membrane and mitochondrial dysfunction since motility is largely dependent on mitochondrial energy production (Amann and Pickett, 1987; Moran *et al.*, 1992; Ruiz-Pesini *et al.*, 1998). The loss of sperm function in the ostrich is not consistent with the findings of Sood *et al.* (2011) who reported a beneficial effect of fast cooling, when a pre-cooled extender set at 5 °C was used for Emu sperm, in terms of avoiding chilling injury and maintaining membrane integrity, morphology, motility and penetration of the egg membrane compared to slow cooling. The detrimental effect of a pre-cooled diluent set at 5 °C on PMOT, compared to 21 and 38 °C, became more pronounced at higher dilution rates of 1:4 to 1:8 compared to dilution rates of 1:1 to 1:2. The latter result confirms the possibility of chilling damage of the cells when larger volumes of a pre-cooled diluent are used that will increase the cooling rate of the cell.

When short-term storage of ≥ 24 hours is considered, the results suggest slow cooling of ostrich semen to a low storage temperature of 5 °C to maintain sperm function in terms of PMOT at a maximum level. Although higher storage temperatures of ~ 20 °C have been suggested for the emu, to avoid cooling it is not always a viable option for commercial use, but rather for on-site AI. Lower storage temperatures of ~ 5 °C are necessary to reduce metabolic activity (Clarke *et al.*, 1982; Wishart, 1982, 1984) for increased storage time of chilled semen for national distribution and retail. Although cryopreserved semen is an alternative option, the sperm function loss associated with cryopreservation is much higher (40 to 70 %), while processing and distribution costs also exceed that of chilled semen. Chilled semen can be stored and transported at 5 °C in a fairly wide variety of inexpensive containers for commercial AI applications.

4.3. The effect of storage time and the interaction effect of storage time and male on sperm traits

Storage time caused a significant decline in all sperm traits measured from 1 to 48 hours of storage at 5 °C. This is consistent with the well-established fact that the fertilizing ability of sperm declined with an increase of storage time in other avian species like chicken and turkey (Garren and Shaffner, 1952; Clarke *et al.*, 1982; Bilgili *et al.*, 1987). The decline in fertilizing ability is related to the loss of sperm function, associated with metabolic changes that are exaggerated as storage time progresses and other storage practices like dilution and storage temperature (Clarke *et al.*, 1982; Dumpala *et al.*, 2006). LIN (62.29 ± 9.33 %) and STR (63.73 ± 10.57 %) were maintained for 48 hours without any decline, suggesting little to no effect of storage time on the quality of sperm swimming abilities. LIVE (85.05 ± 2.52 %) and NORM (55.97 ± 6.89 %) were maintained for 24 hours without any loss. Thereafter, respective sperm losses of 28.29 % and 29.88 % were observed up to 48 hours. The latter result suggests a limited influence of storage time on sperm viability and morphology, although a degree of loss was observed as storage time progressed after 24 hours of storage. This is in accordance with results of Siudzinska and Lukaszewicz (2008) who observed that the quality of semen of four different breeds of domestic fowls declined by ~ 20 % in terms of live and morphological normal sperm over a period of 24 hours. This is however not in agreement with results of Dumpala *et al.* (2006) who observed a linear reduction in sperm viability, expressed as the percentage dead cells over only 8 hours of storage in broiler breeders.

ALH, WOB, BCF were maintained for 5 hours, while a loss of 1.18 μm , 17.31 %, 3.01 Hz respectively was observed after 48 hours of storage. HOS, PMOT, MOT, VCL, VSL, VAP were maintained for 1 hour, but show a marked decline of 30.11 %, 29.61 %, 40.5 %, 23.92 $\mu\text{m/s}$, 16.53 $\mu\text{m/s}$, 21.94 $\mu\text{m/s}$ respectively over the 48 hours of storage suggesting a shorter storage period of 24 hours may limit the loss recorded to 17.20 %, 19.21 %, 23.65 %, 16.97 $\mu\text{m/s}$, 12.24 $\mu\text{m/s}$ and 16.82 $\mu\text{m/s}$ respectively. The linear decline in membrane integrity (HOS), motility (PMOT, MOT) and velocity traits (VCL, VSL, VAP), associated with an increase in storage time may possibly be attributed to the decrease in availability of nutrients as well as oxygen to the sperm cells, the toxic effect of by-product build up and possible oxidative stress on the cells that directly affects membrane integrity and motility and kinematic traits per se (Nevo, 1965; Parker and McDaniel, 2006). Results are however difficult to compare between various avian studies due to differences in storage processing, diluent specifications, dilution rate, dilution temperature, cooling rate, storage temperature and the sperm specific function evaluated, as well as species and individual differences in metabolic requirements and cell sensitivity towards processing.

The variation among males in their ability to maintain sperm function expressed as PMOT, MOT, BCF, NORM, VCL, VSL and VAP when used for *in vitro* short-term storage was evident in this study. Although only four males were used, significant variation was observed between them in terms of sperm function for the different storage periods of 1, 5, 24 and 48 hours. Males can differ in their initial sperm quality, but then show a higher rate of change associated with a loss in sperm function over a storage period of up to 48 hours, while others had the ability to maintain sperm function and being less sensitive towards storage over the 48 hour period. For instance the ejaculate of one specific male would have the potential to maintain morphological normal sperm from 1 to 24 hours, without any significant loss while sperm from another showed a 14 % loss over the same period. Similarly, the sperm of one male showed a 12 % loss in MOT over a period of 24 hours, while another showed a 30 % loss.

A high initial quality of sperm and ability to withstand processing, are two important considerations for successful artificial insemination since fertilization will depend on the number of cells with normal sperm function surviving storage that would reach the female reproductive tract. Such males are ideal for prolonged (24 to 48 hours) chilled semen storage and possibly cryopreservation, as they can be selected on initial semen quality and of their sensitivity towards storage processing, based on the rate of change over the

storage periods. Males with a good initial quality with a high rate of change would be more suitable for storage over shorter periods of 1 to 5 hours, or insemination within a short time after collection and dilution. Ostrich male variation in terms of initial semen quality (Chapter II; Kamar and Badreldin, 1959; Bonato *et al.*, 2010, 2011, 2014) has been well documented, but information on storage capability is limited for the ostrich. However, other studies done in stallions have shown that males can be classified as poor or good coolers based on their suitability for chilled semen survival (Battelier *et al.*, 2001). The variation between males and species can putatively be explained by genetic differences. Documented genetic differences in seminal plasma protein concentration, amidase activity and fatty acid composition could contribute to the variation associated with sperm traits and their ability to withstand storage processing (Cross, 1998; Surai *et al.*, 1998; Ciereszko *et al.*, 2010). Blesbois *et al.* (2005) pointed out the importance of the proportion of cholesterol and phospholipids in avian species relating to their sensitivity or suitability towards storage processing. These metabolites may have a direct influence on the membrane fluidity, an important characteristic of the membrane to survive processing stress, with species and individuals lower in cholesterol/phospholipids having a higher fluidity and stability (Cross, 1996; Blesbois *et al.*, 2005). The biochemical aspects of the ostrich sperm membrane still needs to be investigated to establish fatty acid composition and relations that they are involved in in order to predict behaviour of the cell under certain storage conditions and to improve storage protocols.

5. Conclusion

All ostrich sperm functions were markedly affected by dilution rate. Specifically, this study highlighted the importance to dilute semen immediately upon collection at the most suitable rate of an interpolated value of 1:6 for artificial insemination or if processed for storage (chilled and cryopreserved). Although sperm function was less influenced by dilution temperature, certain functions like sperm motility and progressive motility are better maintained at higher dilution temperatures of ≥ 21 °C. Higher dilution temperatures of ≥ 21 °C are convenient for field processing and suggest sensitivity of ostrich sperm towards fast cooling. Cooling rates should be further investigated to elucidate suitable cooling rates specific for storage (short- and long-term). For chilled semen, 24 hours seemed to be the most suitable period of storage for ostrich semen as minimal deterioration of sperm function took place over this period. Furthermore, males differed in their sensitivity towards storage processing assessed as *in vitro* measured sperm functions. The initial ejaculate quality in terms of sperm function together with the rate of

change in these functions from the quality of the initial ejaculate up to the end of processing should be considered as a convenient tool to estimate possible sperm function maintenance ability and the suitability of semen of specific males for storage. Males that exhibit the ability to maintain sperm function when processed for storage would be most suitable for longer periods of short-term storage and cryopreservation that could enable high rates of *in vivo* fertilization success after artificial insemination. Further studies to establish sire lines with high breeding values for storage suitability would be most beneficial for selection and genetic improvement of ostrich stock for AI.

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CHAPTER V

THE RESPONSE OF OSTRICH SPERM TO COOLING AT 5 °C STORAGE AND IN VIVO APPLICATION THROUGH ARTIFICIAL INSEMINATION

Optimal cooling is one of the fundamentals of a successful chilled stored semen protocol to maintain sperm functionality *in vitro* at above zero temperatures. No information is available regarding the sensitivity of the ostrich sperm to cooling. Therefore a wide range of cooling rates had to be tested to explore the behaviour of ostrich sperm as affected by cooling. This was done by serial dilution of semen samples from nine South African Black ostrich males at room temperature (~ 24 °C) to make 0, 2, 4 and 8x dilutions, and cooling at controlled rates of 43, 23, 5 and 1 °C/minute to 5 °C using a programmable freezer. Following dilution and cooling, semen was evaluated, stored for 24 hours at 5 °C and again evaluated. Results indicated that cooling had an expected detrimental impact on sperm viability (LIVE), membrane integrity (HOS), progressive motility (PMOT), motility (MOT), curve-linear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), lateral head amplitude (ALH), linearity (LIN), straightness of swim (STR), wobble (WOB) and beat -cross frequency (BCF). Slower cooling rates of 1, 5 and 23 °C/minute performed best in terms of the immediate effect exerted on sperm function in terms of PMOT, MOT, VCL, VSL, VAP, LIN and WOB, compared to 43 °C/minute. The elevated ALH values obtained after the cooling stage for semen cooled at 43 °C/min, compared to the non-processed ALH values and that of the slower cooling rates (< 43 °C/min) suggested possible premature hyper activation of sperm cells when fast cooled. The linear decline in VSL, VAP, LIN and WOB from 1 °C/min to 43 °C/min confirmed slower cooling of ≤ 1 °C/min to be more appropriate to maintain ostrich sperm function than faster cooling rates of ≥ 1 °C/min. In vivo testing was conducted by the artificial insemination of 16 females split equally into a treatment group of chilled stored semen and a control group with fresh diluted semen. The control and treatment groups were inseminated with an insemination dose of 800×10^6 sperm cells/mL and 1000×10^6 sperm cells/mL respectively with equal sperm numbers from each male. Females in both groups were inseminated with the same frequency that included 4 consecutive days of insemination with a follow-up insemination every 6-day for 3 consecutive turns. Fertility assessment of eggs included the counting trapped sperm in the outer perivitelline layer (OPVL sperm/mm²) and determining fertilization status through germinal disc evaluation. OPVL sperm was the only fixed effect that contributed to the variation associated with fertilization status. Fertilised eggs had a mean (\pm SE) number of 11.01 ± 2.27 OPVL sperm/mm², compared to 0.92 ± 0.39 OPVL sperm/mm² for non-fertilized eggs. Variation between females for both groups was observed for OPVL sperm number and fertilization status with two females consistently producing eggs with higher OPVL numbers (13.08 ± 4.56 and 7.00 ± 3.45 sperm/mm²), that were fertilized (75 to 100 %). It was evident that fertilised eggs could be obtained from chilled ostrich semen. However, research on female factors affecting OPVL sperm numbers is needed for optimizing of both *in vitro* and *in vivo* protocols.

1. Introduction

The rate of ostrich semen cooling before storage must still be addressed to maximise the maintenance of sperm function after short-term storage for artificial insemination (AI) purposes. The fundamentals of a successful short-term storage and AI program that have been set out for the ostrich, include reliable stress free semen collection (Rybnik *et al.*, 2007), semen collection frequency, timing and output (Bonato *et al.*, 2011, 2014b) as well as the duration of female sperm storage (Malecki *et al.*, 2004). More recently, fundamental studies on ostrich sperm function traits (Chapter II), the development of an ostrich specific semen extender (Chapter III) including chilled semen processing protocols in terms of optimal dilution rates and temperatures (Chapter IV) were established. These results enable a feasible liquid storage protocol to further the application of AI on a commercial basis in the ostrich industry.

Cooling of semen to sub-physiological holding or storage temperatures (≥ 0 °C) is necessary to reduce the sperm metabolism. It has been suggested by Hammerstedt and Andrews (1997) that the metabolic rate of any cell type would halve with every 10 °C decrease in the storage temperature. A low storage temperature would thus reduce the deleterious effects, associated with higher metabolic rates during storage characterized by the consumption and depletion of the bio-energy source (adenosine triphosphate, ATP) and oxygen, increased bacteriospermia, altered enzymatic activity, accumulation of metabolic by-products as well as oxidative stress exerted on the cell by oxygen free radicals that would impair sperm function (Sood *et al.*, 2012). Storage at 5 °C for other avian species like the chicken and turkey were shown to reduce ATP consumption by 75 to 80 %, compared to higher storage temperatures (40 °C) that are closer to the physiological temperatures (Wishart, 1984). Sood *et al.* (2012) demonstrated similar benefits of lower storage temperatures (5 and 10 °C) for emu semen reflected by sperm quality traits such as a higher percentage progressive motility, morphology, fertility and viability. However, sperm function may be lost by cooling the semen to these lower storage temperatures, due to phase transition of the membrane lipids when exposed to the critical zone of temperatures (15 to 20 °C) known as sperm chilling injury or cold shock. This reaction is unique to the biological cell because of specific molecule composition and properties (Watson, 1981; Quinn, 1985; Watson and Morris, 1987; Crowe *et al.*, 1989; Hammerstedt *et al.*, 1990; Drobnis *et al.*, 1993; Arav *et al.*, 1996; Saragusty *et al.*, 2005; Petrunkina, 2007). Blesbois *et al.* (2005) showed a linear increase

in anisotropy values between 40 and 10 °C, indicating a decrease in membrane fluidity associated with cooling of domestic bird semen. Sensitivity of sperm to cooling and cellular response may differ between species most probably due to species-specific membrane composition in terms of fatty acid composition and sterol levels (Watson and Morris, 1987; Parks and Lynch, 1992; White, 1993; Blesbois *et al.*, 2005).

The aim of this study was to develop the initial framework for ostrich sperm thermo-sensitivity and cellular response. This was achieved by applying a wide range of cooling rates to cool semen to a sub-physiological storage temperature of 5 °C. The initial evaluation of different sperm functions at different intervals after treatment enabled quantifying cellular response to a certain extent. The *in vitro* sperm function results allowed the implementation of the first *in vivo* fertility assessments of chilled stored ostrich semen through perivitelline techniques suggested (Malecki and Martin, 2003; Malecki *et al.*, 2005, 2008; Brand *et al.*, 2014). The latter would assist in optimization of the current ostrich short-term storage protocol developed in Chapter IV and to further studies in thermo-sensitivity of ostrich sperm.

2. Material and methods

2.1. Animal population

Animals in the resource population were screened from the commercial ostrich breeding flock maintained at the Oudtshoorn Research Farm on the basis of behavioural attributes rendering them suitable for AI (referred as desirable behaviour as described by Bonato *et al.* (2011), Bonato and Cloete, (2013) and Bonato *et al.* (2014a)). The origin of the ostrich flock and the general management procedures implemented therein has already been described (Van Schalkwyk *et al.*, 1996, 2000; Bunter and Cloete, 2004). Males for both trials were chosen for their reliability in terms of ejaculate quality, as well as their willingness to cooperate during semen collection using the dummy female method (Rybnik *et al.*, 2007). Females displaying the voluntarily crouch behaviour were used for artificial insemination purposes (Malecki and Martin, 2004; Malecki *et al.*, 2008). Although artificial insemination procedures for ostriches have not been optimized, a reliable method has been suggested by Malecki *et al.* (2008). Females were inseminated with a rigid straw (~ 30 cm) that can enter the cloaca by the guidance of the operator's fingers of one hand alongside the female phallus into the vagina orifice to reach the vagina. These docile females housed in single pens produced eggs in the absence of males with a slightly

lower egg production compared to females paired off with males (Bonato and Cloete, 2013).

Experiment 1, ejaculates were collected from nine *South African Black* males aged between 3 and 7 years. Ejaculates were allocated to test the effects of controlled cooling rates (1, 5, 23, 43 °C/minute) at different serial dilution rates (0, 2, 4, 8 x) on sperm function traits.

Experiment 2, ejaculates were collected from eight *South African Black* males aged between 3 and 7 years at different intervals to artificially inseminate a group of 16 *South African Black females*, aged between 2 and 10 years. The trial commenced during the months of August and September, 2015, over a period of 38 days. This period has been characterized as peak egg production months in pairs (Fair *et al.*, 2011) and for single-penned females (Bonato *et al.*, 2014a). The group of 16 females were split into two groups of eight each and allocated to the trial, testing AI with chilled stored semen (treatment group) and fresh diluted semen (control group) on *in vivo* fertility through perivitelline techniques (Malecki *et al.*, 2004).

2.2. Semen preparation

Sperm concentration was obtained in both experiments by means of spectrophotometer (Spectrawave, WPA, S800, Biochrom) with transmittance values that were previously calibrated against actual cell counts using a haemocytometer. Aliquots of 20 µl semen diluted 1:400 (v/v) with phosphate buffered saline solution containing 10 % formalin were used for the latter.

Experiment 1: *The effect of cooling rate of serially diluted semen on sperm function in vitro*

Individual semen samples from nine SAB ostriches were serially diluted with OS1 based on the ostrich seminal plasma composition of macro minerals (Chapter III) at room temperature (~ 24 °C) to make 0, 2, 4 and 8x dilutions, similar to 0, 1:1; 1:3; 1:7, and cooled at controlled rates: 1, 5, 23 and 43 °C/minute to 5 °C using a programmable freezer. Semen was evaluated for sperm specific functions that included motility, viability and membrane integrity after dilution, cooling and 24 h of storage at 5 °C.

Experiment 2: The effect of chilled stored semen on in vivo fertility using perivitelline techniques

Semen samples were diluted immediately upon collection at a dilution rate of 1:6 and a dilution temperature of 21 °C with the OS2, based on the ostrich seminal plasma composition of macro- and micro-elements (Chapter III) diluent. For storage, semen was cooled at a rate of 1 °C/minute to 5 °C, immediately after sperm function evaluation. Semen samples were stored individually for 24 hours in an aerated incubator (BL°CKICE Cooling Block, Techne), set at 5 °C. Semen of x-y males was pooled for insemination to supply the control and treatment female groups with an insemination dose of 800×10^6 sperm cells/mL and 1000×10^6 sperm cells/mL, respectively, with equal sperm numbers from each male. Females in both groups were inseminated at the same frequency that included 4 consecutive days of insemination with a follow-up insemination every 6 days for 3 consecutive cycles.

2.3. In vitro sperm function evaluation**2.3.1. Motility evaluation**

Sperm motility traits were evaluated by means of the Sperm Class Analyzer® (SCA) version 5.3 (Microptic S.L., Barcelona, Spain) after dilution, cooling and after 24 h storage. All sperm motility recordings were made after re-suspension of neat sperm as well as treated sperm in a standard motility buffer using Sodium Chloride (150mM) and TES (20mM) with male specific seminal plasma (2 %) to a final sperm concentration of 20×10^6 . After re-suspension a sample was placed in a 38°C water bath for 1 minute. For motility recording, 2 µl of diluted semen was placed onto a pre-warmed slide covered gently with a cover glass (22 x 22 mm) and allowed to settle for 20 seconds prior to recording. Seven to nine different fields were captured randomly to eliminate bias towards higher sperm concentration or motility, until at least 500 motile sperm were recorded. Sperm motility traits included motility (MOT, %), progressive motility (PMOT, %), curve-linear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), amplitude of lateral head displacement (ALH, µm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat- cross frequency (BCF, Hz).

2.3.2. Viability evaluation

Sperm viability in terms of live sperm (LIVE, %) was measured using the LIVE/DEAD® Sperm Viability Kit (Life technologies) that contained the SYBR® 14 and Propidium Iodide (PI) fluorescent stains. All sperm viability recordings were made after re-suspension of sperm in the standard ostrich diluent (pH7) to a final sperm concentration of 20×10^6 sperm sperm cells/mL. The SYBR® 14 working solution was prepared in a HEPES/NaCl medium to a 1:49 concentration (v/v) SYBR® 14 to HEPES/NaCl solution. Sperm suspension aliquots of 250 µl were re-suspended with 1.5 µl membrane-permeant SYBR® 14 working solution and incubated for 10 minutes at a temperature controlled environment of 38 °C. After incubation 2 µl of the next fluorescent stain, Propidium Iodide (PI), was added and incubated for another 10 minutes, whereafter cells were evaluated. For evaluation of viable (green) and non-viable (red/or green with red) sperm, a 2 µl droplet was placed on a glass slide and covered with a cover glass (22 x 22 mm) and allowed to settle for 20 seconds, prior to recording. The fluorescent sperm was observed and photographed under 10x microscopy with an Olympus BX41 epifluorescent microscope (Olympus Optical Co., Tokyo, Japan), equipped with a filter, camera (ColorView Illu Soft Imaging System) and software package (analysis FIVE, Olympus Soft Imaging Solutions GmbH, Münster) to count viable and non-viable sperm. Nine to ten different fields were randomly captured until at least 500 spermatozoa were evaluated. Distorted fields as well as fields that included drift or debris or clumps of sperm were excluded. The SYBR® 14 nucleic acid stain labels live sperm with green fluorescence, and membrane-impermeable PI labels the nucleic acids of membrane-compromised sperm with red fluorescence.

2.3.3. Membrane integrity evaluation

Sperm membrane integrity in terms of resistance to hypo-osmotic swelling (HOS, %) was measured using the Hypo-osmotic swelling test (Jeyendran *et al.*, 1984), adapted specifically for the ostrich by means of preliminary experimental evaluation. All sperm membrane integrity recordings were made after re-suspension of sperm in a standard salt (NaCl/H₂O) solution, adapted to 25 mOsm to a final sperm concentration of 20×10^6 sperm sperm cells/mL. For HOS recording 2 µl of diluted semen was placed onto a pre-warmed slide, using a heated stage set at 38 °C, covered gently with a cover glass (22 x 22 mm) and allowed to settle for 20 seconds, prior to recording. Sperm was captured using the Sperm Class Analyzer® (SCA) version 5.3 (Microptic S.L., Barcelona, Spain) with a Basler A312fc digital camera (Basler AG, Ahrensburg, Germany) mounted on an

Olympus BX41 microscope (Olympus Optical Co., Tokyo, Japan) equipped with phase contrast optics. Seven to nine different fields were captured randomly until good representation (500 sperm) was reached and biasness towards higher sperm concentration eliminated. Distorted fields as well as fields that included drift or debris or clumps of sperm were excluded.

2.4. In vivo assessment of fertility

2.4.1. Egg handling

Eggs were collected in the mornings and processed according to a standard procedure that included disinfecting and weighing. Details on the methods of egg collection, sanitation and storage on the research farm have been documented (Brand *et al.*, 2007, 2008). Eggs were stored for 48 hours at a temperature of 17 °C and relative humidity (RH) of 75 %, prior to opening for quantitative fertility assessment through perivitelline techniques.

2.4.2. Egg break out and germinal disc evaluation for fertilisation status determination

Egg break out and evaluation of the germinal disc (GD) is an objective method for the evaluation of true egg fertility as 17-20 % of discarded ostrich eggs are caused by embryonic mortality and infertility (Kosin, 1944, 1945; Romanoff and Romanoff, 1949; Wilson, 1991; Brown *et al.*, 1996; Bakst *et al.*, 1998; Malecki and Martin, 2003; Brand *et al.*, 2007, 2014; Malecki *et al.*, 2008). The establishment of early fertility by means of alternative methods like candling is subjective, since the shadowed area of the embryo is still poorly defined before 7 days of age (Deeming, 1995; Brand *et al.*, 2014). Egg fertilization status was observed by means of egg break-out and evaluation of the appearance of the germinal disc where the absence of the blastoderm would indicate infertility and the presence of the blastoderm fertilization (Malecki *et al.*, 2008). The opening of the eggs was done by carefully breaking the eggshell in the region of the air cell and removing excess albumin and membranes. The opened egg was then placed on a pedestal with an extra lighting source (Olympus LG-PS2, Olympus Optical Co., Ltd, Tokyo, Japan) under the stereo microscope (Olympus SZ-61, Olympus Optical Co., Ltd, Tokyo, Japan) to obtain a clear digital image by means of a mounted colour view camera (ColorView IIIU, Soft Imaging System) and image analysis software (analysis FIVE,

Olympus Optical Co., Ltd, Tokyo, Japan). The GD area was classified according to the description given by Malecki *et al.* (2008) and Brand *et al.* (2014) for the ostrich.

2.4.3. Quantifying of sperm trapped in the outer perivitelline layer

The counting of trapped sperm in the outer perivitelline layer (OPVL) near the ovum surface has been found to be a good predictor of fertility and the probability of fertilisation, indication of sperm storage tubule (SST) storage, transport, depletion, the functionality of the processed sperm and thus an indication of adapting sperm processing methods and insemination practices (Wishart, 1997; Wishart and Staines, 1999; Malecki and Martin, 2003). A 2 x 2 cm piece of perivitelline layer overlying the GD area was collected and cleaned of excess yolk with PBS, mounted on a glass slide and stained with 1 µg/mL Diamidino-2-phenylindole (DAPI)/PBS working solution whereafter it was left to dry at room temperature, protected from light (Wishart, 1987). The sperm nuclei were visualised with a fluorescence microscope (Olympus BX40, Olympus Optical Co., Ltd, Tokyo, Japan), using a “U” filter cube with 372 nm excitation and 456 nm emission wavelengths. The number of sperm in the OPVL within the GD area was estimated by counting in a frame placed in the centre and moving it 5 frames away from the centre.

2.5. Statistical analyses

Sperm traits as percentages, and with skewed distribution (as determined by the Shapiro-Wilk test: $P < 0.05$) were transformed using the arc sine of the percentage mean square-root ($\text{degree} \cdot \arcsin \sqrt{\%}$), while the sperm concentration was transformed to natural logarithms. Analyses included a distribution analysis and summary statistics to obtain variance parameters and graphs, describing the sperm traits. The total number of records, means, standard deviation, minimum, maximum and coefficients of variation (CV) was determined for each sperm trait. The contribution of each fixed effect (FE) to a particular sperm trait was evaluated by expressing the sum of squares for such an effect, as a percentage of the total corrected sum of squares (TCSS) (Leighton *et al.*, 1982; Smith, 2010). Least squares means, standard errors (S.E.) and CV's were calculated and subjected to Tukey's multiple range tests to investigate differences between least squares means. Correlations (Pearson) and regressions (linear and non-linear) were used to determine significant ($P < 0.05$) relationships between traits.

Separate general linear mixed models (GLMM) were performed in Experiment 1 for sperm function traits namely motility (PMOT, MOT, VCL, VSL, VAP, ALH, LIN, STR, WOB and BCF), viability (LIVE), and membrane integrity (HOS) which included the fixed effects of semen processing stages (S, neat, diluted or stored), cooling rate (R), dilution rate (D), sperm concentration (C, as a linear covariate) and male (M) as a random effect. GLMM performed in Experiment 2 for *in vivo* fertility assessment traits included fertilization status and number of sperm trapped in the OPVL with the fixed effects of semen treatment (T, fresh diluted and chill stored), female age (A, as a linear covariate), egg weight (M, as a linear covariate), number of OPVL sperm (O, as a linear covariate) and the number of days after last AI (N, as a linear covariate) with appropriate and female (F) as a random effect to account for the repeated sampling of the same females. GLMM were applied to assess the specific effect of females on fertility assessment traits and its interaction with other fixed effects. All analyses were done using the Statistical Analysis System (SAS, version 9.3).

Example of the GLMM fitted with Y being the dependent sperm traits of motility, viability and membrane integrity in Experiment 1:

$$Y_{ijkl} = \mu + M_i + S_j + R_k + D_l + b_0(C)_{ijkl} + e_{ijkl}$$

Where:

- Y_{ijkl} = Sperm trait under assessment
- μ = population mean
- M_i = random effect of the i^{th} male ($i = 1, 2, 3, 4, 5, 6, 7, 8, 9$)
- S_j = fixed effect of the j^{th} processing stage ($j = \text{neat, diluted, stored}$)
- R_k = fixed effect of the k^{th} cooling rate ($k = 1, 5, 23, 43 \text{ } ^\circ\text{C/minute}$)
- D_l = fixed effect of the l^{th} dilution rate ($l = 0, 1:1, 1:3, 1:7$)
- C_{ijkl} = sperm concentration fitted as a linear covariate
- b_0 = regression coefficients of Y_{ijkl} on sperm concentration (C)
- e_{ijkl} = random error

Example of the GLMM fitted with Y being the in vivo fertility assessment traits that include fertilization status, number of OPVL sperm and fertile period in Experiment 2:

$$Y_{ij} = \mu + F_i + T_j + b_0 (A)_{ij} + b_1 (M)_{ij} + b_2 (N)_{ij} + b_3 (O)_{ij} + e_{ij}$$

Where:

- Y_{ij} = Fertilization status
- μ = population mean
- F_i = random effect of the i^{th} female ($i = 1, 2, 3, 4, \dots, 16$)
- T_j = fixed effect of the j^{th} treatment ($j = \text{fresh diluted, chilled stored}$)
- A_{ij} = female age fitted as a linear covariate
- b_0 = regression coefficients of Y_{ij} on female age (A)
- M_{ij} = egg mass fitted as a linear covariate
- b_1 = regression coefficients of Y_{ij} on egg mass (M)
- N_{ij} = number of days after last insemination as a linear covariate
- b_2 = regression coefficients of Y_{ij} on egg mass (N)
- O_{ij} = number of OPVL sperm/mm² as a linear covariate
- b_3 = regression coefficients of Y_{ij} on number of OPVL sperm/mm² (O)
- e_{ij} = random error

3. Results

Experiment 1: *The effect of cooling rate of serially diluted semen on sperm function in vitro*

3.1. Descriptive statistics

Across analyses the mean (\pm SE) sperm concentration was $3.20 \times 10^9 \pm 3.98 \times 10^7$ sperm cells / mL with a CV of 21.08 % over 288 records. The summary statistics including the number of records, mean, standard deviation, minimum, maximum and coefficient of variation for the different sperm function traits are set out in Table 19.

Table 25: Description of sperm traits that included sperm viability (LIVE, %), membrane integrity (HOS, %), progressive motility (PMOT, %) and motility (MOT, %), curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat- cross frequency (BCF, Hz).

Sperm trait	Records	Mean	S.D	Minimum	Maximum	CV (%)
Live (%)	281	76.96	15.48	5.26	98.31	20.12
Hypo- osmotic swelling resistant (%)	282	67.34	22.58	0.00	92.20	33.53
Progressive motile (%)	282	28.44	17.88	0.00	73.00	62.86
Motile (%)	282	73.60	18.37	7.40	96.80	24.96
Curve-linear velocity ($\mu\text{m/s}$)	282	49.82	14.97	14.90	82.10	30.05
Straight-line velocity ($\mu\text{m/s}$)	282	27.62	12.31	2.60	69.10	44.56
Average path velocity ($\mu\text{m/s}$)	282	37.65	15.09	6.10	74.80	40.07
Amplitude of lateral head displacement (μm)	282	2.12	0.65	0.00	3.60	30.63
Linearity (%)	282	52.96	12.85	15.70	84.10	24.27
Straight (%)	282	71.56	9.81	36.20	94.00	13.71
Wobble (%)	282	72.94	11.02	35.90	91.00	15.11
Beat- cross frequency (Hz)	282	8.06	2.65	0.00	12.80	32.94

S.D: Standard deviation; CV: Coefficient of variation (%)

The contribution of each FE with its degrees of freedom (DF), R-square value and CV are shown in

Table 20 for LIVE, HOS, PMOT and MOT while kinematic sperm traits (VCL, VSL, VCL, VAP, ALH, LIN, STR, WOB and BCF) are displayed in Table 21. The least squares means for the different sperm traits are represented in Table 22 and Table 23.

Table 26: The source of variation for the fixed effect of sperm concentration, dilution rate, dilution temperature and storage time with their variation contribution (FE, %) with its degrees of freedom (DF) on sperm viability (LIVE, %), membrane integrity (HOS, %), progressive motility (PMOT, %) and motility (MOT, %).

Variation source	DF	LIVE	DF	HOS	DF	PMOT	MOT
Stage	2	40.62***	2	30.82***	2	64.88***	51.72***
Cooling rate	3	0.82	3	1.03	3	1.13***	1.17**
Dilution rate	3	6.99***	3	23.00***	3	6.89***	4.45***
TCSS	281	34671.97	281	66308.59	281	51195.77	40894.38
Error mean square	273	17906.99	273	29743.23	273	14049.08	17516.74
R²		0.48		0.55		0.73	0.57
CV (%)		13.04		18.85		23.64	13.33

; TCSS: Total corrected sum of squares; CV: Coefficient of variation; *P < 0.05; **P < 0.01; ***P < 0.001

Table 27: The source of variation for the fixed effect of sperm concentration, dilution rate, dilution temperature and storage time with their variation contribution (FE, %) with its degrees of freedom (DF) on sperm kinematic traits, curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat- cross frequency (BCF, Hz).

Variation source	DF	VCL	VSL	VAP	ALH	LIN	STR	WOB	BCF
Stage	2	62.70***	60.01***	62.48***	20.09***	40.32***	18.34***	52.01***	24.50***
Cooling rate	3	1.59***	2.59***	2.42***	0.11	2.31**	1.10	3.00***	1.03
Dilution rate	3	7.34***	7.80***	7.64***	11.86***	8.42***	6.48***	7.94***	7.28***
TCSS	281	62977.26	42566.76	63946.57	118.38	16478.46	11161.05	13853.92	1980.76
Error mean square	273	18099.67	12772.33	17814.35	80.61	8118.32	8288.15	5180.20	1333.94
R ²		0.71	0.70	0.72	0.32	0.51	0.26	0.63	0.33
CV (%)		16.34	24.76	21.46	25.64	11.68	9.49	7.34	27.43

TCSS: Total corrected sum of squares; CV: Coefficient of variation; *P < 0.05; **P < 0.01; ***P < 0.001

Table 28: The least square means (\pm S.E) of sperm concentration, dilution rate, dilution temperature and storage time on sperm viability (LIVE, %), membrane integrity (HOS, %), progressive motility (PMOT, %) and motility (MOT, %).

Variation source	LIVE	HOS	PMOT	MOT
Stage	***	***	***	***
Fresh	86.18 \pm 1.47 ^a	77.94 \pm 1.98 ^a	42.16 \pm 2.16 ^a	84.81 \pm 2.38 ^a
Cool	81.84 \pm 1.47 ^b	72.94 \pm 1.98 ^b	33.96 \pm 2.16 ^b	80.37 \pm 2.39 ^b
Stored	61.69 \pm 1.48 ^c	48.91 \pm 1.98 ^c	9.05 \pm 2.16 ^c	54.87 \pm 2.39 ^c
Cooling rate	P > 0.05	P > 0.05	**	**
1	76.61 \pm 1.76 ^a	64.79 \pm 2.40 ^a	30.75 \pm 2.33 ^a	76.47 \pm 2.62 ^a
5	76.62 \pm 1.70 ^a	64.32 \pm 2.33 ^a	31.15 \pm 2.29 ^{ba}	74.09 \pm 2.57 ^{ba}
23	78.78 \pm 1.47 ^a	68.87 \pm 1.98 ^a	28.38 \pm 2.15 ^{cba}	74.14 \pm 2.38 ^{cba}
43	74.29 \pm 1.60 ^a	68.35 \pm 2.19 ^a	23.29 \pm 2.23 ^d	68.70 \pm 2.49 ^d
Dilution rate	***	***	***	***
0	69.98 \pm 1.62 ^a	49.96 \pm 2.22 ^a	20.64 \pm 2.23 ^a	68.10 \pm 2.49 ^a
1:1	76.92 \pm 1.59 ^{bcd}	63.78 \pm 2.16 ^b	29.51 \pm 2.22 ^{bcd}	72.06 \pm 2.47 ^{bc}
1:3	80.08 \pm 1.59 ^{cbd}	74.12 \pm 2.16 ^c	31.50 \pm 2.22 ^{cbd}	75.40 \pm 2.47 ^{cd}
1:7	79.31 \pm 1.59 ^{dcb}	78.46 \pm 2.16 ^d	31.91 \pm 2.22 ^{dcb}	77.83 \pm 2.48 ^{dc}

*P < 0.05; **P < 0.01; ***P < 0.001; Means with different superscripts differ (P < 0.05)

Table 29: The least square means (\pm S.E) of sperm concentration, dilution rate, dilution temperature and storage time on the curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat- cross frequency (BCF, Hz).

Variation source	VCL	VSL	VAP	ALH	LIN	STR	WOB	BCF
Stage	***	***	***	***	***	**	***	***
Fresh	60.44 \pm 1.70 ^a	36.26 \pm 1.31 ^a	48.31 \pm 1.69 ^a	2.36 \pm 0.07 ^a	59.67 \pm 1.33 ^a	74.98 \pm 1.26 ^a	79.26 \pm 1.06 ^a	8.94 \pm 0.32 ^a
Cool	56.14 \pm 1.71 ^b	32.27 \pm 1.31 ^b	44.16 \pm 1.70 ^b	2.28 \pm 0.07 ^{ba}	57.25 \pm 1.34 ^{ba}	73.32 \pm 1.26 ^{ba}	77.88 \pm 1.06 ^{ba}	8.91 \pm 0.33 ^{ba}
Stored	33.49 \pm 1.70 ^c	14.38 \pm 1.31 ^c	21.25 \pm 1.69 ^c	1.71 \pm 0.07 ^c	41.21 \pm 1.33 ^c	65.23 \pm 1.26 ^c	61.74 \pm 1.06 ^c	6.15 \pm 0.33 ^c
Cooling rate	***	***	***	P > 0.05	***	P > 0.05	***	P > 0.05
1	51.49 \pm 1.85 ^a	29.27 \pm 1.45 ^a	39.50 \pm 1.84 ^a	2.15 \pm 0.09 ^{dc}	54.11 \pm 1.57 ^a	72.17 \pm 1.48 ^{dbc}	73.77 \pm 1.24 ^a	8.54 \pm 0.38 ^a
5	53.32 \pm 1.82 ^{ba}	30.37 \pm 1.42 ^{ba}	41.76 \pm 1.81 ^{ba}	2.11 \pm 0.09 ^a	54.42 \pm 1.52 ^{ba}	71.16 \pm 1.44 ^a	75.22 \pm 1.20 ^{ba}	7.71 \pm 0.37 ^a
23	49.62 \pm 1.77 ^{ca}	27.58 \pm 1.31 ^{ca}	37.61 \pm 1.69 ^{ca}	2.13 \pm 0.07 ^a	53.20 \pm 1.32 ^{cba}	71.62 \pm 1.25 ^a	73.35 \pm 1.06 ^{cba}	8.15 \pm 0.32 ^a
43	45.67 \pm 1.77 ^d	23.34 \pm 1.37 ^d	32.76 \pm 1.76 ^d	2.06 \pm 0.08 ^a	49.11 \pm 1.44 ^d	69.76 \pm 1.36 ^a	69.50 \pm 1.14 ^d	7.61 \pm 0.35 ^a
Dilution rate	***	***	***	***	***	**	***	***
0	42.95 \pm 1.77 ^a	21.63 \pm 1.37 ^a	30.63 \pm 1.76 ^a	1.76 \pm 0.08 ^a	46.68 \pm 1.45 ^a	66.97 \pm 1.37 ^a	67.87 \pm 1.15 ^a	7.05 \pm 0.35 ^a
1:1	51.47 \pm 1.75 ^b	28.51 \pm 1.36 ^b	39.09 \pm 1.74 ^b	2.07 \pm 0.08 ^b	52.72 \pm 1.42 ^b	71.23 \pm 1.34 ^b	72.81 \pm 1.13 ^b	7.59 \pm 0.35 ^{ba}
1:3	53.35 \pm 1.75 ^{cb}	30.09 \pm 1.36 ^{cb}	41.10 \pm 1.74 ^{cb}	2.32 \pm 0.08 ^c	54.32 \pm 1.42 ^{cb}	72.19 \pm 1.34 ^{cb}	74.56 \pm 1.13 ^{cb}	8.46 \pm 0.35 ^{cb}
1:7	52.33 \pm 1.76 ^{dc}	30.32 \pm 1.36 ^{dc}	40.81 \pm 1.75 ^{dc}	2.30 \pm 0.08 ^{dc}	57.12 \pm 1.43 ^{dc}	74.32 \pm 1.35 ^{dc}	76.59 \pm 1.13 ^{dc}	8.89 \pm 0.35 ^{dc}

*P < 0.05; **P < 0.01; ***P < 0.001; Means with different superscripts differ (P < 0.05)

3.2. *The effect of stage on sperm traits*

All sperm traits measured (LIVE, HOS, PMOT, MOT, VCL, VSL, VAP, ALH, LIN, STR, WOB, BCF) were detrimentally influenced ($P < 0.05$;

Table 20 and

Table 21) by the processing stages of cooling and storing. The variation associated with processing stages ranged from 64.88 % for PMOT to 18.34 % for STR and was the highest, compared to the other fixed effects of cooling rate and dilution rate. Fresh, cooled and stored sperm differed significantly for most sperm traits, namely LIVE, HOS, PMOT, MOT, VCL, VSL and VAP, with the highest sperm function measured directly after collection (fresh), compared to cooled and stored processed sperm (Table 22 and Table 23). PMOT was most sensitive to processing with a severe reduction of 33.11 % after cooling and storage. Overall, cooling had a smaller impact on sperm function, compared to that of storage. ALH, LIN, STR, WOB and BCF were unaffected by cooling ($P > 0.05$) but significantly declines of 0.65 μm , 18.46 %, 9.75 %, 17.52 %, 2.79 Hz respectively were recorded after storage.

3.3. The effect of cooling rate on sperm traits

Cooling rate contributed to the variation associated with PMOT (FE = 1.13 %; $P < 0.0001$), MOT (FE = 1.17 %; $P = 0.0011$), VCL (FE = 1.59 %; $P < 0.0001$), VSL (FE = 2.59 %; $P < 0.0001$), VAP (FE = 2.42 %; $P < 0.0001$), LIN (FE = 2.31 %; $P = 0.0040$) and WOB (FE = 3.00 %; $P < 0.0001$). PMOT, MOT, VCL, VSL, VAP, LIN and WOB was significantly compromised by the fastest cooling rate of 43 °C/min, compared to 1, 5, 23 °C/min that did not differ significantly. Although cooling rate did not contribute significantly ($P = 0.06$) to the variation associated with LIVE, the same trend was displayed with the lowest (74.29 ± 1.60 %) percentage of viable sperm observed when cooled at 43 °C/min compared to slower cooling rates. No significant Pearson's correlation coefficients were obtained for PMOT, MOT and VCL with cooling rate with regression analyses for these traits being discarded (Table 30).

Table 30: Pearson's correlations among ostrich sperm traits namely progressive motile (PMOT, %); motility (MOT, %), curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), beat- cross frequency (BCF, Hz), sperm viability (LIVE, %) and membrane integrity (HOS, %) with cooling rate.

	LIVE	HOS	PMOT	MOT	VCL	VSL	VAP	ALH	LIN	STR	WOB	BCF
CR	-0.03	-0.11	-0.09	-0.09	-0.10	-0.14*	-0.13*	0.01	-0.13*	-0.06	-0.14*	-0.05

CR: Cooling rate; *P < 0.05; **P < 0.01; ***P < 0.001

The Pearson's correlation coefficients between cooling rate and VSL ($r = -0.14$; $P = 0.021$), VAP ($r = -0.13$; $P = 0.0317$), LIN ($r = -0.13$; $P = 0.0303$) and WOB ($r = -0.14$; $P = 0.022$) indicated weak negative relationships. Linear regressions ($Y = \alpha + \beta_1 X$; $P < 0.05$) depicting the relationships of cooling rate amounted to $Y = 29.66 - 0.106X$; $P = 0.0210$; $R^2 = 0.02$ for VSL, to $Y = 39.98 - 0.121X$; $P = 0.0317$; $R^2 = 0.02$ for VAP, to $Y = 54.96 - 0.104X$; $P = 0.0303$; $R^2 = 0.02$ for LIN and to $Y = 74.75 - 0.09X$; $P = 0.0220$; $R^2 = 0.03$ for WOB (Figure 40). The negative linear regressions of VSL and VAP on cooling rate indicate reductions of respectively 0.11 and 0.12 $\mu\text{m/s}$ with every 1 $^\circ\text{C}/\text{min}$ increase in cooling rate. Accordingly, a decrease of 0.1 % for both LIN and WOB can be expected with every 1 $^\circ\text{C}/\text{min}$ increase in cooling rate.

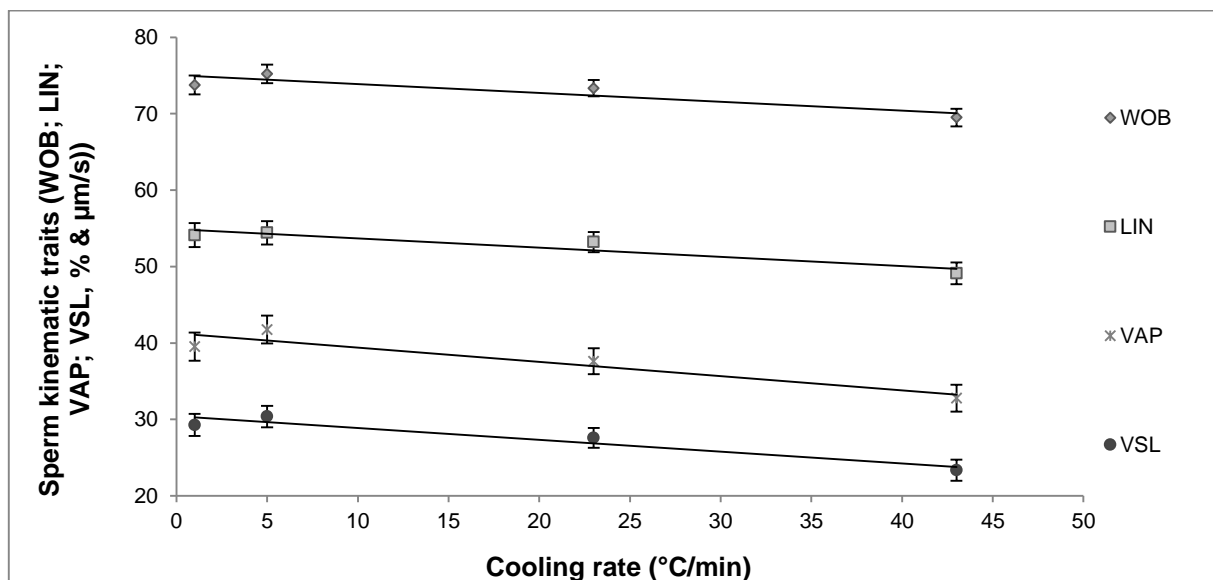


Figure 40: The effect of cooling rate on sperm kinematic traits namely, sperm wobble (WOB, %), linearity (LIN, %), average path velocity (VAP, $\mu\text{m/s}$) and straight-line velocity (VSL, $\mu\text{m/s}$). Standard error is indicated by vertical bars at the mean..

3.4. The effect of dilution rate on sperm traits

Dilution rate (neat; 1:1; 1:3; 1:7) as a FE contributed to the variation associated with all sperm traits measured and ranged from 4.45 % for MOT to 23 % for HOS. Sperm function expressed as LIVE, HOS, PMOT, MOT, VCL, VSL, VAP, ALH, LIN, STR and WOB improved ($P < 0.05$) after dilution compared to the neat state. Only HOS continuously increased with an increase in dilution rate with each rate being significantly different from each other. LIVE, PMOT, VSL, VSL, VAP, ALH, LIN, STR and WOB were not different between the different dilution rates. MOT was slightly higher ($P < 0.05$) at a 1:7 dilution compared to 1:1, but did not significantly differ from 1:3. BCF was unaffected by dilution rates ($P > 0.05$). The relationship between all traits measured and dilution was confirmed ($P < 0.0001$) by Pearson correlation coefficients ranging from 0.19 for PMOT to 0.44 for HOS (Table 31).

Table 31: Pearson's correlation coefficients among ostrich sperm traits, progressive motile (PMOT, %); motility (MOT, %), curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), beat- cross frequency (BCF, Hz), sperm viability (LIVE, %) and membrane integrity (HOS, %) with dilution rate.

	PMOT	MOT	VCL	VSL	VAP	ALH	LIN	STR	WOB	BCF	LIVE	HOS
Dilution	0.19 ***	0.19 ***	0.18 ***	0.21 ***	0.20 ***	0.28 ***	0.26 ***	0.24 ***	0.26 ***	0.25 ***	0.19 ***	0.44 ***

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

A quadratic relationship ($Y = \alpha + \beta_1X + \beta_2X^2$; $P < 0.0001$) was best to describe the relationship between HOS ($Y = 50.01 + 8.53X - 0.61X^2$; $P < 0.0001$; $R^2 = 0.23$) and dilution rate (Figure 41).

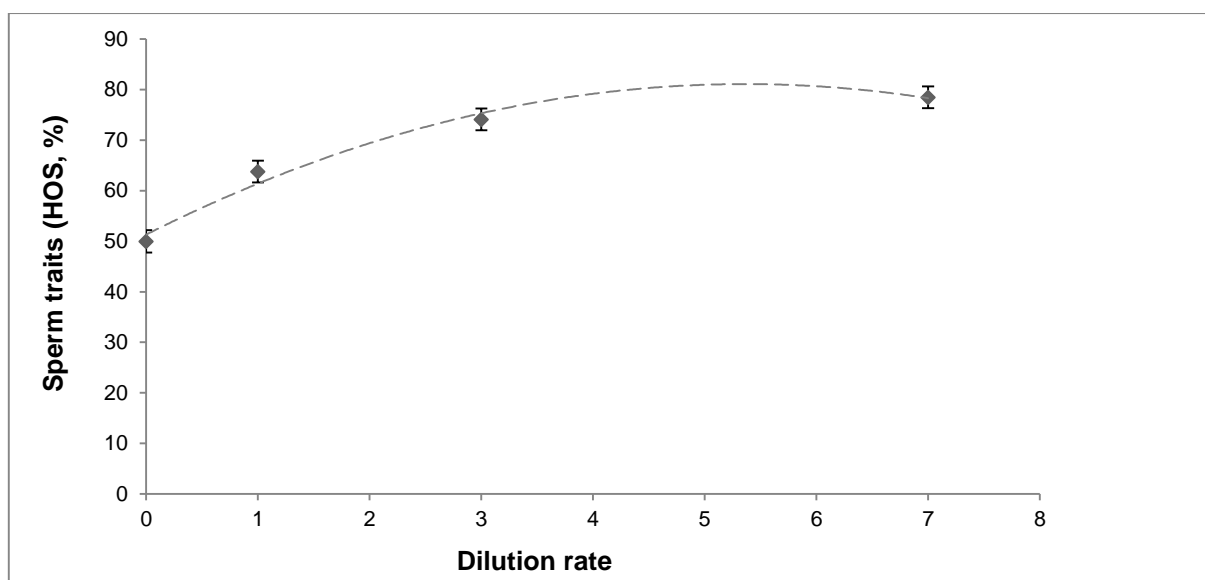


Figure 41: The quadratic relationship between sperm membrane integrity (HOS, %) with dilution (0, 1:1, 1:3, 1:7; semen to diluent). Standard error is indicated by vertical bars at the mean.

The inflection point ($X = \pm (\beta_1/2\beta_2)$) where HOS would be maximized (79.83 %) occurred where $X = 6.99$. This finding confirms previous results that a dilution rate of 1:6 to 1:7, semen to diluent, maximizes sperm membrane integrity.

3.5. The interaction of cooling rate with processing stage and cooling rate with dilution rate on sperm traits

The interaction of cooling rate with processing stage influenced ($P = 0.0067$) only the sperm trait ALH and contributed 36.05 % of the variation associated with this trait (Figure 42). ALH for neat semen in the fresh samples were not different from each other ($P > 0.05$), irrespective of cooling treatments. Slightly lower ($P > 0.05$) ALH values lower than that of fresh semen were observed in the cooled samples for 1, 5 and 23 °C/min treatments, except for 43 °C/min that displayed the highest ($P > 0.05$) ALH after cooling. A linear increase amounting to $Y = 2.13 + 0.01X$; $P = 0.0004$; $R^2 = 0.13$ was derived for cooling rates from 1 to 43 °C/min was observed for cooled ALH values. ALH evaluated after storage was lowest ($P < 0.05$) for a rate of 5, 23 and particularly 43 °C/min, compared to fresh and cooled samples except for 1 °C/min that maintained ALH at the same level as fresh and cooled semen. A linear decrease ($Y = 1.92 - 0.01X$; $P = 0.046$; $R^2 = 0.04$) was derived between stored ALH values for the different cooling treatments of 1 to 43 °C/min, with the mean ALH at 1 °C/min being significantly higher than at 43 °C/min.

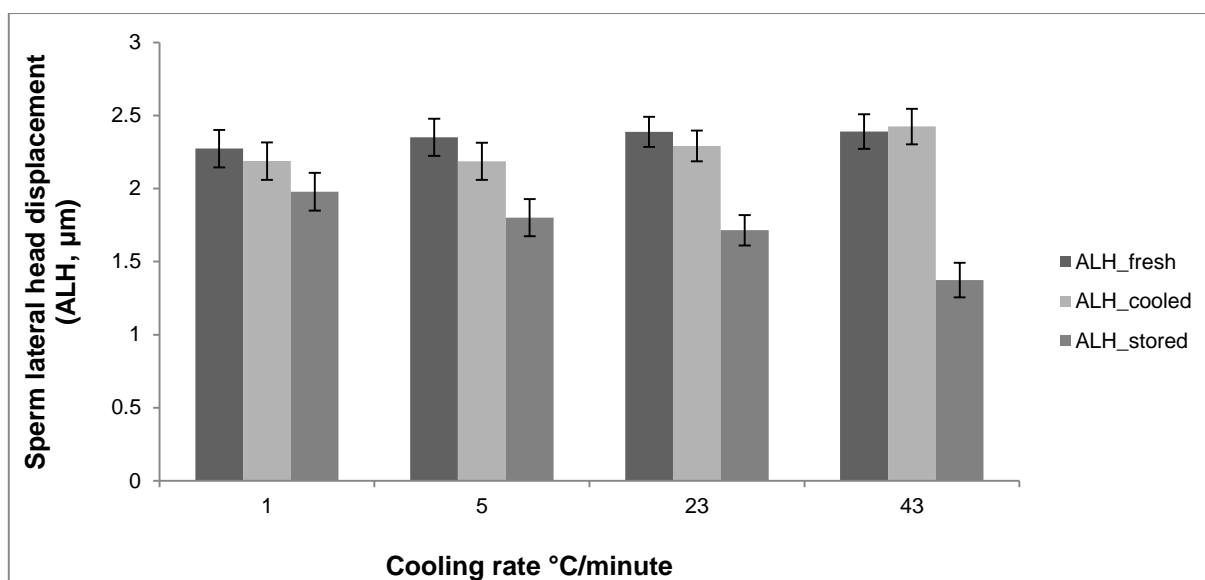


Figure 42: The interaction of cooling rate with processing stage on the amplitude of lateral head displacement (ALH, μm). Standard error is indicated by vertical bars at the mean.

Experiment 2: The effect of chilled stored semen on *in vivo* fertility through perivitelline techniques

3.6. Descriptive statistics

Across the analyses the mean (\pm SE) female age amounted to 5.58 ± 0.27 years with a mean (\pm SE) egg weight of 1270.92 ± 25.98 grams (g) with CV's of respectively 35.71 % and 14.88 % over 53 records. A minimum of 0 OPVL sperm/ mm^2 and a maximum of 22.16 OPVL sperm/ mm^2 were obtained across 49 records with a mean OPVL number of 2.98 ± 0.80 sperm/ mm^2 and a CV of 187.78 %. The contribution of each fixed effect (FE, %) with its degrees of freedom (DF), R-square value and CV is shown in Table 32 for fertilization status and number of OPVL sperm

Table 21.

Table 32: The source of variation for the fixed effect of semen treatment (fresh diluted, chilled stored), female age, egg mass, OPVL sperm number and the number of days after the last insemination with their variation contribution (FE, %) with its degrees of freedom (DF) on fertility assessment traits that include fertilization status and OPVL sperm number.

Variation Source	DF	Fertilization status	DF	OPVL sperm
Semen treatment	1	0.88	1	7.24
Female age	1	2.48	1	0.03
Egg mass	1	1.43	1	0.13
OPVL sperm	1	51.39***	1	n/a
Number of days after last AI	1	0.16	1	0.12
TCSS	48	7.96	48	1502.21
Error mean square	43	3.41	44	1371.16
R ²		0.57		0.09
CV(%)		138.10		187.38

OPVL sperm: the number of sperm trapped in the outer perivitelline layer; CV: Coefficient of variation; TCSS: Total corrected sum of squares; n/a: fixed effect not applicable for the specific model *P < 0.05; **P < 0.01; ***P < 0.001

3.7. The effect of the number of OPVL sperm, semen treatment, female age, egg mass and days after last AI on fertility assessment traits

The number of OPVL sperm was the only fixed effect that contributed ($P < 0.001$) to the variation (FE = 51.39 %) in fertilization status. Female age, egg weight and semen treatment (fresh diluted or chilled stored), had no significant effect on the fertilization status of eggs obtained through AI. A strong positive Pearson correlation coefficient ($r = 0.73$; $P < 0.0001$) was obtained between OPVL sperm and fertilization status, indicating that fertilization would improve with a higher number of OPVL sperm. Fertilised eggs had a mean number of 11.01 ± 2.27 OPVL sperm/mm², while non-fertilized eggs had a mean number of 0.92 ± 0.39 OPVL sperm/mm². The fixed effects of semen treatment, female age, egg weight and days after last AI had no effect ($P > 0.05$) on the number of OPVL sperm obtained with further analyses discarded.

3.8. The effect of female on fertility assessment traits

Female identity fitted as a fixed effect contributed significantly to the variation associated with fertilization status, OPVL sperm number and egg weight. Two females across the two treatment groups consistently produced fertilized (75 and 100% respectively) eggs with a higher mean weight (1311.50 ± 35.54 and 1357.67 ± 14.14 g respectively) than the average (1270.92 ± 25.98 g) and higher OPVL sperm (13.08 ± 4.56 and 7.00 ± 3.45 sperm/mm²) than average (2.98 ± 0.80 sperm/mm²), compared to the other 13 females that either did not produce eggs at all, or produced eggs inconsistently with variable weight and sperm numbers.

4. Discussion

4.1. The effect of processing stage, cooling rate, the interaction of cooling rate with processing stage and dilution rate on sperm function traits

The results indicated that ostrich semen is sensitive towards processing in the form of cooling and storing. The significant reductions in all sperm traits measured namely LIVE, HOS, PMOT, MOT, VCL, VSL, VAP, ALH, LIN, STR, WOB and BCF when processed indicated that cooling, as well as semen storing had a detrimental impact on sperm function. The harmful impact of processing varied between the measured traits with some being more sensitive namely PMOT, MOT, WOB, LIN, LIVE and HOS that declined on average with 4.30 %, when cooled and by 21.13 % when stored. Processing sensitive kinematic traits namely VCL, VSL and VAP decreased with 4.15 μ m/s when cooled and 21.15 μ m/s when stored. The recorded reduction between processing stages for process sensitive traits implies that ostrich sperm is less sensitive towards the effects of the cooling stage than that of the state of storage or due to the indirect effect of cooling on sperm function that is only visible once stored.

Even though cooling of ostrich semen decreased sperm function only to a limited extent, the rate at which cooling took place significantly accentuated this effect for certain motility traits. These results are consistent with the findings of Rasul *et al.* (2001), that differential cooling of buffalo semen only affects sophisticated or fine parameters of sperm motion and not overall motility. PMOT, MOT, VCL, VSL, VAP, LIN and WOB was reduced by faster cooling rates of 43 °C/min, compared to 1, 5 and 23 °C/min in the present study. Although no significant difference was obtained between slower rates of 1, 5 and 23 °C/min, the significant linear decrease from 1 °C/min to 43 °C/min for VSL, VAP, LIN and

WOB confirmed slower cooling rates of ≤ 1 °C/min to be more appropriate to maintain ostrich sperm function, compared to the faster rates of ≥ 1 °C/min. This is in agreement with the findings of Amann and Pickett (1987), Ashrafi *et al.* (2011) and Martorana *et al.* (2014) that slower cooling rates < 1 °C/min reduced sperm injury in chilling sensitive species like the stallion, ram and monkey respectively. A similar result was evident in the study on ostriches (Chapter IV) where a permanent loss of progressive motility (PMOT) was found when semen was diluted immediately upon collection at a low dilution temperature of 5 °C that resulted in fast cooling compared to higher dilution temperatures of 21 and 38 °C. Martorana *et al.* (2014) reported significant decreases for rhesus macaque sperm in terms of motility, but also for membrane integrity when cooled at fast rates exceeding 0.5 °C/min from physiological temperature to above zero. HOS was however unaffected in this study by cooling rate and the possible reasoning would be that the linear decrease in other sperm functions associated with increased cooling rate was not high enough (> 43 °C/min) to observe the severe effects exerted on the membrane or the test was not suitable to detect the limited amount of membrane changes.

The loss of sperm function in specific motility traits observed in this study are caused by chilling injury, also referred to as cold-shock, that takes place when semen is abruptly cooled from physiological or room temperature to storage temperatures ≥ 0 °C (Drobnis *et al.*, 1993; Arav *et al.*, 1996; Saragusty *et al.*, 2005). The severity of chilling injury damage on the cell thus depends on the final storage temperature and the rate of cooling to reach this final temperature. The cooling process generally affects normal chemical and physical cellular conditions that disrupt homeostasis and metabolism that needs to be restored (Martorana *et al.*, 2014). The stress response of the cell can thus be characterised by the direct effect of altered membrane permeability, loss of intracellular components, decreased metabolism and motility, with eventual cell death. The loss of sperm velocity observed for the ostrich sperm when fast cooled may reflect mitochondrial disruption and more specifically the loss of intracellular components that include adenine nucleotides, which would result in depleted ATP stores that would compromise motility traits as a result of chilling injury (Parks and Arion, 1987; Rasul *et al.*, 2001).

When the semen was cooled from room temperature (~ 24 °C) to a final liquid storage temperature of 5 °C the cell passed through the critical temperature zone of 15 to 20 °C, reaching the phase transition temperature and increasing the vulnerability of the cell to chilling injury (Karnovsky *et al.*, 1982; Quinn, 1985; Hammerstedt *et al.*, 1990). At this

temperature the physical properties of the cell membrane are changed from an ordered gel phase to the disordered liquid crystalline phase and phospholipids re-orient themselves into a different configuration that disrupts membrane function, permeability and fluidity (Karnovsky *et al.*, 1982; Quinn, 1985; Hammerstedt *et al.*, 1990; Drobnis *et al.*, 1993). Blesbois *et al.* (2005) showed a linear increase in anisotropy values between 40 and 10 °C indicating a reduction in membrane fluidity associated with cooling. Membrane fluidity and permeability are important functions to resist chilling injury and for the sperm cell to restore physiological homeostasis after cooling (Parks and Lynch, 1992; Blesbois *et al.*, 2005).

The sensitivity of ostrich sperm towards cooling and proneness to chilling injury would depend mainly on the complexity and uniqueness of the membrane structure in terms of hydrogen-carbon length, degree of membrane phospholipid fatty acid saturation and unsaturation, cholesterol content and chargehead group species that will directly influence membrane behaviour under thermal changes as well as the phase transition temperature of the specie (Quinn, 1985; Ladha, 1998; Surai *et al.*, 1998; Blesbois and Hermier, 2003; Voet and Voet, 2004; Blesbois *et al.*, 2005). Species with a higher proportion of polyunsaturated fatty acids (PUFA) will maintain functionality of the protective layer at lower temperatures better, since polyunsaturated fatty acids are more fluid and better equipped to lower the phase transition temperature (Drobnis *et al.*, 1993; Saragusty *et al.*, 2005). PUFA are also important structural components of the membrane in terms of ion transport and thus membrane permeability. Membranes with a higher proportion of saturated fatty acids are compressed by decreasing temperatures turning them dense and rigid (Drobnis *et al.*, 1993; Saragusty *et al.*, 2005). The ratio of PUFA to saturated fatty acids has been given as 0.9 and 1.1 respectively, in the chicken, recognized for its high resistance towards processing stress, and the turkey (Ravie and Lake, 1985). Anisotropy values, a measure of membrane rigidity, obtained by Blesbois *et al.* (2005) confirmed species differences for the chicken that had a more fluid membrane compared to the turkey and even more so than the guinea fowl which is known to be highly sensitive towards thermo-processing. The sensitivity of ostrich semen towards cooling may also be due to the sperm plasma membrane's sterol composition and the ratio of sterols, more specifically cholesterol, to membrane phospholipids since it has been found to differ among species, individuals and ejaculates (Parks *et al.*, 1981; Ladha, 1998; Gadella *et al.*, 1999; Blesbois *et al.*, 2005). The different regions of the plasma membrane can also differ in their cholesterol/phospholipid ratio. For instance, the

acrosomal membrane of bull sperm has a lower ratio, compared to other regions of the plasma membrane making some parts more vulnerable than others (Parks and Arion, 1987; Gadella *et al.*, 1999). Cholesterol, also a powerful decapacitation factor, has been found to maintain the balance of the membrane by acting as a buffer and preventing lower temperatures from inhibiting fluidity and preventing higher temperatures from increasing fluidity (Davis, 1980; Parks and Lynch, 1992; Aitken and Nixon, 2013). Higher cholesterol/phospholipid ratios have been identified in the thermo-sensitive Guinea fowl (0.33 ± 0.03) compared to the chicken (0.26 ± 0.02) that is less thermo-sensitive. Cholesterol and phospholipids have a steric interaction with one another in modulating bilayer fluidity, stability and permeability (Parks *et al.*, 1981; Ladha, 1998; Blesbois *et al.*, 2005). The cholesterol molecules are packed in among the phospholipid molecules where they prevent fatty acid chains from packing together and crystallizing, a process that markedly reduce membrane fluidity. The cholesterol/phospholipid ratio present in the ostrich sperm plasma membranes may be a contributory reason for the significant interaction effect of cooling rate with processing stage on the sperm trait ALH in this study (Sherwood, 2004).

The variation in sperm ALH associated with ostrich semen processing steps (dilution and cooling) has been observed in other species like the buffalo (Rasul *et al.*, 2001) and the bull (Bailey *et al.*, 1994), but with no reference to different cooling rates during these processing stages. Elevated ALH values were obtained after the cooling stage for semen cooled at 43 °C/min, compared to the non-processed ALH values and that of the slower cooling rates (< 43 °C/min).

The elevated ALH values obtained directly after cooling associated with the faster cooling rate of 43 °C/min are probably the cause of hyper-activation that plays a part during the capacitation process. Although the process of capacitation is still a debatable subject in avian species, it is a well-known occurrence in mammalian sperm and is characterized by cholesterol efflux from the sperm plasma membrane, together with an increased membrane fluidity and permeability to a calcium influx that results in hyper-activated movement (Davis *et al.*, 1979). The efflux of cholesterol has also been suggested in avian species by Blesbois *et al.* (2005) who observed a drastic reduction of cholesterol/phospholipids portions in the semen of thermo-sensitive guinea fowls and turkeys, compared to the chicken after thermo-processing. Hyper-activated movement can be characterized by a vigorous and non-linear movement caused by increased

amplitude of flagellar beats, but has also been found difficult to evaluate. Since the CASA system does not allow the direct analysis of flagellar movements that generate sperm hyperactivity (Yanagimachi, 1994; Mortimer, 1997). However, Schmidt and Kamp (2004) suggested evaluating kinematic parameters of sperm head movements that are correlated with characteristic changes in flagellar movements to identify hyperactivity. ALH, as the measure of the lateral sperm head's deviation from the average path of progression, is known to increase during hyperactivation. It has been nominated to be a satisfactory tool for identifying hyperactivated sperm and a potential marker of intracellular Ca^{2+} levels that is known to also increase during hyperactivation (Robertson *et al.*, 1988; Bailey *et al.*, 1994; Mortimer, 1997; Rasul *et al.*, 2001; Schmidt and Kamp, 2004). The ALH of boar sperm has been found to increase by as much as six times when hyperactivation was induced *in vitro* (Schmidt and Kamp, 2004). Sperm capacitation and thus hyperactivated movement would take place under normal *in vivo* circumstances as the sperm ascend the female reproductive tract and reaches the site of fertilization. However hyperactivated movement can be induced prematurely by chilling injury and processing stress that will trigger an intrinsic apoptotic cascade of events. The latter has been confirmed in other mammalian species like the bull where premature capacitation-like changes, due to processing stress, have been reported by Cormier *et al.* (1997). Thermo-sensitive species are typically high in PUFA, characterized by multiple double bonds is a source of methylene bridges (-CH₂-) containing reactive hydrogens most suitable for peroxidation and thus makes them vulnerable to oxidative stress (Alvarez and Storey, 1982; Aitken *et al.*, 2004). The increased concentration of reactive oxygen species (ROS) generated, specifically hydrogen peroxide, inducing tyrosine kinase activity, is a known trigger for the onset of capacitation and thus hyperactivated movement that will eventually induce irreversible motility loss and cell apoptosis through the continuum of an intrinsic apoptotic cascade (Alvarez and Storey, 1982; de Lamirande and Gagnon, 1993; Koppers *et al.*, 2011; Aitken and Nixon, 2013). The markedly decreased ALH values obtained after storage for the faster cooling rate of 43 °C/min confirms the irreversible loss of ALH.

Dilution of ostrich semen has a beneficial effect on maintaining sperm function traits better reflected by LIVE, HOS, PMOT, MOT, VCL, VSL VAP, ALH, LIN, STR and WOB compared to neat semen that has not been diluted. The latter is in agreement with earlier studies done (Chapter IV) for ostrich semen specifically investigating the effect of dilution. In this study no difference in sperm function maintenance for LIVE, PMOT, MOT, VCL,

VSL, VAP, ALH, LIN, STR, WOB and BCF was observed when serial dilutions were applied at different rates (1:1; 1:3; 1:7) except for HOS that was highest at a 1:7 dilution rate. The maintenance of maximum membrane integrity by diluting the ejaculate at higher rates is consistent with that reported in Chapter IV. However the non-significant improvement of other functional sperm traits when diluted at higher rates is not in agreement with earlier studies where optimum sperm function of all traits measured, namely LIVE, HOS, PMOT, MOT, VCL, VSL VAP, ALH, LIN, STR, WOB and BCF was obtained at higher dilution rates of 1:6 to 1:7, as shown in Chapter IV. The reason for the differences obtained between studies is mainly based on the time of dilution after semen collection and thus the extent of sperm function deterioration. In this study delayed lab semen dilution initiated the onset of sperm function deterioration much earlier whereafter the lost sperm function could not be revived even when diluted at higher rates. Field dilution of semen immediately upon collection, similar to that of Chapter IV, limits the extent of sperm function deterioration. A higher proportion of intact sperm may be maintained when diluted at 1:6 - 1:7 rates with a carry-over effect of survivability, when processed for storage. Sperm function deterioration associated with delayed dilution is the result of high cell concentrations and semen agglutination *in vitro*. During normal breeding conditions the ejaculated semen would be deposited in the female to dilute with fluids of the female reproductive tract to migrate through the genital tract for fertilization and storage in specialized storage tubules (Bakst, 1998; Bezuidenhout *et al.*, 1995). High cell concentrations have been associated with nutrient and energy depletion, decreased O₂ and increased CO₂ concentrations, toxicity because of increased waste-product concentrations, an acidic pH, a change in electrolyte, H₂O and salt concentrations, with associated volume and pressure changes *in vitro* (Nevo, 1965; Amann and Hammerstedt, 1980; Clarke *et al.*, 1982; Wishart 1984; Bilgili *et al.*, 1987; Thomson and Wishart, 1991; Voet and Voet, 2004). Ciereszko *et al.* (2010) confirmed the deterioration of undiluted ostrich semen quality due to the occurrence of agglutination while Bollwein *et al.* (2004) described agglutinated porcine semen to lose motility with the incidence of agglutination disappearing once diluted.

4.2. The effect of the number of OPVL sperm, semen treatment, female age, egg weight, days after last AI and female identity on fertility assessment traits

The number of sperm trapped in the outer perivitelline membrane was the only trait that had a very strong correlation with the fertilization status of the eggs collected after artificial insemination with either fresh, diluted or chilled, stored semen. The strong correlation

between fertilization probability and trapped OPVL sperm is not a new finding and has been supported by research on poultry by Wishart (1987), Brillard and Bakst (1990), Brillard (1993), and for the emu (Malecki and Martin, 2002, 2003, 2004), as well as the ostrich (Malecki and Martin, 2003; Malecki *et al.*, 2004). Wishart (1987) showed that fertility increased linearly with increased OPVL sperm numbers. However, it has been pointed out that this trend is only valid for a certain maximum of OPVL sperm, whereafter fertility will not improve. The number of OPVL sperm depended on certain male and female factors (Ogasawara *et al.*, 1966; Bakst, 1989; Brillard, 1993). Male factors include sperm fitness suitable for female selection and SST storage (Ogasawara *et al.*, 1966; Bakst, 1989; Brillard, 1993). Steele and Wishart (1992) found that processed chicken sperm may be viable *in vitro*, but were unable to traverse the female reproductive tract *in vivo* and limit storage of such sperm in the SST.

Insemination of ostrich females resulted in very low (mean \pm S.E; 2.98 ± 0.80 sperm/mm²) OPVL sperm numbers, compared to Malecki and Martin (2003) who reported a corresponding mean of 253 ± 18 OPVL sperm/mm², with a range of 0 to 1330 in eggs of females during natural mating. However it has been reported in other avian studies that dramatic losses in sperm numbers occur during insemination with less than 1 % of inseminated sperm reaching the SST (Allen and Grigg, 1957; Howarth, 1971; Brillard, 1993). The low number of OPVL sperm is thus no reason for apprehension since it has been shown that avian eggs can be fertilized even when only a few sperm are present or even when no sperm can be found in the OPVL of the GD region (Malecki and Martin, 2002) or when only one sperm has entered the egg (Bramwell *et al.*, 1995; Wishart, 1997). Excess sperm has been shown to have a negative impact on embryonic development (Fofanova, 1965; Van Krey *et al.*, 1966; Bramwell *et al.*, 1997). Even though sperm functionality can be altered during processing practices for storage and artificial insemination, no significant difference was observed in this study for the number of trapped OPVL sperm obtained from eggs produced via fresh, diluted or chilled, stored semen inseminations. The latter indicates that processing stress experienced by the sperm associated with the current protocol for chilled storage semen was limited and that other factors influencing the OPVL sperm numbers should be explored. Female factors influencing OPVL sperm numbers reported in the literature include specific selection requirements of sperm, female age, oviposition and timing between inseminations, as well as the onset of egg production (Brillard and Bakst, 1990; Brillard, 1993). These female factors mainly reside back to the specialized functionality of the SST, which is a

unique feature with specific capability between females of different species, to efficiently store high sperm numbers. The number of OPVL sperm obtained in other avian females like the turkey correlated strongly positively ($r = 0.85$) with the number of sperm stored in the SST (Brillard and Bakst, 1990). The SST of the ostrich has been described by Bezuidenhout *et al.* (1995) with a fertile period of up to 28 days post-coitus (Bezuidenhout *et al.*, 1995; Swan and Sicouri, 1999; Malecki *et al.*, 2004; Dzoma, 2010). By incorporation of these female factors during artificial insemination practices one can potentially increase OPVL sperm numbers and thus fertility rates with the potential to gain more knowledge about semen processing treatments for optimization purposes. For instance inseminating prior to the onset of egg production has been found to increase the number of SST stored sperm and thus OPVL sperm numbers in chickens and turkeys (Brillard and Bakst, 1990; Brillard, 1993). Females laid fertilised eggs for up to 11 days, with either treatment after the last insemination while sperm were still visible in the perivitelline membrane after a maximum of 13 and 24 days after the last insemination for fresh, diluted and chilled, stored semen in this study. Together with the results of other ostrich studies, including a fertile period of 9 days for females bred artificially with 3 billion sperm on five consecutive days (Bonato and Cloete, 2013) and a fertile period of 28 days when bred naturally (Malecki *et al.*, 2004), suggests insemination less frequent with higher initial dosages of highly functional sperm to boost SST numbers and to support a consistent sperm release without the stress associated with weekly inseminations and increased male collection frequency. This is specifically important for a recently domesticated species like the ostrich female that often displays a high flight reaction that may have an indirect impact on OPVL numbers and thus fertility after insemination.

The variability between females in this study was evident for OPVL sperm numbers and fertilization status thus complicating *in vivo* evaluation. Although 14 of the 16 females produced eggs, only 50 % of females produced eggs with OPVL sperm and only 20 % of females produced eggs with a consistently higher OPVL number that were fertilized. However, the low numbers of these reliable females limit *in vivo* fertility tests for processed semen and need to be addressed. The variability in fertility rates and inadequate genetic improvement associated with ostrich breeding are, however, constraints that limited genetic progress of the ostrich industry for many generations (Deeming, 1996; Cloete *et al.*, 1998; Dzoma and Motshegwa, 2009). A functional *in vivo* fertility test allows assessment of reproduction efficiency in females, as well as the identification and selection of suitable individuals or lines for artificial insemination practices.

5. Conclusion

Evidence obtained from this study indicates that different cooling rates impact on ostrich sperm function that becomes more evident after the different processing steps. The effect of the different cooling rates on different sperm motility traits indicate that cooling rates of 1 °C /min is the most appropriate to cool semen from collection temperature (~ 24 °C) to 5 °C for liquid storage, compared to the higher cooling rates tested. The results strengthen the case for further study of slow cooling rates (< 1 °C/min), which resemble those of a thermo-sensitive species, as well as membrane characteristics that would enable expounding the effects of cooling on ostrich semen. Specific objectives would be to determine the degree of fatty acid saturation and the cholesterol/phospholipid ratio of the sperm plasma membrane that would allow prediction of membrane behaviour at reduced temperatures. *In vivo* application of the chilled stored semen protocol through artificial insemination has been shown to result in fertile eggs in this study. Future *in vivo* tests should be directed towards less frequent inseminations with higher initial dosages to support a consistent sperm release. Special attention should be focussed on fertile female selection. Refinement of the artificial insemination protocol to maximize OPVL numbers should also be undertaken.

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CHAPTER VI

SENSITIVITY OF OSTRICH SPERM TOWARDS CRYOPROTECTANT TOXICITY

The identification of the cryoprotectant (CP) of choice and the level of application has been recognized as a vital step to allow the cryopreservation of semen for indefinite storage at $-196\text{ }^{\circ}\text{C}$. These prerequisites for a suitable CP have not yet been determined for the ostrich. Semen from six South African Black ostrich males was individually diluted with an ostrich specific diluent (OS1) immediately after collection at a 1:1 semen/diluent ratio and placed in a $24\text{ }^{\circ}\text{C}$ water bath within 30 minutes of collection and subsequent evaluation. The diluted sample was cooled in a programmable freezer (Asymptote, Grant, EF600) at a rate of $1^{\circ}\text{C}/\text{minute}$ to a final temperature of $5\text{ }^{\circ}\text{C}$. The cooled aliquots were then diluted (1:1) with pre-cooled ($5\text{ }^{\circ}\text{C}$) CPs (dimethylacetamide, methylacetamide and dimethylsulfoxide) at different concentrations of 4 to 16 %. CP/semen equilibration was left for 15 minutes in an aerated incubator set at $5\text{ }^{\circ}\text{C}$. After equilibration, secure plastic straws were loaded, sealed and frozen at a standardized rate of $5^{\circ}\text{C}/\text{minute}$ from 5°C to $-20\text{ }^{\circ}\text{C}$, nucleation at $-20\text{ }^{\circ}\text{C}$ and from $-20\text{ }^{\circ}\text{C}$ to $-80\text{ }^{\circ}\text{C}$ at a rate of $10^{\circ}\text{C}/\text{minute}$. Thereafter, the straws were removed from the programmable freezer and plunged into liquid nitrogen at $-196\text{ }^{\circ}\text{C}$. Samples were thawed 24 hours later for 1 minute and 20 seconds at $5\text{ }^{\circ}\text{C}$. A panel of sperm function tests was conducted to evaluate semen, including motility and kinematic parameters measured by the Sperm Class Analyzer®, percentage live sperm measured by fluorescence SYBR14®/PI (LIVE/DEAD®), percentage of sperm able to resist the hypo-osmotic swelling (HOS) stress test. Sperm morphology was determined using samples fixed in 2.5 % buffered glutaraldehyde, stained with Wrights' stain (Rapidiff®) and evaluated using a 100x oil immersion objective and phase contrast illumination. Sperm functions reflected by normal morphology (NORM), membrane integrity (HOS), membrane viability (LIVE), progressive motility (PMOT), motility (MOT), curve-linear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP) were maintained at the same level after CP addition and equilibration during the pre-processing stages when DMA was used at a level of 16 %. Although freezing and thawing reduced sperm function markedly by ~ 50 %, the highest sperm functionality (LIVE and HOS) was maintained by DMA 16 %.

1. Introduction

Avian cryopreservation has been studied for more than 50 years with a variety of groundbreaking procedures that have been developed (Schaffner *et al.*, 1941; Lake, 1986; Foote, 1998, 2002). Maximal survival of sperm after cryopreservation, however, remains problematic, due to the many steps that are involved, many of which sperm are not adapted, with variable survival responses from species and individuals (Hammerstedt, 1995; Blanco *et al.*, 2000, 2011; Holt, 2000; Blesbois *et al.*, 2005). Cryopreservation can be defined as the process whereby cells or whole tissues are preserved indefinitely for future use by cooling to sub-zero temperatures, such as $-196\text{ }^{\circ}\text{C}$ (the boiling point of liquid nitrogen). A state referred to as cryptobiosis occurs where the cell enters an ametabolic state in response to an adverse environmental condition such as freezing. Cryptobiosis

represents a very useful concept to conserve genetic material *ex situ* for future application, involving a process termed cryobanking or biobanking. Biobanking can be of significant value to the ostrich industry where avian influenza represents a substantive risk to valuable genetic resources. Cryopreservation has been successfully applied for other cell types, embryos and primordial germ cells and may also benefit avian semen cryopreservation (Tajima, 2002; Blesbois *et al.*, 2005). Cryopreserved semen may also offer benefits such as increasing fertilization rates when ostrich females are inseminated during high egg production months (July/August), when males produce low quality semen (Lambrechts, 2004; Bonato *et al.*, 2014; Chapter II) or for combating sexual incompatibility and the maintenance of heterozygosity in small populations.

Cryopreservation methods involve the reduction of temperature, cellular dehydration, as well as freezing and thawing. This sequence of events exposes the sperm cell to severe osmotic, mechanical and thermal changes (Tselutin *et al.*, 1999; Squires *et al.*, 2004; Blesbois and Brillard, 2007). For the cryopreservation process to be successful, the cell must be able to maintain the plasma membrane. The cell structure being most prone to cryopreservation damage, integrity, configuration and function to retain metabolic activities for cell functions important for fertilization in the thawed cell (Parks, 1992; Watson, 2000; Gee *et al.*, 2004). Cell structure and composition is specie- and individual-specific with unique molecular composition and properties that determine the sensitivity of the cell towards the cryopreservation processes demanding specialized protocols for optimal *in vitro* and *in vivo* survival post thaw (Ansah and Buckland, 1982; Parks and Lynch, 1992; White, 1993; Blanco *et al.*, 2000; Blesbois *et al.*, 2005).

Preservation of sperm function through cryopreservation is dependent on interrelated factors that form the foundation of semen storage (short- and long-term), together with specialized factors that only apply to cryopreservation. Fundamental prerequisites for the ostrich in terms of reliable stress-free semen collection methods (Rybnik *et al.*, 2007), semen collection frequency, timing and output (Bonato *et al.*, 2011, 2014) and the duration of female sperm storage (Malecki *et al.*, 2004) have been met. More recently, the foundation of sperm function traits (Chapter II), the development of an ostrich specific semen extender (Chapter III) with accompanying semen processing protocols in terms of optimal dilution rates and temperatures (Chapter IV) as well as a suitable slow cooling regime after collection to 5 °C (Chapter V) also established the basis for developing a long-term storage protocol. However cryopreservation-specific guidelines for ostrich

semen cryopreservation are lacking in the literature. Tselutin *et al.* (1999) pointed out the importance of cryoprotectant type, and its usage during cryopreservation since it is the only supplement protecting the cell against water freezing injury. Approximately 100 compounds have been investigated and identified as potential cryoprotectants (CP's). However, the few compounds routinely used can be subdivided into penetrating and non-penetrating categories (Doebbler, 1966; Karow, 1969; Meryman, 1971). These two types can be used separately, or in combination to obtain better results (Blanco *et al.*, 2011). Penetrating CP's, best and most commonly used, can penetrate the plasma membrane of the sperm cell and act both intracellularly and extracellularly. The molecular mass of these molecules is small and typically less than 100 daltons (Wowk, 2007). Gao *et al.* (1993), Holt (2000) and Purdy (2006) described penetrating CP's to cause membrane lipid and protein rearrangements resulting in increased membrane fluidity, to displace intracellular water thus establishing greater dehydration of the cell and reduce intracellular ice formation at low temperatures, also by lowering the freezing point of the medium to a temperature much lower than that of water, thus ultimately aiding cryo-survival. Only a few CP's, namely glycerol, dimethylsulfoxide (DMSO), dimethylacetamide (DMA), methylacetamide (MA) are routinely used in avian cryopreservation (Karow, 1969; Lake and Ravie, 1984; Gee *et al.*, 1985; Tselutin *et al.*, 1999; Han *et al.*, 2005; Sasaki *et al.*, 2010; Malecki and Kadokawa, 2011; Sood *et al.*, 2011). Glycerol is generally an effective cryoprotectant for most avian sperm, but is contraceptive for intra-vaginally inseminated chicken and turkey sperm that have to be removed by centrifugation before insemination, thereby increasing processing stress (Lake, 1986; Donoghue and Wishart, 2000). DMA and DMSO were suggested by Tai *et al.* (2001) as a replacement for glycerol to avoid the contraceptive effect of glycerol, if not removed before AI and also to simplify avian AI protocols. MA was the only CP formally tested on ostrich semen, but the percentage of live and motile ostrich sperm after thawing remained unsatisfactory although higher concentrations (12 %) of MA proved to be more effective than lower concentrations (Malecki and Kadokawa, 2011). Sood *et al.* (2011) found DMA (9 %) optimal for maximum post-thaw viability of emu sperm. Since the emu is a close relative of the ostrich, this CP may also play a role in the cryopreservation of ostrich semen. It is thus important that the most appropriate CP concentration is identified to limit toxicity, since these CP's are non-physiological chemicals, limiting extreme osmotic gradients on the cell, while also maintaining effectiveness by limiting ice formation (Fahy *et al.*, 1990; Hammerstedt *et al.*, 1990; Tselutin *et al.*, 1999; Blanco *et al.*, 2000).

The aim of this study was therefore to identify an appropriate cryoprotectant type, at a suitable concentration as one of the first two crucial specialized elements needed to progress in the application of ostrich semen cryopreservation.

2. Material and methods

2.1. Animal population

Ejaculates were collected from six South African Black (SAB) males, aged between 3 and 7 years, using the dummy female method (Rybnik *et al.*, 2007). They were allocated to a standardized factorial experiment, evaluating the CP's methylacetamide (MA), dimethylacetamide (DMA) and dimethylsulfoxide (DMSO) at different concentrations of 4, 8, 12, and 16 % in a 3 x 4 factorial experimental design. All males were repeated for the three CP's. Males were chosen for their reliability in terms of ejaculate quality and willingness to mount the dummy female. Males in the resource population were screened from the commercial ostrich breeding flock maintained at the Oudtshoorn Research Farm on the basis of behavioural attributes rendering them suitable for AI (referred to as desirable behaviour repertoires and described by Bonato and Cloete, 2013). The origin of the ostrich flock and the general management procedures implemented therein were described previously (Van Schalkwyk *et al.*, 1996; Bunter and Cloete, 2004; Cloete *et al.*, 2008).

2.2. Semen preparation

Sperm concentration was obtained in both experiments by means of spectrophotometer (Spectrawave, WPA, S800, Biochrom) transmittance values that were previously calibrated against actual cell counts using a haemocytometer. Aliquots of 20 µL semen diluted 1:400 (v/v) with phosphate buffered saline solution containing 10 % formalin were used for the latter.

An initial 1:1 field dilution with OS1, the ostrich semen diluent based on the ostrich seminal plasma composition of macro minerals (Chapter III), was performed and placed in a 24 °C water bath within 30 minutes of collection and evaluated. The diluted sample was split into 250 µL aliquots and cooled in a programmable freezer (Asymptote, Grant, EF600), at a rate of 1 °C/minute to a final temperature of 5 °C. The aliquots were then mixed and diluted with the pre-cooled (5 °C) CP set at different concentrations of 4 to 16

%, at a dilution rate 1:1. Mixed semen cryoprotectant aliquots were left to equilibrate for 15 minutes in an aerated incubator (BL°CKICE Cooling Block, Techne), set at 5 °C. After equilibration the 250 µL secure plastic straws were loaded, sealed and frozen at standardized rates of 5 °C/minute from 5 °C to nucleation at -20 °C and from -20 °C to -80 °C at a rate of 10 °C/minute. Thereafter, the straws were removed from the programmable freezer and plunged into liquid nitrogen at -196 °C. The samples were thawed 24 hours later for 1 minute and 20 seconds at 5 °C. Straws were chosen because they are the standard packaging used in cryobanking (Blesbois, 2011). Sampling for sperm function evaluation took place simultaneously for the neat ejaculate and at different intervals after specific processing steps that included dilution, cooling, CP addition and equilibration, as well as thawing.

2.3. *In vitro* sperm function evaluation

2.3.1. *Motility evaluation*

Sperm motility traits were evaluated by means of the Sperm Class Analyzer® (SCA) version 5.3 (Microptic S.L., Barcelona, Spain) at different intervals, that included the neat ejaculate, after dilution, cooling, after CP addition/equilibration and after 24 h cryopreservation/thawing. All sperm motility recordings were made after re-suspension of neat sperm, as well as treated sperm in a standard motility buffer using Sodium Chloride (150mM) and TES (20mM) with male specific seminal plasma (2 %) to a final sperm concentration of 20×10^6 sperm cells/mL. After re-suspension a sample was placed in a 38 °C water bath for 1 minute. For sperm motility recording, 2 µL of diluted semen was placed onto a pre-warmed slide covered gently with a cover glass (22 x 22 mm) and allowed to settle for 20 seconds prior to recording. Seven to nine different fields were captured randomly to eliminate bias towards higher sperm concentration, or motility until at least 500 motile sperm were presented. Sperm motility traits included motility (MOT, %), progressive motility (PMOT, %), curve-linear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), amplitude of lateral head displacement (ALH, µm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat-cross frequency (BCF, Hz).

2.3.2. *Viability evaluation*

Sperm viability in terms of live sperm (LIVE, %) was measured using the LIVE/DEAD® Sperm Viability Kit from Life technologies that contains the SYBR® 14 and Propidium

Iodide (PI) fluorescent stains. All sperm viability recordings were made after re-suspension of sperm in the standard OS1 diluent at pH7 to a final sperm concentration of 20×10^6 sperm cells/mL. The SYBR® 14 working solution was prepared in a HEPES/NaCl medium to a 1:49 concentration (v/v) SYBR® 14 to HEPES/NaCl solution. Sperm suspension aliquots of 250 μ L were re-suspended with 1.5 μ L membrane-permeant SYBR® 14 working solution and incubated for 10 minutes at a temperature-controlled environment of 38 °C. After incubation 2 μ L of the next fluorescent stain, Propidium Iodide (PI), was added and incubated for 10 minutes before cells were evaluated. For evaluation of viable (green) and non-viable (red/or green with red) sperm, a 2 μ L droplet was placed on a glass slide and covered with a cover glass (22 x 22 mm) and allowed to settle for 20 seconds, prior to recording. The fluorescent sperm was observed and photographed under 10x microscopy with an Olympus BX41 epifluorescent microscope (Olympus Optical Co., Tokyo, Japan) equipped with a filter, camera (ColorView Illu Soft Imaging System) and software package (analysis FIVE, Olympus Soft Imaging Solutions GmbH, Münster) to count viable and non-viable sperm. Nine to ten different fields were randomly captured until at least 500 spermatozoa were evaluated. Distorted fields, as well as fields that included drift or debris or clumps of sperm, were excluded. The SYBR® 14 nucleic acid stain labels live sperm with green fluorescence, and membrane-impermeable PI labels the nucleic acids of membrane-compromised sperm with red fluorescence.

2.3.3. Membrane integrity evaluation

Sperm membrane integrity assessed as hypo-osmotic swelling resistant sperm (HOS, %) was measured using the Hypo-osmotic swelling test (Jeyendran *et al.*, 1984) adapted specifically for the ostrich by means of preliminary experimental exploration. All sperm membrane integrity recordings were made after re-suspension of sperm in a standard salt (NaCl/H₂O) solution, adapted to 25 mOsm to a final sperm concentration of 20×10^6 cells/mL. For HOS recording, 2 μ L of diluted semen was placed onto a pre-warmed slide, using a heated stage set at 38 °C, covered gently with a cover glass (22 x 22 mm) and allowed to settle for 20 seconds prior to recording. Sperm was captured using the Sperm Class Analyzer® (SCA) version 5.3 (Microptic S.L., Barcelona, Spain) with a Basler A312fc digital camera (Basler AG, Ahrensburg, Germany), mounted on an Olympus BX41 microscope (Olympus Optical Co., Tokyo, Japan) equipped with phase contrast optics. Seven to nine different fields were captured randomly until good representation (500 sperm) was reached and biasness towards higher sperm concentration eliminated.

Distorted fields, as well as fields that included drift or debris or clumps of sperm were excluded.

2.3.4. Morphology evaluation

Sperm morphology in terms of the percentage of normal (NORM, %) and abnormal (ABNRM, %) sperm was determined by semen sampling after the specific processing steps and fixed in 2.5% buffered glutaraldehyde at a ratio of 1:10. Smears were prepared from the fixed cell suspensions, air-dried for a minimum period of 24 h and stained with Wright's stain (Rapidiff[®], Clinical Sciences Diagnostics, Johannesburg, South Africa) in Coplin jars as previously described by Du Plessis and Soley (2014). Smears were examined with an Olympus BX63 light microscope (Olympus Corporation, Tokyo, Japan) using a 100x oil immersion objective (phase contrast illumination). The incidence of normal and defective sperm was determined for each sample by evaluating 300 cells per slide. Sperm images were digitally recorded using the Olympus cellSens Imaging Software (Olympus Corporation, Tokyo, Japan) and classified (NORM and ABNRM) according to the description given specifically for the ostrich by Du Plessis *et al.* (2014).

2.4. Statistical analyses

Sperm traits as percentages, and with skewed distribution (as determined by the Shapiro-Wilk test: $P < 0.05$) were transformed using the arc sine of the percentage mean square-root ($\text{degree} \cdot \arcsin \sqrt{\%}$), while the sperm concentration was transformed to natural logarithms. Analyses included a distribution analysis and summary statistics to obtain variance parameters and graphs, describing the sperm traits. The total number of records, means, standard deviation, minimum, maximum and coefficients of variation (CV) was determined for each sperm trait. The contribution of each fixed effect (FE) to particular sperm traits was evaluated by expressing the sum of squares for such an effect, as a percentage of the total corrected sum of squares (TCSS) (Leighton *et al.*, 1982; Smith, 2010). Least squares means, standard errors (S.E.) and CV's were calculated and subjected to Tukey's multiple range tests to investigate differences between least squares means. Correlations (Pearson) and regressions (linear and non-linear) were used to determine significant ($P < 0.05$) relationships between traits. The Statistical Analysis System (SAS, version 9.3) was used for all the analyses performed.

Separate general linear mixed models (GLMM) were performed for sperm function traits namely motility (PMOT, MOT, VCL, VSL, VAP, ALH, LIN, STR, WOB and BCF), viability

(LIVE), membrane integrity (HOS) and sperm morphology (NORM and ABNRM) which included the FE of semen processing stage (S; neat, diluted, cooled, CP addition and equilibration, thawed), CP type (R; MA, DMA, DMSO), CP concentration (D; 4, 8, 12, 16 %), sperm concentration (C as a linear covariate) and male (M) as a random effect.

Example of the GLMM fitted with Y being the dependent sperm traits of motility, viability, membrane integrity and sperm morphology:

$$Y_{ijkl} = \mu + M_i + S_j + R_k + D_l + b_0(C)_{ijkl} + e_{ijkl}$$

Where: Y_{ijkl} = Sperm trait under assessment

μ = population mean

M_i = random effect of the i^{th} male ($i = 1, 2, 3, 4, 5, 6$)

S_j = fixed effect of the j^{th} processing stage ($j = \text{neat, diluted, cooled, CP addition/equilibration, thawed}$)

R_k = fixed effect of the k^{th} CP type ($k = \text{MA, DMA, DMSO}$)

D_l = fixed effect of the l^{th} CP concentration ($l = 4, 8, 12, 16 \%$)

C_{ijkl} = sperm concentration fitted as a linear covariate

b_0 = regression coefficients of Y_{ijkl} on sperm concentration (C)

e_{ijkl} = random error

3. Results

3.1. Descriptive statistics

Across the analyses the mean (\pm SE) sperm concentration was $3.14 \times 10^9 \pm 1.76 \times 10^7$ sperm cells/ mL, with a CV of 15.19 % over 735 records (Table 19). Respective means for NORM = $67.75 \pm 1.33 \%$, LIVE = $63.64 \pm 1.04 \%$, HOS = $51.85 \pm 1.02 \%$, PMOT = $29.99 \pm 0.62 \%$, MOT = $70.83 \pm 0.84 \%$, VCL = $54.76 \pm 0.52 \mu\text{m/s}$, VSL = $28.77 \pm 0.36 \mu\text{m/s}$, VAP = $41.03 \pm 0.50 \mu\text{m/s}$, ALH = $2.41 \pm 0.02 \mu\text{m}$, LIN = $52.05 \pm 0.36 \%$, STR = $70.19 \pm 0.33 \%$, WOB = $73.13 \pm 0.39 \%$ and BCF = $9.04 \pm 0.23 \text{ Hz}$ were recorded. Fewer records, 236, were evaluated for NORM compared to other measured sperm traits, since these samples were evaluated offsite by an independent party leading to the exclusion of neat samples. CV's of sperm traits ranged from 11.98 to 49.61 %. Summary statistics including the number of records, mean, standard deviation, minimum, maximum and CV for each trait are set out in Table 19.

Table 33: Descriptive statistics for sperm traits that included sperm normal morphology (NORM, %), viability (LIVE, %), membrane integrity (HOS, %), progressive motility (PMOT, %) and motility (MOT, %), curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat-cross frequency (BCF, Hz).

Sperm trait	Records	Mean	SD	Minimum	Maximum	CV (%)
Normal (%)	236	67.75	20.37	7.00	93.00	30.07
Live (%)	678	63.64	27.12	1.00	96.00	42.61
Hypo-osmotic swelling resistant (%)	639	51.85	25.72	0.00	90.67	49.61
Progressive motile (%)	639	29.99	15.63	0.00	68.00	52.11
Motile (%)	639	70.83	21.13	13.00	96.20	29.83
Curve-linear velocity ($\mu\text{m/s}$)	639	54.76	13.14	18.90	87.90	23.99
Straight-line velocity ($\mu\text{m/s}$)	639	28.77	9.10	2.90	62.90	31.62
Average path velocity ($\mu\text{m/s}$)	639	41.03	12.64	7.60	70.90	30.81
Amplitude of lateral head displacement (μm)	639	2.41	0.50	0.00	3.60	20.88
Linearity (%)	639	52.05	9.02	11.50	80.40	17.32
Straight (%)	639	70.19	8.41	32.80	90.00	11.98
Wobble (%)	639	73.13	9.91	8.35	90.30	13.55
Beat-cross frequency (Hz)	639	8.54	1.59	0.00	12.30	18.58

SD: Standard deviation; CV: Coefficient of variation

The contributions of each fixed effect (FE, %) with its degrees of freedom (DF), R-square value and CV are shown in

Table 20 for NORM, LIVE, HOS, PMOT and MOT. The kinematic sperm traits (VCL, VSL, VCL, VAP, ALH, LIN, STR, WOB and BCF) are displayed in

Table 21. The least squares means for the main effects are provided in Table 22 and Table 23 for the respective sperm traits.

Table 34: The source of variation for the fixed effects of sperm concentration, dilution rate, dilution temperature and storage time with their variation contribution (FE, %) with its degrees of freedom (DF) on sperm viability (LIVE, %), membrane integrity (HOS, %), progressive motility (PMOT, %) and motility (MOT, %).

Variation Source	DF	NORM	DF	LIVE	DF	HOS	PMOT	MOT
CP type	1	1.19**	2	0.81***	2	1.42***	0.25	0.73***
CP conc	2	0.12	3	0.06	3	0.71	0.10	0.05
Stage	4	57.30***	4	59.59***	4	40.24***	43.37***	49.19***
CP type x CP conc	n/a	n/a	6	0.94***	6	3.68***	0.27	0.36
CP type x Stage	8	2.66**	8	2.46***	8	2.85***	2.66***	2.81***
CP type x CP conc x Stage	8	0.24	36	1.36*	36	2.59*	1.48	2.25
TCSS	252	132917.21	677	212444.7	638	179291.47	75719.05	120667.50
Error mean square	228	40454.33	618	36139.66	579	48154.15	29899.45	35035.21
R ²		0.70		0.83		0.73	0.61	0.71
CV (%)		27.48		14.31		19.93	22.34	13.32

CP: Cryoprotectant; Conc: Concentration; CV: Coefficient of variation; TCSS: Total corrected sum of squares; n/a: not applicable to the sperm function since data was limited for this function; *P < 0.05; **P < 0.01; ***P < 0.001

Table 35: The source of variation for the fixed effect of sperm concentration, dilution rate, dilution temperature and storage time with their variation contribution (FE, %) with its degrees of freedom (DF) on sperm kinematic traits, curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat-cross frequency (BCF, Hz).

Variation source	DF	VCL	VSL	VAP	ALH	LIN	STR	WOB	BCF
CP type	2	1.51***	0.29	1.15**	0.16	0.25	0.23	0.96*	0.18
CP conc	3	0.08	0.11	0.08	0.03	0.31	0.26	0.12	0.20
Stage	4	34.06***	27.62***	31.13***	7.95***	6.00***	0.32	10.85***	3.22***
CP type x CP conc	6	0.57	0.36	0.41	0.86	2.17	2.45	1.01	2.66
CP type x Stage	8	2.06***	2.34***	1.83**	3.62**	2.80	3.28	1.77	3.07
CP type x CP conc x Stage	36	1.00	1.50	1.06	3.83	1.12	1.78	1.13	1.94
TCSS	640	110628.27	52912.05	44072.37	161.48	24475.33	27880.83	36639.98	1592.17
Error mean square	581	57240.46	32593.50	57151.47	129.61	20639.58	25494.15	28840.17	1405.72
R ²		0.48	0.38	0.44	0.20	0.16	0.09	0.21	0.12
CV (%)		18.12	26.04	24.18	19.63	12.93	11.62	11.96	18.33

CP: Cryoprotectant; CPConc: Cryoprotectant concentration; CV: Coefficient of variation; TCSS: Total corrected sum of squares; *P < 0.05; **P < 0.01; ***P < 0.001

Table 36: The least square means (\pm S.E) of sperm concentration, dilution rate, dilution temperature and storage time on sperm viability (LIVE, %), membrane integrity (HOS, %), progressive motility (PMOT, %) and motility (MOT, %).

Variation source	NORM	LIVE	HOS	PMOT	MOT
CP type	**	***	***	P > 0.05	***
DMA	68.99 \pm 3.81 ^a	64.56 \pm 1.13 ^a	52.91 \pm 2.13 ^a	30.09 \pm 2.53 ^a	71.70 \pm 2.19 ^a
DMSO	63.89 \pm 3.81 ^b	60.33 \pm 1.14 ^b	49.54 \pm 2.13 ^a	28.11 \pm 2.54 ^a	67.28 \pm 2.20 ^b
MA	62.62 \pm 3.98 ^b	57.03 \pm 1.23 ^c	44.02 \pm 2.22 ^b	27.43 \pm 2.56 ^a	67.20 \pm 2.22 ^b
CPconc	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
4	66.78 \pm 4.46	61.34 \pm 1.31	47.61 \pm 2.27	28.22 \pm 2.60	68.25 \pm 2.27
8	65.49 \pm 4.48	60.62 \pm 1.31	47.75 \pm 2.27	28.34 \pm 2.60	68.98 \pm 2.27
12	71.28 \pm 4.44	59.44 \pm 1.31	48.19 \pm 2.27	29.01 \pm 2.60	69.03 \pm 2.27
16	67.64 \pm 3.99	61.35 \pm 1.31	51.74 \pm 2.15	28.59 \pm 2.54	68.63 \pm 2.20
Stage	***	***	***	***	***
Neat	n/a	78.48 \pm 1.35 ^a	58.55 \pm 2.32 ^a	32.13 \pm 2.63 ^a	80.86 \pm 2.31 ^a
Diluted	86.48 \pm 3.88 ^a	76.04 \pm 1.37 ^{ba}	64.87 \pm 2.32 ^b	39.56 \pm 2.62 ^b	79.07 \pm 2.29 ^{ba}
Cooled	67.76 \pm 3.88 ^b	75.21 \pm 1.35 ^{cb}	60.11 \pm 2.32 ^{cb}	37.05 \pm 2.62 ^{cb}	80.59 \pm 2.29 ^{cba}
CP added	62.62 \pm 3.99 ^{cb}	60.19 \pm 1.37 ^d	45.96 \pm 2.34 ^d	26.52 \pm 2.63 ^d	70.37 \pm 2.31 ^d
Thawed	43.82 \pm 3.98 ^d	13.24 \pm 1.37 ^e	16.62 \pm 2.34 ^e	7.44 \pm 2.63 ^e	32.74 \pm 2.31 ^e
CP type x CP conc	n/a	***	***	P > 0.05	P > 0.05
CP type x Stage	**	***	***	***	***
CP typexCPconcxStage	P > 0.05	*	*	P > 0.05	**

CP: Cryoprotectant; DMA: dimethylacetamide; MA: methylacetamide; DMOS; dimethylsulfoxide; CPConc: Cryoprotectant concentration; n/a: not applicable to the sperm function since data was not recorded; *P < 0.05; **P < 0.01; ***P < 0.001; Means with different superscripts differ (P < 0.05)

Table 37: The least square means (\pm S.E) of sperm concentration, dilution rate, dilution temperature and storage time on the curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat-cross frequency (BCF, Hz).

Variation source	VCL	VSL	VAP	ALH	LIN	STR	WOB	BCF
CP type	***	P > 0.05	**	P > 0.05	P > 0.05	P > 0.05	*	P > 0.05
DMA	55.32 \pm 2.07 ^a	28.37 \pm 1.54 ^a	41.03 \pm 2.13 ^a	2.41 \pm 0.04 ^a	50.92 \pm 1.35 ^a	69.57 \pm 1.34 ^a	72.71 \pm 1.11 ^a	8.53 \pm 0.23 ^a
DMSO	51.07 \pm 2.08 ^b	27.66 \pm 1.54 ^a	38.25 \pm 2.14 ^b	2.35 \pm 0.04 ^a	53.44 \pm 1.36 ^a	72.47 \pm 1.35 ^a	73.38 \pm 1.13 ^{ba}	8.57 \pm 0.23 ^a
MA	54.24 \pm 2.10 ^{ca}	28.46 \pm 1.56 ^a	41.23 \pm 2.15 ^{ca}	2.38 \pm 0.04 ^a	52.10 \pm 1.38 ^a	69.91 \pm 1.37 ^a	75.17 \pm 1.16 ^c	8.72 \pm 0.23 ^a
CP conc	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
4	53.69 \pm 2.14	27.62 \pm 1.59	39.85 \pm 2.19	2.39 \pm 0.05	50.87 \pm 1.43	69.84 \pm 1.42	72.80 \pm 1.23	8.51 \pm 0.25
8	53.89 \pm 2.14	28.14 \pm 1.59	40.49 \pm 2.19	2.37 \pm 0.05	51.69 \pm 1.43	69.88 \pm 1.42	73.84 \pm 1.23	8.66 \pm 0.25
12	53.51 \pm 2.14	28.63 \pm 1.59	40.50 \pm 2.20	2.38 \pm 0.05	53.24 \pm 1.37	71.48 \pm 1.42	74.81 \pm 1.24	8.69 \pm 0.25
16	53.08 \pm 2.08	28.27 \pm 1.55	39.84 \pm 2.14	2.37 \pm 0.05	52.80 \pm 1.44	71.39 \pm 1.36	73.56 \pm 1.14	8.55 \pm 0.23
Stage	***	***	***	***	***	P > 0.05	***	***
Neat	54.30 \pm 2.17 ^a	28.47 \pm 1.62 ^a	40.92 \pm 2.23 ^a	2.12 \pm 0.05 ^a	52.20 \pm 1.48 ^a	69.95 \pm 1.46	73.51 \pm 1.29 ^a	8.99 \pm 0.26 ^a
Diluted	61.23 \pm 2.16 ^b	33.03 \pm 1.61 ^b	46.89 \pm 2.21 ^b	2.55 \pm 0.05 ^b	54.10 \pm 1.48 ^a	71.04 \pm 1.44	76.51 \pm 1.27 ^{ba}	8.66 \pm 0.33 ^{ba}
Cooled	62.06 \pm 2.16 ^{cb}	33.49 \pm 1.61 ^{cb}	48.29 \pm 2.21 ^{cb}	2.51 \pm 0.05 ^{cb}	54.15 \pm 1.46 ^a	69.59 \pm 1.44	77.58 \pm 1.27 ^{cb}	8.10 \pm 0.33 ^{cab}
CP added	53.28 \pm 2.17 ^{da}	27.94 \pm 1.62 ^{da}	39.91 \pm 2.22 ^{da}	2.47 \pm 0.05 ^{dcb}	52.41 \pm 1.46 ^a	70.87 \pm 1.46	74.43 \pm 1.29 ^{dabc}	7.98 \pm 0.26 ^{dabc}
Thawed	36.86 \pm 2.17 ^e	17.89 \pm 1.61 ^e	24.85 \pm 2.22 ^e	2.23 \pm 0.05 ^{ea}	47.90 \pm 1.48 ^e	71.79 \pm 1.46	66.74 \pm 1.29 ^e	9.26 \pm 0.65 ^e
CP type x CPconc	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
CP type x Stage	***	***	**	***	P > 0.05	P > 0.05	P > 0.05	P > 0.05
CPtypexCPconcStage	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05

CP: Cryoprotectant; DMA: dimethylacetamide; MA: methylacetamide; DMOS; dimethylsulfoxide CPConc: Cryoprotectant concentration; *P < 0.05; **P < 0.01; ***P < 0.001; Means with different superscripts differ (P < 0.05)

3.2. *The effect of semen processing stage on sperm traits*

Processing stage contributed ($P < 0.05$) most to the variation associated with FE's on bulk of sperm traits assessed (NORM, LIVE, HOS, PMOT, MOT, VCL, VSL, VAP, ALH, LIN, WOB, BCF), the magnitude of the effect ranging from 59.59 % (LIVE) to 6.00 % (STR) (

Table 20 and

Table 21). NORM was the only sperm trait compromised ($P < 0.05$) by more than 18 % when cooled in comparison to CP addition and equilibration that imposed no further reduction ($P > 0.05$) (Figure 43). Although HOS and PMOT was significantly higher after dilution, compared to LIVE and MOT that were unaffected ($P > 0.05$) by dilution, all these traits (LIVE, HOS, PMOT and MOT) showed limited deterioration ($P > 0.05$) from pre-treatment processing (dilution and cooling) until after CP addition and equilibration (Figure 43). LIVE, HOS, PMOT and MOT was reduced significantly by 10 to 15% after CP addition and equilibration.

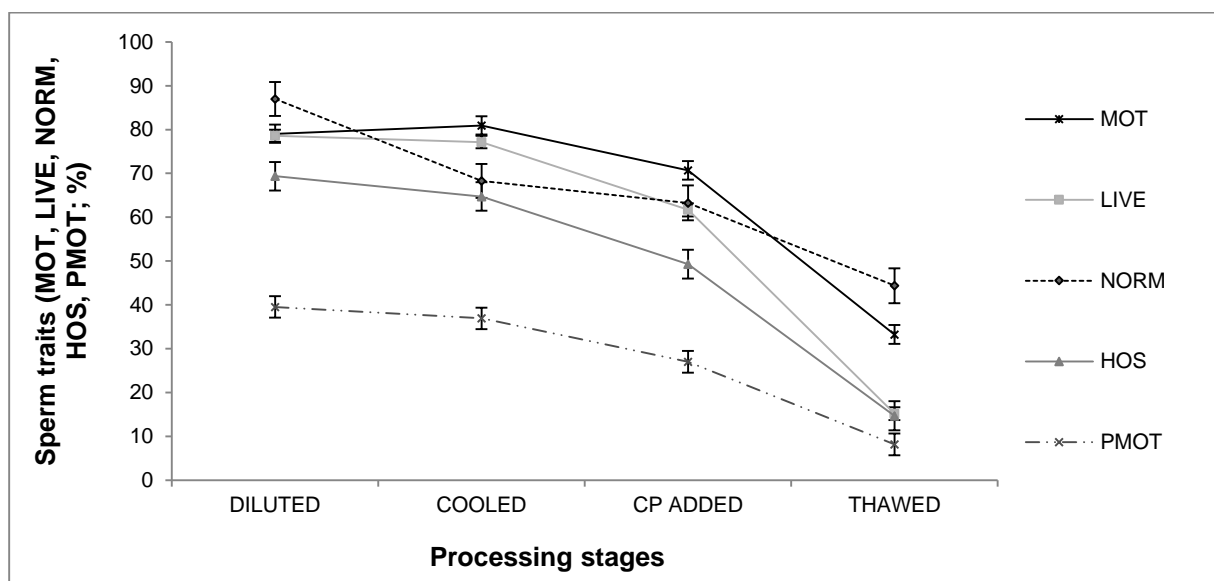


Figure 43: Least squares means depicting the effect of processing stages (dilution, cooled, CP addition and equilibration, thawing) on sperm traits namely sperm motility (MOT, %), viability (LIVE, %), morphology (NORM, %), membrane integrity (HOS, %) and progressive motility (PMOT, %) across the respective CPs added. Standard error is indicated by vertical bars at the mean.

Kinematic traits namely VCL, VSL, and VAP were higher ($P < 0.05$) after dilution, with no significant decrease when cooled, but decreased ($P < 0.05$) after CP addition and equilibration. VCL, VSL and VAP levels after CP addition and equilibration were not different ($P > 0.05$) from that obtained in the neat samples. LIN, WOB and BCF were the only traits that were not reduced ($P > 0.05$) by CP addition with the same values after CP addition and equilibration than those measured pre-treatment. Thawing significantly reduced all sperm traits (NORM, LIVE, HOS, PMOT, MOT, VCL, VSL, VAP, ALH, LIN, WOB, BCF) affected by processing to their lowest level with only ALH at thawing, not differing ($P > 0.05$) from the ALH in neat samples.

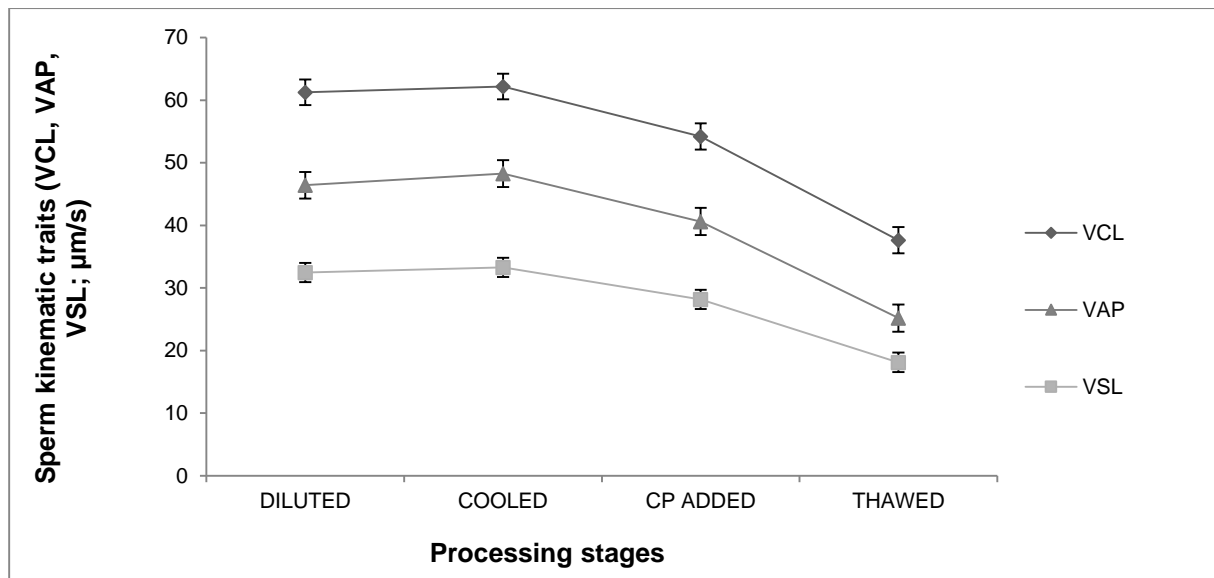


Figure 44: Least squares means depicting the effect of processing stages (dilution, cooled, CP addition and equilibration, thawing) on sperm kinematic traits namely sperm curve line velocity (VCL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$) and straight-line velocity (VSL, $\mu\text{m/s}$). Standard error is indicated by vertical bars at the mean.

3.3. The effect of CP type and CP concentration on sperm traits

Sperm traits, namely NORM, LIVE, HOS, MOT, VCL, VAP and WOB varied significantly when treated with the different CPs (DMA, DMSO and MA). The contribution of CP type to the variation in different sperm traits ranged from 1.42 % for HOS to 0.73 % for MOT (

Table 20 and

Table 21). A higher ($P < 0.05$) percentage of morphologically normal sperm (68.99 ± 3.81 %) was observed when DMA was used, compared to DMSO and MA that increased sperm abnormalities with > 6 % (Table 22). Overall, LIVE and MOT were highest ($P < 0.05$) when DMA was used, compared to DMSO and MA that compromised these traits. The highest ($P < 0.05$) HOS was observed when DMA and DMSO were used with no difference ($P > 0.05$) between the latter two, compared to MA that impaired HOS ($P < 0.05$). Overall, the addition of MA resulted in higher ($P < 0.05$) WOB-values, compared to DMA. VCL and VAP was highest ($P < 0.05$) when DMA and MA was used with no difference ($P > 0.05$) between these two CPs. In contrast, DMSO compromised ($P < 0.05$) velocity traits. CP concentration had no effect ($P > 0.05$) on any of the sperm traits measured.

3.4. The interaction effect of CP type with processing stage on sperm traits

The interaction effect of CP type with processing stage contributed ($P < 0.05$) towards the variation associated with NORM (Figure 45), HOS (Figure 46), LIVE (Figure 47), PMOT (Figure 48), MOT (Figure 49), VCL (Figure 50), VSL, VAP and ALH, R^2 values ranging from 1.83 to 3.62 %. As expected, no differences ($P > 0.05$) were observed at the initial processing stages of dilution and cooling among sperm traits for the different CPs. Significant differences between CPs could only be observed during the stages of CP addition and equilibration as well as after thawing. The CP addition and equilibration stage was understandably the most important stage for evaluating the true effect of CP type on sperm function.

DMA was the only CP that did not reduce the sperm functions of NORM, LIVE, PMOT, MOT, VCL, VSL and VAP after CP addition and equilibration. HOS was the only function that was reduced by the addition and equilibration of DMA, but to a limited extent (17.0 ± 3.21 %). Sperm functions of NORM, LIVE, HOS, PMOT and MOT declined ($P < 0.05$) with the addition and equilibration of MA and DMSO.

NORM (71.95 ± 4.38 %; Figure 45) and HOS (56.04 ± 3.01 %; Figure 46) was significantly higher after DMA was added as CP, compared to MA and DMSO that did not differ ($P > 0.05$) from each other. MA and DMSO reduced ($P < 0.05$) NORM by 11 to 14 % and HOS by 20 to 25 %.

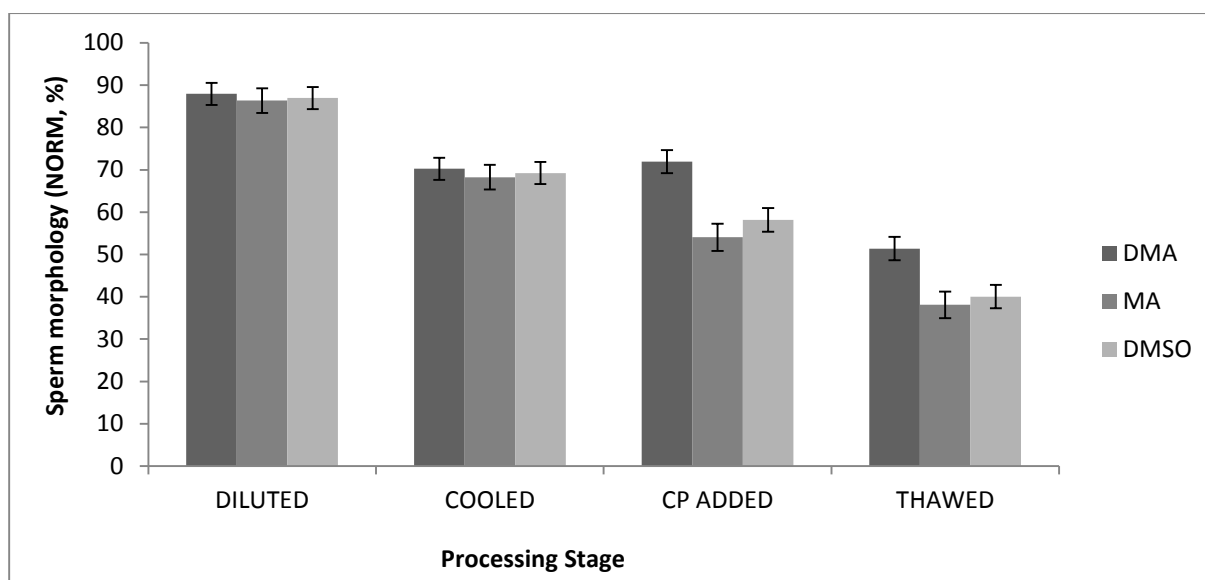


Figure 45: Least squares means depicting the interaction effect of CP type (DMA: dimethylacetamide; MA: methylacetamide; DMSO; dimethylsulfoxide) with processing stages (diluted, cooled, CP added and equilibrated, thawed) on normal sperm morphology (NORM, %). Standard error is indicated by vertical bars at the mean.

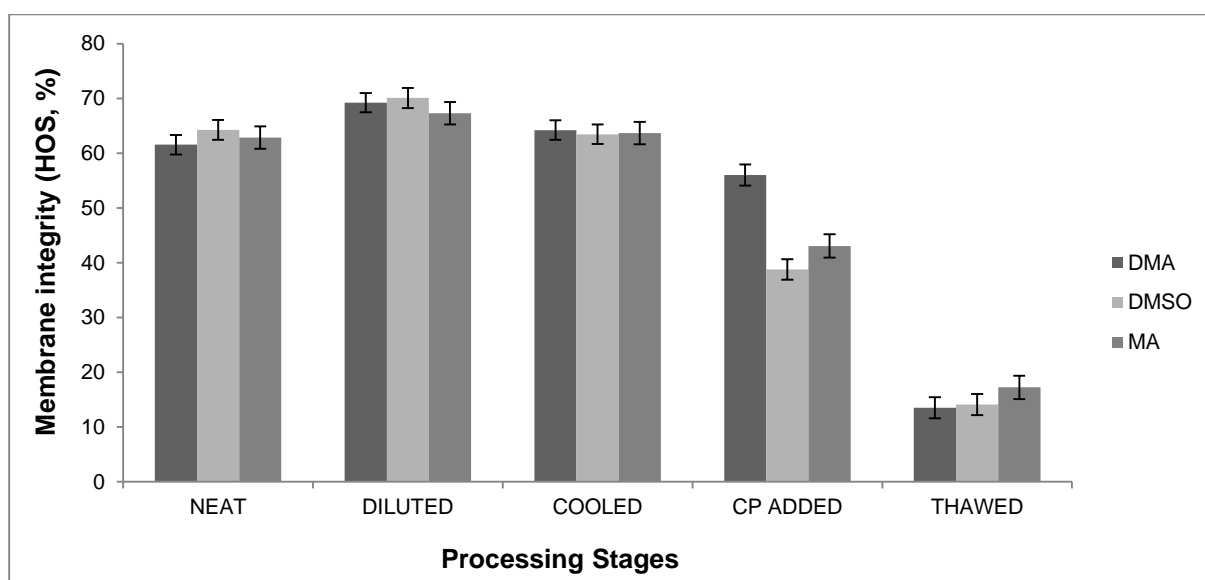


Figure 46: Least squares means depicting the interaction effect of CP type (DMA: dimethylacetamide; MA: methylacetamide; DMSO; dimethylsulfoxide) with processing stages (neat, diluted, cooled, CP added and equilibrated, thawed) on sperm membrane integrity (HOS, %). Standard error is indicated by vertical bars at the mean.

DMA also maintained significantly higher LIVE (74.79 ± 2.05 %; Figure 47), PMOT (33.56 ± 1.91 %; Figure 48) and MOT (80.83 ± 2.76 %; Figure 49) after addition and equilibration, compared to MA and DMSO. MA maintained slightly higher ($P < 0.05$) PMOT (26.90 ± 3.06 %) and MOT (71.46 ± 2.81 %) compared to DMSO, but was not significantly different from DMSO in terms of LIVE.

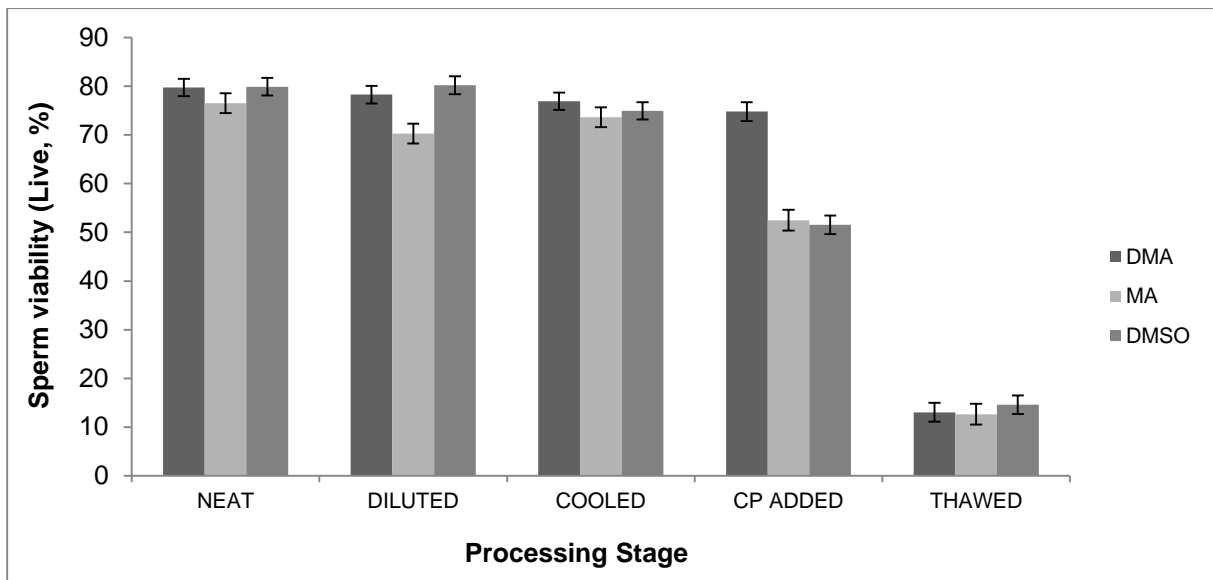


Figure 47: Least squares means depicting the interaction effect of CP type (DMA: dimethylacetamide; MA: methylacetamide; DMSO; dimethylsulfoxide) with processing stages (neat, diluted, cooled, CP added and equilibrated, thawed) on sperm viability (LIVE, %). Standard error is indicated by vertical bars at the mean.

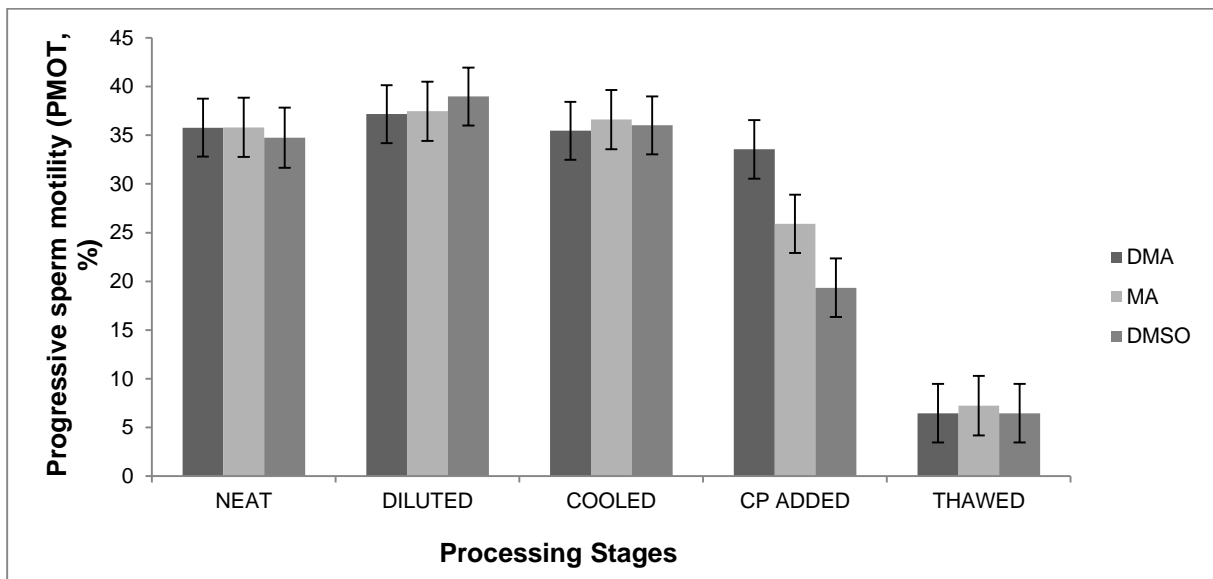


Figure 48: Least squares means depicting the interaction effect of CP type (DMA: dimethylacetamide; MA: methylacetamide; DMSO; dimethylsulfoxide) with processing stages (neat, diluted, cooled, CP added and equilibrated, thawed) on progressive sperm motility (PMOT, %). Standard error is indicated by vertical bars at the mean.

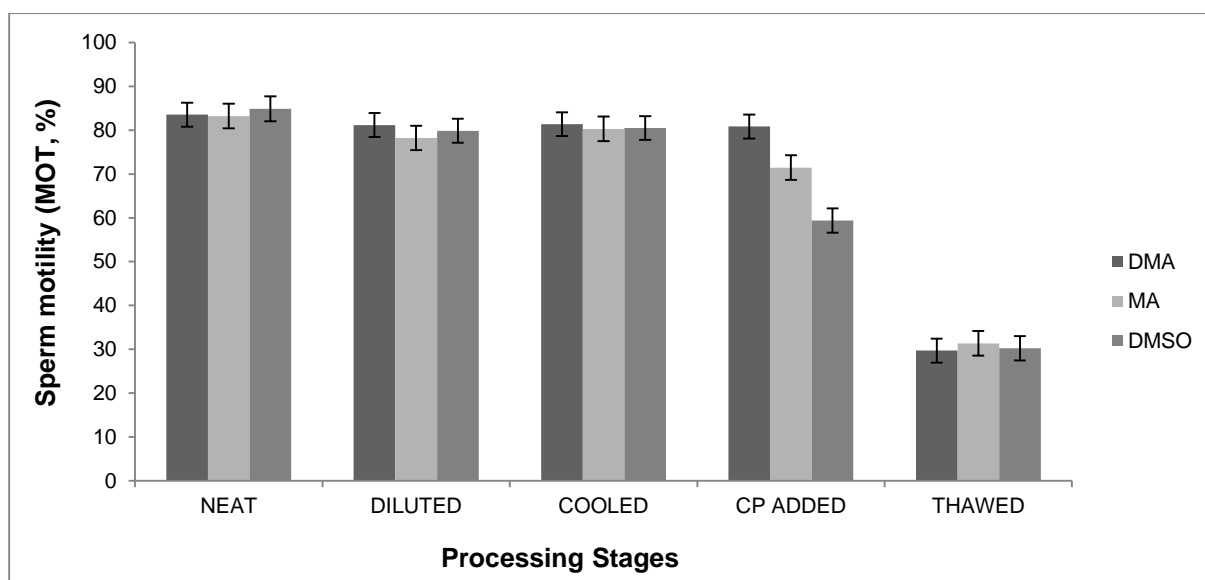


Figure 49: Least squares means depicting the interaction effect of CP type (DMA: dimethylacetamide; MA: methylacetamide; DMSO; dimethylsulfoxide) with processing stages (neat, diluted, cooled, CP added and equilibrated, thawed) on sperm motility (MOT, %). Standard error is indicated by vertical bars at the mean.

DMA and MA maintained significantly higher sperm velocity traits reflected by VCL (Figure 50), VSL (Figure 51) and VAP (Figure 52) after their addition and equilibration, with no difference ($P > 0.05$) between the latter two CP's when compared to DMSO. ALH was the only sperm trait that was the highest after CP addition and equilibration when treated with either DMA or DMSO compared to MA that had significantly lower ($P < 0.05$) ALH values than with the latter CP's.

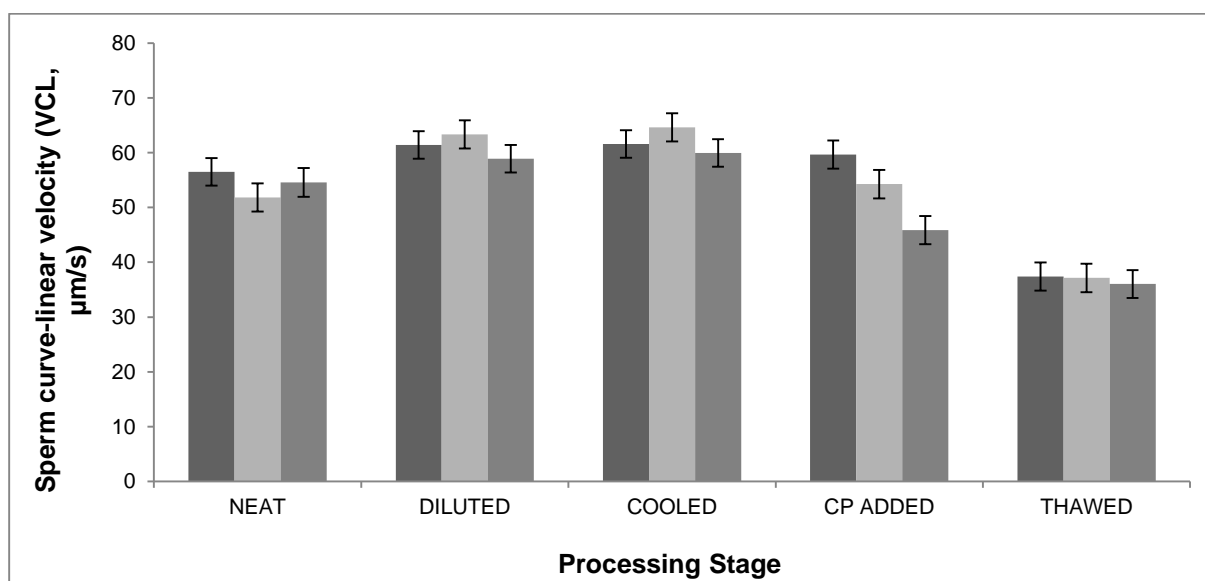


Figure 50: Least squares means depicting the interaction effect of CP type (DMA: dimethylacetamide; MA: methylacetamide; DMSO; dimethylsulfoxide) with processing stages (neat, diluted, cooled, CP added and equilibrated, thawed) on sperm curve-linear velocity (VCL, $\mu\text{m/s}$). Standard error is indicated by vertical bars at the mean.

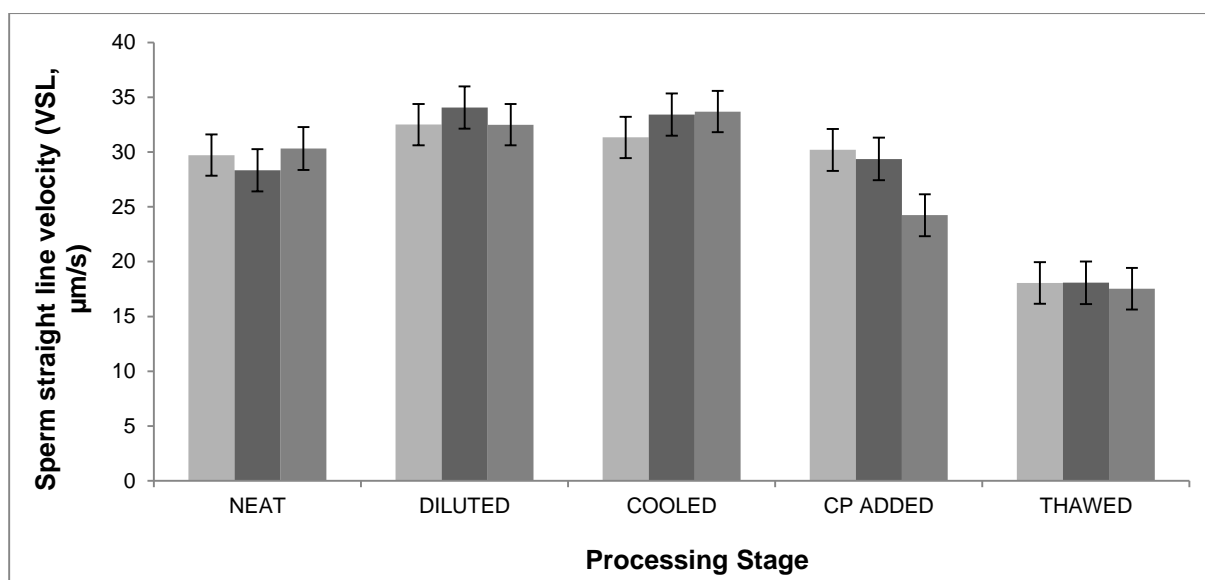


Figure 51: Least squares means depicting the interaction effect of CP type (DMA: dimethylacetamide; MA: methylacetamide; DMOS; dimethylsulfoxide) with processing stages (neat, diluted, cooled, CP added and equilibrated, thawed) on sperm straight-line velocity (VSL, $\mu\text{m/s}$). Standard error is indicated by vertical bars at the mean.

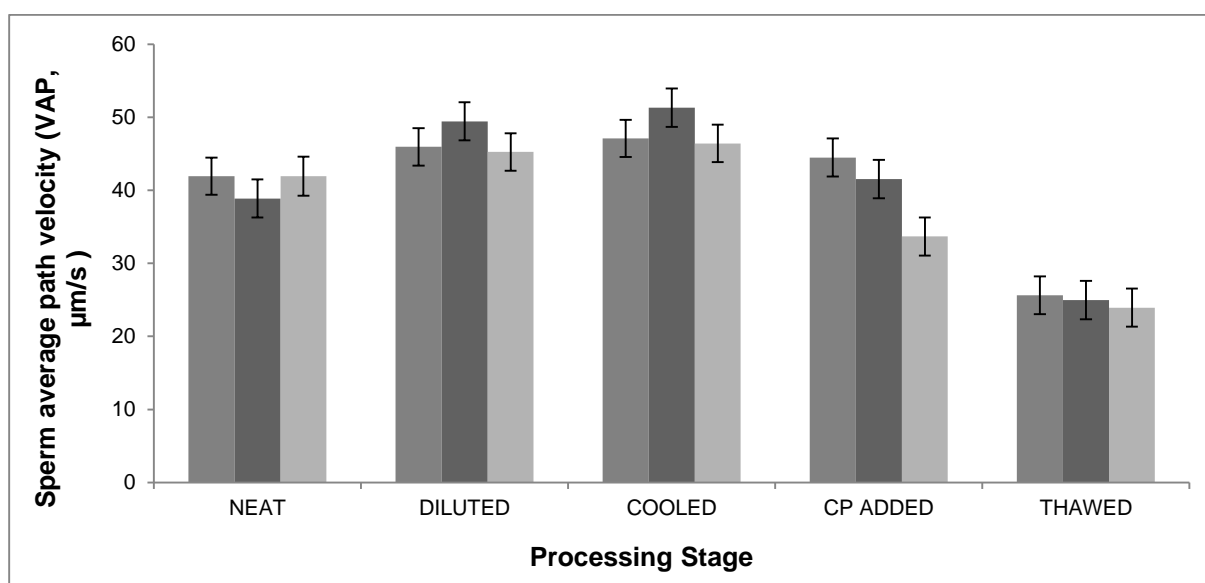


Figure 52: Least squares means depicting the interaction effect of CP type (DMA: dimethylacetamide; MA: methylacetamide; DMOS; dimethylsulfoxide) with processing stages (neat, diluted, cooled, CP added and equilibrated, thawed) on sperm average path velocity (VAP, $\mu\text{m/s}$). Standard error is indicated by vertical bars at the mean.

After the thaw stage it was evident that DMA maintained significantly higher NORM ($51.40 \pm 4.38\%$), compared to MA and DMSO that did not differ from each other ($P > 0.05$). In contrast, LIVE ($13.41 \pm 2.11\%$), HOS ($14.98 \pm 3.08\%$), PMOT ($6.70 \pm 3.02\%$), MOT ($30.42 \pm 2.78\%$), VCL ($36.86 \pm 2.57\ \mu\text{m/s}$), VSL ($17.89 \pm 1.92\ \mu\text{m/s}$), VAP ($24.85 \pm 2.60\ \mu\text{m/s}$) and ALH ($2.23 \pm 0.09\ \mu\text{m}$) was maintained at the same ($P > 0.05$) level for all three CP's after thawing.

3.5. *The interaction effect of CP type with CP concentration and processing stage on sperm traits*

The three-factor interaction effect of CP type with CP concentration and processing contributed towards the variation associated with LIVE (1.36 %) and HOS (2.59 %) sperm (

Table 20 and Table 22). DMA was found to be the only CP able to maintain LIVE after CP addition at all concentrations of 4, 8, 12 and 16 %. In contrast, LIVE declined with CP concentration in the other CPs after CP addition (Figure 53). At lower CP concentrations of 4 % there was no difference ($P > 0.05$) in LIVE between DMA, DMSO and MA. However, at higher concentrations (8, 12, 16 %), DMA outperformed DMSO and MA for maintaining LIVE. The regression of LIVE on CP concentration for DMSO and MA after CP addition and equilibration was best quantified by the respective linear regressions $Y = 72.66 - 2.45 X$ ($P < 0.0001$; $R^2 = 0.42$) and $Y = 68.13 - 1.26 X$ ($P = 0.0179$; $R^2 = 0.16$). The equations indicate respective reductions of 2.45 % and a 1.26 % in LIVE with every 1 % increase in DMSO and MA concentration.

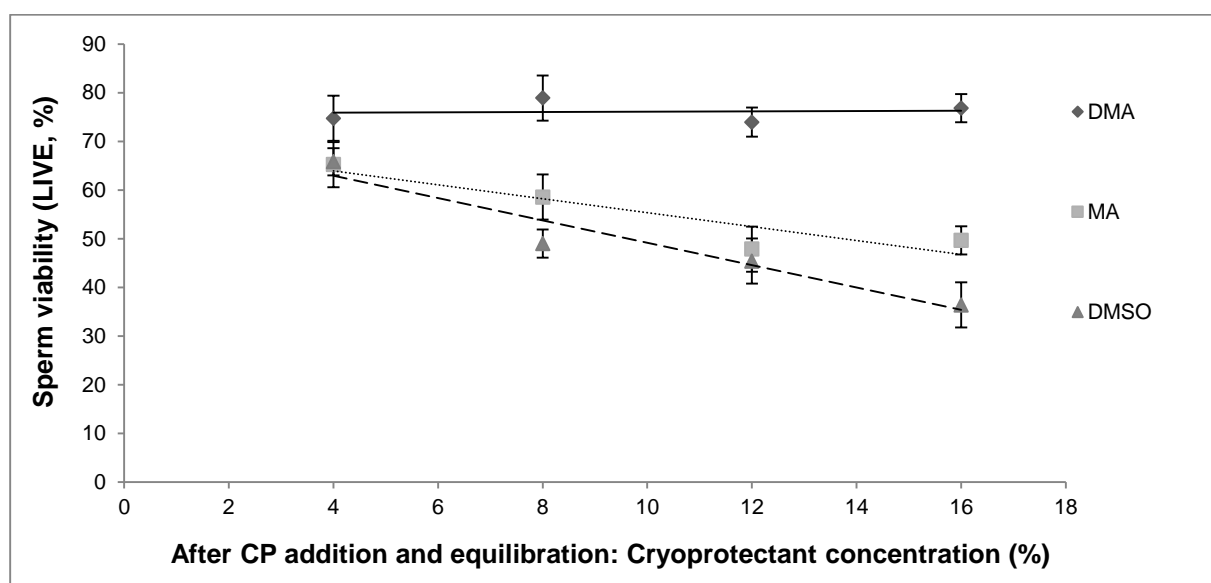


Figure 53: The interaction effect of CP type (DMA: dimethylacetamide; MA: methylacetamide; DMSO; dimethylsulfoxide) with CP concentration (4, 8, 12, 16 %) at the processing stage CP added and equilibrated on the sperm trait sperm viability (LIVE, %). Standard error is indicated by vertical bars at the mean.

An increased DMA concentration of 16 % maintained the highest level of HOS resistant sperm compared to lower concentrations of 4 and 8 % ($P < 0.05$) (Figure 54). The linear increase in HOS associated with increased DMA concentrations was best described by the linear regression $Y = 36.76 + 1.77 X$ ($P = 0.0045$; $R^2 = 0.19$), indicating a 1.77 % increase in HOS for every 1 % increase in DMA concentration (Figure 54). At lower CP concentrations of 4 to 8 % no significant differences were observed between DMA, DMSO and MA for HOS. At CP concentrations $> 8\%$ the difference between CP's became significantly different with DMA, maintaining the highest levels of HOS. DMSO showed a significant reduction in its ability to maintain HOS as CP concentration increased. The linear regression of HOS on CP concentration was described by the equation $Y = 57.92$

– 2.06 X ($P = 0.0003$; $R^2 = 0.23$), suggesting a 2.06 % reduction in HOS with every 1 % increase in DMSO concentration. MA did not show any significant difference in its ability to maintain HOS at CP concentrations of 4 to 16 %.

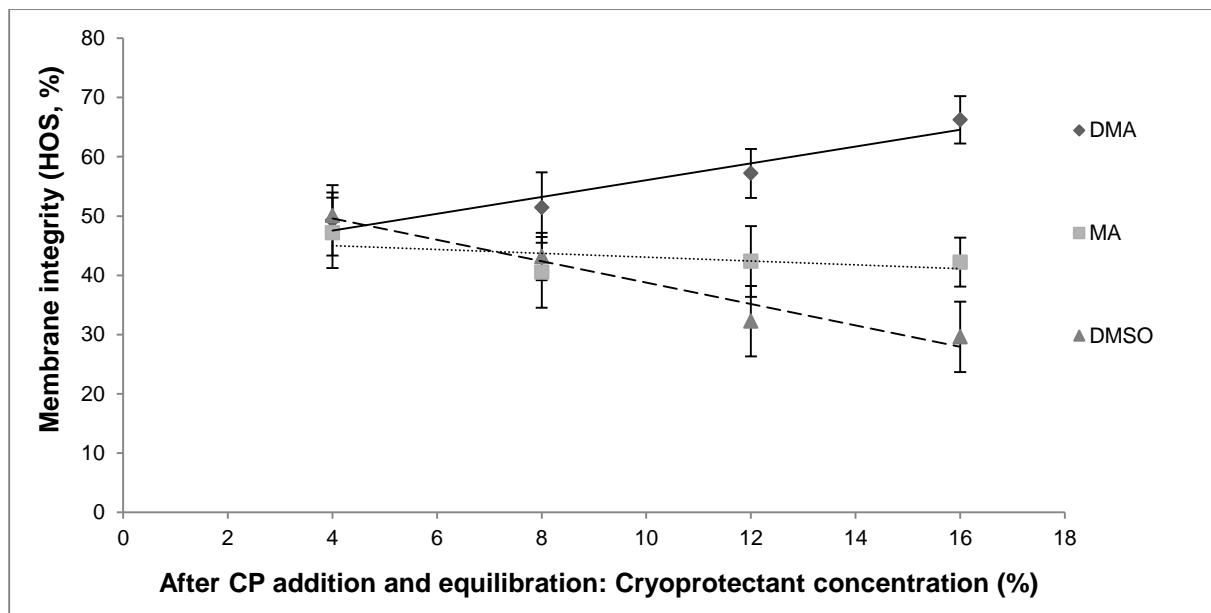


Figure 54: The interaction effect of CP type (DMA: dimethylacetamide; MA: methylacetamide; DMSO; dimethylsulfoxide) with CP concentration (4, 8, 12, 16 %) and processing stage (CP added and equilibrated) on the sperm trait sperm membrane integrity (HOS, %). Standard error is indicated by vertical bars at the mean.

At the highest DMA concentration (16 %), HOS was not significantly reduced by CP addition and equilibration, compared to the other CPs that reflected decreased sperm function after addition and equilibration, even at optimal concentrations. The beneficial effect of DMA 16 % in terms of HOS is presented in Figure 55

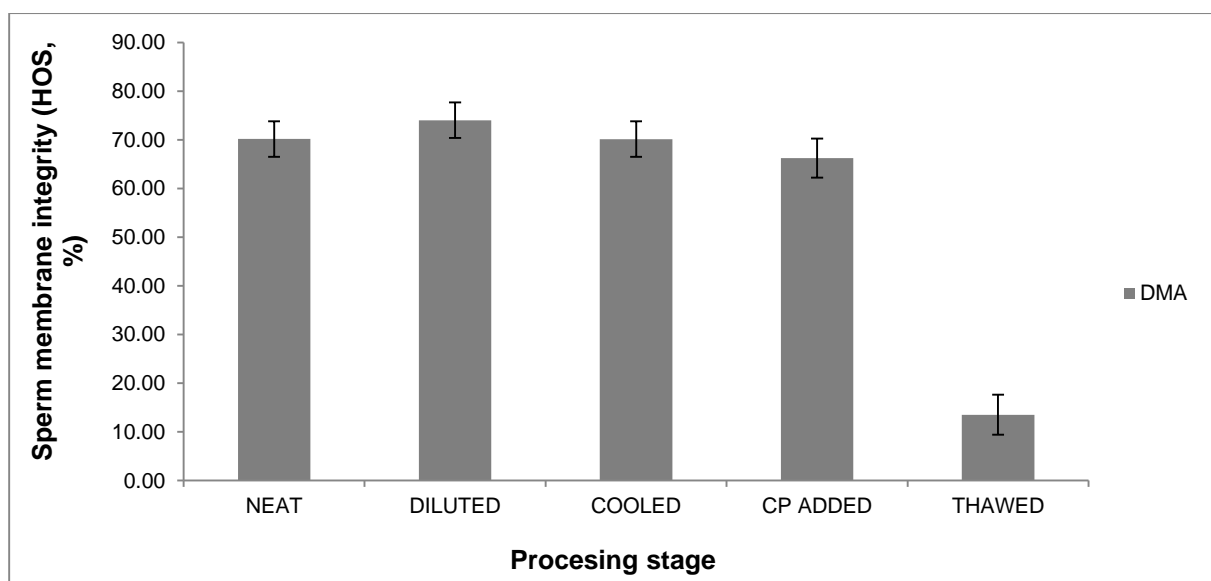


Figure 55: The effect of DMA (dimethylacetamide) at a concentration of 16 % after the different processing stages (neat, diluted, cooled, CP added and equilibrated, thawed) on sperm membrane integrity (HOS, %). Standard error is indicated by vertical bars at the mean.

After thawing the three CP's (DMA, DMSO, MA) at different concentrations did not differ significantly from each other for either LIVE or HOS. A linear increase of $Y = 1.17 + 1.06 X$ ($P = 0.0118$; $R^2 = 0.15$) in LIVE was observed with increased concentrations of DMA with 16 % being significantly better in maintaining LIVE, than 4 % (Figure 56). A comparable favourable trend was observed for HOS with increased DMA concentrations in absolute terms, but this was not significant ($P > 0.05$).

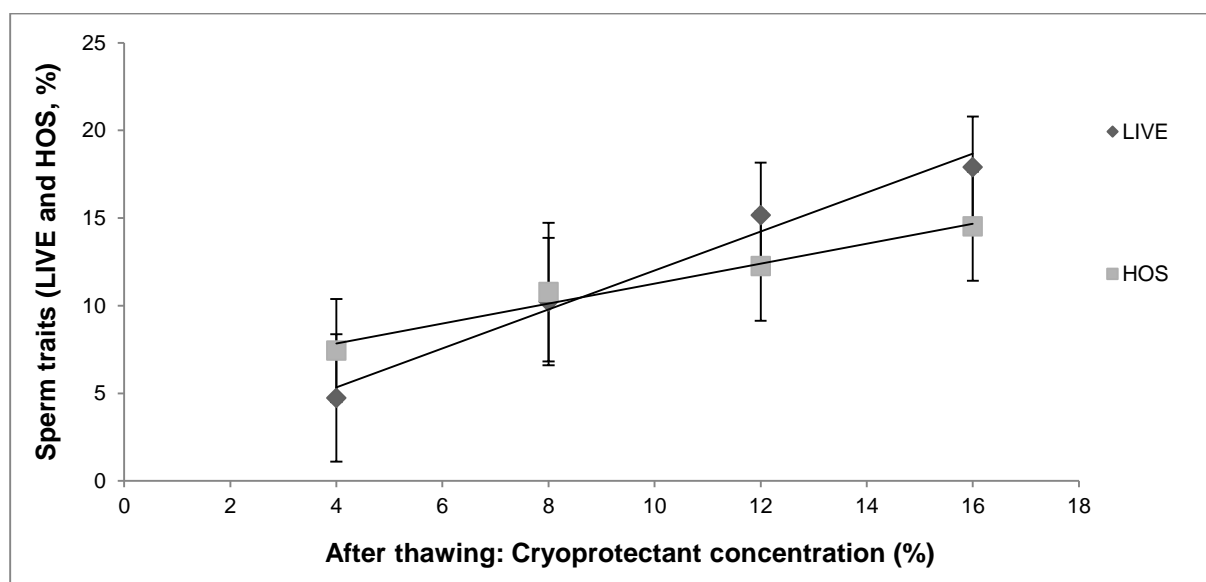


Figure 56: The effect of applying DMA at different concentrations (4, 8, 12, 16 %) after thawing on sperm viability (LIVE, %). Standard error is indicated by vertical bars at the mean.

4. Discussion

4.1. *The effects of semen processing stage, CP type and CP concentration on sperm traits.*

Semen processing stage had a significant influence on sperm function with specific sperm functions being more sensitive to certain processing stages. The quantification of the extent of damage on sperm function during the different processing stages is an important stepping stone for the adaption and improvement of the cryopreservation protocol. Ostrich sperm morphology, in terms of the percentage morphological normal cells, was compromised distinctly when cooled compared to the CP addition and equilibration stage that did not deteriorate sperm morphology any further. This result could possibly be ascribed to the temperature-induced plasma membrane phase transition changes that occurred when sperm was cooled, as suggested by Hammerstedt *et al.* (1990). However, since cooling did not have an effect on any of the other sperm traits considered the degree of membrane damage is questionable and can possibly only be observed during later stages of processing for these traits. Hammerstedt *et al.* (1990) revealed that damage induced in one processing step is not necessarily expressed until the cell has undergone further treatments. It is sometimes very difficult to detect this damage at the stage where it was induced because of the low temperatures. Therefore, it is often necessary to complete the entire process of cryopreservation, before evaluating its impacts on the cell.

LIVE, HOS, PMOT, MOT, VCL, VSL, VAP, ALH, LIN, WOB and BCF were reduced by 10 to 15 % by CP addition and equilibration, compared to the processing stages of dilution and cooling that did not adversely affect these traits. Moce *et al.* (2010) demonstrated that the most marked alteration of sperm function stems from contact between sperm cells and the CP. The decrease in these sperm traits associated with CP addition and equilibration is partially due to exposure of the cell to shifting hypertonic conditions, first extracellular then intracellular, that is associated with the movement of the CP into the cell since all CP's tested, fall into the penetrating category. These hypertonic conditions cause dehydration and shrinkage of the cell followed by rehydration and swelling that can induce irreversible damage to the sperm membrane even after returning to isotonic conditions (Du *et al.*, 1992; Blanco *et al.*, 2000). It has been shown that damage to the sperm membrane may result in the impairment of other sperm functions due to the formation of reactive oxygen species (ROS) and the loss of antioxidant enzymes and could contribute to further damage post-thaw (Chatterjee and Gagnon, 2001; Park *et al.*,

2003; Guthrie *et al.*, 2008; Partyka *et al.*, 2012). High levels of ROS can cause destruction of nucleic acids, proteins, lipids and carbohydrates that may ultimately result in cell death (Agarwal *et al.*, 2005; Peris *et al.*, 2007). A decline in mitochondrial activity and thus sperm motility is secondary observations associated with increased ROS formation (Guthrie *et al.*, 2008). The magnitude of cell injury when exposed to hypertonic conditions is dependent on equilibration time and temperature, type of CP and CP concentration (Gao *et al.*, 1992, 1993; Blanco *et al.*, 2000).

A distinct difference between the three CP's was observed when fitted as a main effect and was specifically interpreted after the CP addition and equilibration processing stage, that is the best stage to assess true CP effectiveness. DMA was superior in maintaining higher percentages of the sperm functions NORM, LIVE, HOS, PMOT, MOT, VCL, VSL and VAP compared to the other CPs. MA managed to maintain only VSL and VAP at the same level as DMA, but was superior to DMSO for LIVE, PMOT, MOT and VCL. NORM was the only trait maintained at a higher level after thawing when DMA was used, compared to MA and DMSO. All other sperm functions was severely reduced after thawing with no conclusive differences between the three CP's. CP evaluation after the thawing stage is of little value in this specific study due to CPs tested during a standardised freezing-thawing protocol and not in combination with optimal freezing-thawing rates for each CP. CP type and the subsequent freezing-thawing protocol has been shown highly dependent on each other to maintain optimal sperm function (Seigneurin *et al.*, 2013). Reduction in sperm function after thawing is not a result of CP type as such, but rather an overall outcome of cryo-injury associated with the whole process.

The main effect of CP concentration did not significantly contribute to differences in sperm function even at higher concentrations. However, CP concentration in combination with CP type after the CP addition and equilibration stage influenced the percentages of LIVE and HOS sperm. Higher concentrations of DMA were superior in maintaining these two sperm functions, with a lower concentration of 4 % DMA being similar to 16 % in maintaining LIVE while a higher concentration of 16 % was best to maintain HOS. Similar success (59.5 % survivability) was reported in the guinea fowl when 6 % DMA was used (Seigneurin *et al.*, 2013). It was evident that DMSO reduced both LIVE and HOS linearly with an increase in concentration, while MA was superior to DMSO at high (16 %) concentrations in maintaining these functions. As mentioned before, increased CP

concentrations directly affect the degree of hypertonicity that the cell is exposed to. This is only evident for increased DMSO concentrations, but not for increased concentrations of DMA and MA. These results can be elucidated by the osmolarity range presented by Tselutin *et al.* (1999) in a cryopreservation study done for the fowl for DMSO and DMA. At the same CP addition concentrations (4 to 11 %) DMSO consistently displayed a significantly higher osmolarity, at each level compared to DMA with DMSO reducing the number of viable fowl sperm at a higher concentration, compared to DMA (Tselutin *et al.*, 1999). Tolerance to hyperosmotic conditions is variable for avian species as demonstrated by Blanco *et al.* (2000) by assessing viable sperm %. The phenomena observed in this study for the interaction of CP type with CP concentration could possibly be related to results reported by the latter authors. CP toxicity of non-physiological chemicals is another factor to consider in relation to CP concentration that may cause reduced sperm function through its action upon the actin cytoskeleton that may compromise the capacity of the sperm to cope with osmotic stress (Correa *et al.*, 2007). Although the exact mechanism of CP toxicity is poorly understood it has been referred to numerous times in the literature as a factor compromising sperm function with specific reference to DMSO (Fahy *et al.*, 1990; Fahy, 2010). DMSO (8 %) has been shown the CP of choice for certain avian species like the Blue rock pigeon (Sontakke *et al.*, 2004). However, DMSO (6 %) has been found to damage chromatin and reduce plasma membrane integrity of bull sperm (Tasdemir *et al.*, 2013), and also to reduce the percentage live and morphological normal sperm in the fowl at 4 to 11% concentrations (Tselutin *et al.*, 1999).

The deterioration in all sperm traits measured (NORM, LIVE, HOS, PMOT, MOT, VCL, VSL, VAP, ALH, LIN, WOB, BCF) after thawing, is ostensibly a result of the different types of stressors (mechanical, osmotic and thermal) exerted on the cell during the different stages of the cryopreservation process. Ostrich sperm traits markedly compromised after thawing included NORM, LIVE, as well as HOS that was reduced by percentages ranging from 40 to 60 %. This range is consistent with the norm of 40 to 50 % reduction of the initial sperm function reported in mammals as well as both domestic and non-domestic avian species for sperm traits after thawing. Such reductions mainly stem from damage of the cellular structures like the plasma membrane, nucleus, mitochondria and flagellum (Park and Graham, 1992; Watson, 1995; Donoghue and Wishart, 2000; Gee *et al.*, 2004; Blesbois *et al.*, 2005; Moce *et al.*, 2010; Partyka *et al.*, 2010, 2012). The biophysical appearance of the cell is a major contributor to its susceptibility to cryopreservation

sensitivity because of the low cytoplasmic content and relatively large surface of membranes exposed to the adverse micro-environmental conditions associated with cryopreservation (Blanco *et al.*, 2000; Blesbois and Brillard, 2007). Partyka *et al.* (2012), for instance, reported a 59.1 % loss in live sperm, with a further reduction of 62.3 % in mitochondrial activity for chicken sperm. Watson (2000) suggested that a post-thaw motility of 50% is the norm even under the best experimental conditions. Sperm function after thaw *in vitro* does not always predict the outcome of *in vivo* fertility trials accurately (Donoghue and Wishart, 2000; Moce *et al.*, 2010).

Apart from damage induced at other stages (dilution, cooling, CP addition and equilibration) of the cryopreservation process, the main two reasons for sperm damage during the freezing-thawing stage suggested in the literature are as follows: Firstly, it is reported that mechanical damage from the internal ice crystal formation results in sperm structure alterations and physical damage to the sperm. Secondly, the damage caused by chemical and osmotic effects due to increased solute (salt) concentrations in the residual unfrozen water between the crystals, possibly to toxic levels (Watson, 1981; Watson and Morris, 1987; Hammerstedt *et al.*, 1990; Holt, 2000; Isachenko, 2003). The latter is due to the water content that is withdrawn to form both intracellular and extracellular ice crystals (Salisbury *et al.*, 1978; Mazur, 1984, Isachenko *et al.*, 2003). It has been suggested by Blanco *et al.* (2008) that low temperatures could detrimentally affect physical properties of the sperm membrane due to prolonged exposure to un-isosmotic conditions similar to those discussed earlier with CP addition and equilibration. A more recent study with mammalian sperm confirmed that the cellular damage encountered by sperm at low temperatures is due to an osmotic imbalance and not intracellular ice formation.. The latter is due to the high intracellular protein concentration together with osmotic shrinkage associated with extracellular ice formation that leads to intracellular vitrification during rapid cooling/freezing rates (Morris *et al.*, 2012). These conditions may influence resistance to frictional forces and are species-specific, depending on sensitivity to osmotic pressure. Morris *et al.* (2012) suggested that the sensitivity of sperm from different species to cryopreservation depends on their ability to withstand the osmotic shock, which occurs at low temperatures. Factors contributing to osmotic resistance include the membrane permeability to water and permeating CPs, time of exposure, minimal critical volume, cell size and morphology to influence the magnitude of freezing injury in the avian (Blanco *et al.*, 2000). Blesbois *et al.* (2005) stressed the importance of the biochemical composition of the contribution of the

cholesterol/phospholipid ratios in the membrane to the fluidity of the cell and its ability to survive the cryopreservation process.

5. Conclusion

The cryopreservation of sperm reduced the most important traits (NORM, LIVE, HOS, PMOT, MOT, VCL, VSL, VAP, ALH, LIN, WOB, BCF) by an average of 50 %. The adverse effect of CP addition can be reduced by using a suitable CP agent at an appropriate level. DMA has proved to be better in sustaining the most important ostrich sperm functions (NORM, LIVE, HOS, PMOT, MOT, VCL, VSL, VAP), compared to the other CP's tested (MA and DMSO). The addition of DMA did not result in a reduction after CP addition and equilibration, compared to a 10 to 15 % reduction with other CP's. Sperm function deterioration during the CP addition stage can possibly be reduced for MA at higher CP addition and equilibration temperatures that may decrease the magnitude of severe osmotic conditions. This may be achieved by the addition of the CP to the previously diluted semen at ~ 24 °C before the cooling stage. In contrast, it does not seem possible to ameliorate the effects of DMSO toxicity. Supplementation with exogenous cholesterol, which has been shown beneficial in other species, can possibly also increase membrane permeability to CPs and thereby reduce osmotic stress endured by cell. The next step would be to study more suitable freezing and thawing rates specific to DMA 16 % for improving sperm survival after the thawing stage and for further optimization of the ostrich sperm cryopreservation protocol.

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CHAPTER VII

CRYOPRESERVATION OF OSTRICH SPERM THROUGH FAST FREEZING AND ARTIFICIAL INSEMINATION OF FROZEN-THAWED SEMEN

Optimal freezing is one of the fundamentals of a successful cryopreserved semen protocol to maintain sperm functionality *in vitro* at sub-zero temperatures indefinitely. No information is available regarding the thermo-dynamics of the ostrich sperm during freezing therefore a wide range of freezing rates and strategies had to be tested to explore responses of ostrich sperm to freezing. This was done by selecting four suitable males known for their resistance towards storage processing stress and exposing them to slower and faster freezing rates obtained through a programmable freezer and in liquid nitrogen (LN) vapour, respectively. A 1:1 extension, with the ostrich specific diluent (OS1) at room temperature (~ 24 °C) was performed and cooled at a controlled rate of 1 °C/minute to 5 °C, using a programmable freezer. Following dilution and cooling, semen aliquots were diluted 1:1 in a pre-cooled dimethylacetamide (DMA) solution as cryoprotectant (CP) to a final concentration of 16% DMA. After CP addition and equilibration for 15 minutes at 5° C, 250 µl secure plastic straws were loaded, sealed and frozen. Slow freezing rates were achieved by using a programmable freezer testing 1 and 10 °C/minute to different end temperatures of -19, -22, -28, -30, -60 and -80 °C, before LN immersion at -196 °C. Faster rates were achieved by setting plates at different heights in liquid nitrogen vapour in an enclosed Styrofoam box for 5 minutes before LN immersion. Straws prepared by both methods were thawed 24 hours later in a pre-cooled water bath set at 5 °C for 12 seconds. Evaluation of sperm function after each processing stage (neat, diluted, cooled, CP added and equilibrated, freezing/thawing, without LN immersion and freezing/thawing with LN immersion). After evaluation of the different freezing rates the best method in terms of sperm function maintenance was chosen to test *in vivo* through the artificial insemination of 10 females split randomly into two groups, including a control group inseminated with fresh diluted semen and a treatment group inseminated with cryopreserved semen. Semen of six males was pooled for insemination to supply the control and treatment groups with respective insemination doses of 800×10^6 and 1000×10^6 sperm cells/mL, with equal sperm numbers from each male. Females in both groups were inseminated at the same frequency, namely on 3 consecutive days with a follow-up insemination every 6 days for 3 consecutive cycles. Fertility assessment of eggs included counting trapped sperm in the outer perivitelline layer (OPVL sperm/mm²) and determining fertilization status through germinal disc evaluation. Results indicated that freezing and thawing had an expected detrimental impact on progressive motility (PMOT), motility (MOT), curve-linear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), lateral head amplitude (ALH), linearity (LIN), straightness of swim (STR), wobble (WOB) and beat-cross frequency (BCF). Evaluation at the different end temperatures when controlled freezing was implemented through the programmable freezer, without LN immersion, suggested a decline in PMOT and MOT after -22 °C with a further steady decline from -28 to -80 °C. The freezing rate of 10 °C/minute reduced these losses in PMOT and MOT over the different end temperatures compared to 1 °C/minute. Evaluation after LN immersion at the different end temperatures indicated that 10 °C/minute was superior to 1 °C/minute with -30 °C being the most suitable end temperature to immerse in LN. Faster freezing rates in LN vapour were even better than those applied in the programmable freezer for the maintenance of PMOT and MOT. The fastest freezing rate obtained using

the lowest 3.5 cm plate height being superior in terms of PMOT and MOT maintenance after LN immersion and thawing with a reduction of only 20 to 26 % from the initial motility of sperm. Cryopreservation of semen using the latter method with additional evaluation of sperm functions revealed strong positive Pearson correlation coefficients ($r = 0.5$ to 0.7) between sperm viability (LIVE), membrane integrity (HOS), PMOT and MOT suggesting that the cryopreservation protocol was reasonably successful. Artificial insemination with fresh diluted and cryopreserved semen was implemented to compare *in vitro* with *in vivo* results of cryopreserved semen. OPVL sperm was the only fixed effect that contributed to the variation associated with fertilization status. Fertilised eggs had a mean (\pm SE) number of 191.10 ± 120.57 OPVL sperm/mm², compared to 3.09 ± 0.87 OPVL sperm/mm² for non-fertilized eggs. Artificial insemination with cryopreserved ostrich semen thus resulted in fertilised eggs, proving the principle that the protocol could be refined for commercial application.

1. Introduction

Cryopreservation, the indefinite storage of sperm cells through cryptobiosis, offers the opportunity to biobank valuable genetic resources that are threatened by the avian influenza epidemic experienced by the ostrich industry. The variable fertility rates experienced in the ostrich industry (Deeming, 1996; Cloete *et al.*, 1998; Dzoma and Motshegwa, 2009) and the poor sperm supply stemming from sexual incompatibility (Bertschinger *et al.*, 1992; Hemberger *et al.*, 2001; Malecki and Martin, 2003; Malecki *et al.*, 2008) stand to benefit from artificial insemination (AI) with cryopreserved semen. Such AI may also benefit fertilization when ostrich females are inseminated during high egg production months (July/August), when males generally produce low quality semen (Lambrechts, 2004; Bonato *et al.*, 2014b; Chapter II). The dissemination of germplasm from genetically superior males for the genetic improvement of production and reproduction traits of distant populations may also be facilitated by AI with cryopreserved semen. Studies on genotype x environmental interactions will also become feasible with a protocol allowing for reliable AI with cryopreserved semen.

Successful cryopreservation depends on interrelated factors that lay the foundation of semen storage (short and long) together with specific factors only applicable to cryopreservation. The fundamental prerequisites for the ostrich in terms of reliable stress-free semen collection methods (Rybnik *et al.*, 2007), optimized semen collection frequencies, timing and output (Bonato *et al.*, 2011, 2014b) and the duration of female sperm storage (Malecki *et al.*, 2004) have largely been met. More recently, the foundation of sperm function traits (Chapter II), the development of an ostrich-specific semen extender (Chapter III) with accompanying semen processing protocols in terms of optimal dilution rates and temperatures (Chapter IV), a suitable slow cooling regime after

collection to 5 °C (Chapter V) as well as the suitability of the cryoprotectant DMA at 16 % for protecting cells during cryopreservation (Chapter VI) also established the basis for developing a long term storage protocol. However, specific guidelines for ostrich semen cryopreservation are lacking in the literature. It is known that a number of factors affect sperm cryosurvival. Information on freezing and thawing rate is still needed to complete a viable cryopreservation protocol and to maximise the maintenance of sperm function after cryopreservation. The cooling/freezing method, cooling/freezing rate as well as the temperature zone where the latter is applied and the temperature where cooling/freezing is terminated are important factors to consider in a protocol (Farrant *et al.*, 1977; Sexton, 1980; Morris *et al.*, 1999). Different methods of obtaining suitable freezing rates have been suggested, among others the cooling and freezing of semen at different rates, 1 to 10 °C per minute (°C/min), from 5 °C to -80 °C in a programmable freezer after which the specimen is plunged into liquid nitrogen (LN) or by manually placing the sample for 5 to 30 minutes at a certain height in LN vapour prior to LN submersion (Schuster *et al.*, 2003).

The bulk of function loss associated with cryopreservation rests on the sperm cell's biological complexity with species-specific properties that can either make them more sensitive or resistant towards cryopreservation (Ansah and Buckland, 1982; Parks and Lynch, 1992; Gao *et al.*, 1993; White, 1993; Blanco *et al.*, 2000; Blesbois *et al.*, 2005). The cell becomes dehydrated and shrinks during freezing. In contrast, volume expansion and swelling of the cell is induced upon thawing, when the water re-equilibrates. These volumetric changes are governed by the law of osmosis and the survival of the cell depends on the magnitude of these changes and the capacity of the cell to withstand these changes (Gao *et al.*, 1993; Blanco *et al.*, 2000). The permeability of the CPs and water that could result in osmotic stress of the cell is temperature dependent and stresses the importance of optimal freezing rates (Martorana *et al.*, 2014). The optimal equilibrium freezing rate for a given cell must result in a tolerable volume and osmotic excursions which do not lead to cell injury, specifically the plasma membrane that is most prone, and impairs cell function (Parks and Lynch, 1992; Watson, 2000; Gee *et al.*, 2004; Sieme *et al.*, 2008; Partyka *et al.*, 2012). Freezing rate should be slow enough to allow the cells to minimize chemical potential and osmolarity gradients across the plasma membrane and to dehydrate, without being exposed to severe or prolonged hypertonic conditions (Mazur *et al.*, 1972; Sieme *et al.*, 2008). Too slow or too fast freezing rates can result in cryo-injury. Rapid freezing leads to intracellular ice formation that may osmotically or mechanically damage the cell. The latter is due to the high water content within cells that

cannot transfuse out of the cell in sufficient quantities because of suppressed hydraulic permeability caused by the high freezing rates (Mazur *et al.*, 1972; Mazur, 1984; Morris *et al.*, 2012). At very slow freezing rates, which results in external ice crystallisation, the cell may become severely dehydrated over a long period of time that prolongs the exposure of cells to these extreme volumetric and osmotic changes (Lovelock, 1953; Mazur *et al.*, 1972; Morris *et al.*, 2012). Cellular damage can be minimized by controlling the freezing rate since ice crystal formation is a function of freezing rate and freezing rate a function of membrane permeability (Mazur, 1984; Amann and Pickett, 1987; Hammerstedt *et al.*, 1990; Parks and Lynch, 1992; Devireddy *et al.*, 2000). An inverted “U-shape” curve has been proposed for cell survival plotted as a function of freezing rate (Mazur *et al.*, 1972). The temperature range and end points at which freezing are applied has been shown to be species-specific in terms of *in vitro* and *in vivo* success (Sexton, 1978, 1980; McGann, 1979; Seigneurin and Blesbois, 1995). It has been reported that each cell type is specific to the freezing rate and temperature point for maximal recovery upon thaw after cryopreservation (Leibo *et al.*, 1978). The comparison between species-specific cryopreservation protocols for freezing and thawing is thus of little benefit since slow or fast rates may not correspond to needs of another species in the absence of direct comparisons between cell structure properties and processing principles.

Although prediction of freezing rates for certain cell types has been proposed, the complex sperm cell structure only partially complies with the laws of thermo-dynamics and makes mathematical prediction of optimal freezing and thawing rates complicated, sometimes resulting in less desirable outcomes (Curry, 1994; Gao *et al.*, 1997; Morris *et al.*, 1999; Sieme *et al.*, 2008). Mathematical predictions rely on cell size, osmotic behaviour and tolerable excursions, fractional osmotically inactive cell volume, water permeability of the plasma membrane, permeability coefficients of relevant solubles, activation energy and initial soluble concentrations (Sieme *et al.*, 2008). These cell properties, which are unavailable for the ostrich, differed between supra- and sub-zero temperatures mostly because of membrane changes that can occur during temperature shifts associated with cooling, freezing and thawing, as well as through the complexity of cryoprotectant action (Devireddy *et al.*, 1999, 2000, 2002, 2004; Petrunkina, 2007).

The aim of this study was to establish a viable cryopreservation protocol for ostrich semen. This was achieved by applying a wide range of freezing rates to cryopreserve semen to an ametabolic state in liquid nitrogen at -196 °C, for indefinite storage. The

evaluation of sperm motility after the different processing stages involved quantifying cellular response. The *in vitro* sperm function results allowed the implementation of the first *in vivo* fertility assessments of cryopreserved ostrich semen through perivitelline techniques (Malecki and Martin, 2003; Malecki *et al.*, 2005, 2008; Brand *et al.*, 2014). The latter would assist in optimizing the current ostrich long-term storage protocol developed in Chapter VI and to further study thermo-sensitivity of ostrich sperm.

2. Material and methods

2.1. Animal population

Animals in the resource population were screened from the commercial ostrich breeding flock maintained at the Oudtshoorn Research Farm on the basis of behavioural attributes rendering them suitable for AI (referred as desirable behaviour as described by Bonato *et al.* (2011), Bonato and Cloete (2013) and Bonato *et al.* (2014a). The origin of the ostrich flock and the general management procedures implemented therein has been described previously (Van Schalkwyk *et al.*, 1996, 2000; Bunter and Cloete, 2004; Cloete *et al.*, 2008). Males for both trials were chosen for their reliability in terms of ejaculate quality, as well as their willingness to cooperate during semen collection using the dummy female method (Rybnik *et al.*, 2007). Females displaying the voluntarily crouch behaviour were used for artificial insemination purposes (Malecki *et al.*, 2004, 2008). Although artificial insemination procedures for ostriches have not been optimized, a reliable method has been suggested by Malecki *et al.* (2008). Females were inseminated with a rigid straw (~30 cm) that can enter the cloaca by the guidance of the operator's fingers of one hand alongside the female phallus into the vaginal orifice to reach the vagina. These docile females housed in single pens produced eggs in the absence of males with a slightly lower egg production, compared to contemporary females paired off with males (Bonato and Cloete, 2013).

Experiment 1, the effect of controlled freezing rates on *in vitro* sperm motility traits was studied. Ejaculates were collected from four *South African Black* males aged between 3 and 7 years during peak season in terms of ejaculate quality (December 2013 and January 2014). Ejaculates were allocated to test the effects of controlled freezing rates of 1 and 10 °C/minute respectively, at different end temperatures (-19, -22, -28, -30, -60, -80 °C), followed by immersion in liquid nitrogen at these different end temperatures on sperm motility traits.

Experiment 2, the effect of non-controlled fast freezing rates was studied on *in vitro* sperm motility traits. Ejaculates were collected from the same four *South African Black* males aged between 3 and 7 years as used in Experiment 1 during the peak season (December 2013 and January 2014). Ejaculates were allocated to test the effects of faster freezing rates through different plate heights (3.5, 7, 10 cm) in liquid nitrogen vapour and immersion in liquid nitrogen at these different plate heights on sperm motility traits.

Experiment 3, ejaculates were collected from six *South African Black* males aged between 3 and 7 years at different intervals and used to artificially inseminate a group of 10 *South African Black females* aged between 2 and 10 years. The two trials commenced during the months of September to October and November to December 2014 over a period of 54 days (27 days for each trial). These periods have been characterized as peak egg production months in pairs (Fair *et al.*, 2011) and for single-penned females (Bonato *et al.*, 2014a). The group of 10 females was split into two groups of five each and randomly allocated to an experiment testing insemination with cryopreserved semen (treatment group) and fresh diluted semen (control group) on *in vivo* fertility by using the perivitelline egg membrane techniques (Malecki *et al.*, 2004).

2.2. Semen preparation

Sperm concentration was obtained in both experiments using a spectrophotometer (Spectrawave, WPA, S800, Biochrom) with transmittance values that has been previously calibrated against actual ostrich sperm cell counts, using a haemocytometer. Aliquots of 20 µl semen diluted 1:400 (v/v) with phosphate buffered saline solution containing 10 % formalin were used for the latter.

Experiment 1: The effect of controlled freezing rates on *in vitro* sperm motility function.

An initial 1:1 extension, with the ostrich specific diluent (OS1) at room temperature (~ 24 °C), was performed immediately after collection. The diluted sample was split into equal aliquots and cooled in a programmable freezer (Asymptote, Grant, EF600) at a rate of 1°C/minute to a final temperature of 5 °C. After cooling samples were transferred to an incubator (BL°CKICE Cooling Block, Techne), set at 5 °C. The aliquots were then gently mixed and diluted with the pre-cooled (5 °C) cryoprotectant (CP) stock solution of 32 % Dimethyl acetamide (DMA) at a rate of 1:1 to obtain a final concentration of 16 %. The

CP stock solution of 32 % DMA was freshly prepared by thoroughly mixing 1.6 ml of crude DMA with 3.4 ml OS1 at room temperature in a glass tube with a final stock volume of 5 ml. After CP addition and equilibration for 15 minutes at 5° C, the straws were loaded, sealed and frozen. After CP addition and equilibration for 15 minutes at 5° C the 250 µl secure plastic straws were loaded, sealed and frozen at a controlled rate of 1 °C/minute and 10 °C/minute (the fastest rate that was achievable in the programmable freezer) from 5 °C to different end temperatures of -19, -22, -28, -30, -60 and -80 °C whereafter one straw was evaluated and the other immersed in liquid nitrogen (-196 °C). Thereafter, the straws were removed from the programmable freezer and plunged into liquid nitrogen at -196 °C. The straws were thawed 24 hours later in a water bath at 5 °C for 12 seconds. Straws were chosen because they are the standard packaging used in cryobanking (Blesbois. 2011). Evaluation of sperm function was done for the neat, diluted, cooled, after CP addition and equilibration, different end points (-19, -22, 28, -30, -60 and -80 °C) after freezing without being LN immersed and after thawing at the different end temperatures (-19, -22, 28, -30, -60 and -80 °C), after freezing and LN immersion.

Experiment 2: The effect of non-controlled fast freezing rates on *in vitro* sperm motility function.

Semen samples were cooled to a final temperature of 5 °C, aliquoted, mixed with DMA and sealed in straws as described for Experiment 1. Faster freezing rates were achieved by freezing the straws for five minutes in liquid nitrogen vapour on plates set at 3.5 cm, 7 cm or 10 cm above the level of liquid nitrogen in an enclosed Styrofoam box. The straws were then plunged into liquid nitrogen. The samples were thawed 24 hours later as described for Experiment 1. Evaluation of sperm function was done for neat, diluted and cooled semen, again after CP addition and equilibration, after freezing at different plate heights (3.5, 7.0, 10.0 cm), without being immersed in the LN and after thawing at the different plate heights (3.5, 7.0, 10.0 cm) after freezing and LN immersion.

Experiment 3: The effect of cryopreserved semen on *in vivo* fertility through perivitelline techniques.

An initial 1:1 extension of collected semen with the ostrich specific diluent (OS1) at room temperature (~ 24 °C) was performed immediately after collection. These were cooled to a final temperature of 5 °C, aliquoted, mixed with DMA and sealed in straws as

described for Experiment 1. The straws were frozen by placing the straws on the most suitable plate height (3.5 cm) from Experiment 2, for 5 minutes in liquid nitrogen vapour directly above the liquid nitrogen in an enclosed Styrofoam box. The straws were then plunged into liquid nitrogen (-196 °C). The samples were thawed 24 hours later in a pre-cooled water bath set at 5 °C for 12 seconds. Thawed sperm was evaluated for motility, viability and membrane integrity. The semen of six males was pooled for insemination to supply the control and treatment female groups with respective insemination doses of 800×10^6 sperm cells/mL and 1000×10^6 sperm cells/mL with equal sperm numbers from each male. Females in both groups were inseminated at the same frequency including 3 consecutive days of insemination with a follow-up insemination every 6 days for 3 consecutive cycles.

2.3. *In vitro* sperm function evaluation

2.3.1. *Motility evaluation*

All sperm motility traits were evaluated by means of the Sperm Class Analyzer® (SCA) version 5.3 (Microptic S.L., Barcelona, Spain) at different processing stages. Recordings were made after re-suspension of neat sperm, as well as treated sperm in a standard motility buffer using Sodium Chloride (150 mM) and TES (20 mM) with 2% male specific seminal plasma to a final sperm concentration of 20×10^6 sperm cells/ml. After re-suspension a sample was placed in a 38 °C water bath for 1 minute. For sperm motility recording 2 µl of diluted semen was placed onto a pre-warmed microscope slide, covered gently with a cover glass (22 x 22 mm) and allowed to settle for 20 seconds prior to recording. Seven to nine different fields were captured randomly to eliminate bias towards higher sperm concentrations or motility until at least 500 motile sperm was recorded. Sperm motility traits included motility (MOT, %), progressive motility (PMOT, %), curve-linear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), amplitude of lateral head displacement (ALH, µm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat-cross frequency (BCF, Hz).

2.3.2. *Viability evaluation*

Sperm viability expressed as live sperm (LIVE, %) was measured using the LIVE/DEAD® Sperm Viability Kit (Life technologies) that contained the SYBR® 14 and Propidium Iodide (PI) fluorescent stains. All sperm viability recordings were made after re-suspension of sperm in the standard Ostrich diluent at pH 7 to a final sperm concentration of 20×10^6

sperm sperm cells/mL. The SYBR® 14 working solution was prepared in a HEPES/NaCl medium to a 1:49 concentration (v/v) SYBR® 14 to HEPES/NaCl solution. Sperm suspension aliquots of 250 µl were re-suspended with 1.5 µl membrane-permeant SYBR® 14 working solution and incubated for 10 minutes, at a temperature controlled environment of 38 °C. After incubation 2 µl of the next fluorescent stain, Propidium Iodide (PI), was added and incubated for another 10 minutes whereafter cells were evaluated. For evaluation of viable (green) and non-viable (red/or green with red) sperm a 2 µl droplet was placed on a glass slide, covered with a cover glass (22 x 22 mm) and allowed to settle for 20 seconds prior to recording. The fluorescent sperm was observed and photographed under 10x microscopy with an Olympus BX41 epifluorescent microscope (Olympus Optical Co., Tokyo, Japan), equipped with a filter, camera (ColorView IIIu Soft Imaging System) and software package (analysis FIVE, Olympus Soft Imaging Solutions GmbH, Münster) to count viable and non-viable sperm. Nine to ten different fields were randomly captured until at least 500 sperm were evaluated. Distorted fields as well as fields that included drift or debris or clumps of sperm were excluded. The SYBR® 14 nucleic acid stain labels live sperm with green fluorescence, and membrane-impermeable PI labels the nucleic acids of membrane-compromised sperm, with red fluorescence.

2.3.3. Membrane integrity evaluation

Sperm membrane integrity expressed as resistance to hypo-osmotic swelling (HOS, %) was measured using the Hypo-osmotic swelling test (Jeyendran *et al.*, 1984), adapted specifically for the ostrich by means of preliminary experimental exploration. All sperm membrane integrity recordings were made after re-suspension of sperm in a standard salt (NaCl/H₂O) solution, adapted to 25 mOsm to a final sperm concentration of 20 x 10⁶ sperm sperm cells/mL. For HOS recording 2 µl of diluted semen was placed onto a pre-warmed slide, using a heated stage set at 38 °C, covered gently with a cover glass (22 x 22 mm) and allowed to settle for 20 seconds prior to recording. Sperm was captured using the Sperm Class Analyzer® (SCA) version 5.3 (Microptic S.L., Barcelona, Spain) with a Basler A312fc digital camera (Basler AG, Ahrensburg, Germany), mounted on an Olympus BX41 microscope (Olympus Optical Co., Tokyo, Japan) equipped with phase contrast optics. Seven to nine different fields were captured randomly until good representation (500 sperm) were reached and to eliminate biasness towards a higher sperm concentration. Distorted fields as well as fields that included drift or debris or clumps of sperm were excluded.

2.4. In vivo assessment of fertility

2.4.1. Egg handling

Eggs were collected in the mornings and processed according to a standard procedure that included disinfecting and weighing. Details on the methods of egg collection, sanitation and storage on the research farm have been previously documented (Brand *et al.*, 2007, 2008). Eggs were stored for 48 hours at a temperature of 17 °C and a relative humidity (RH) of 75 % prior to opening for quantitative fertility assessment using perivitelline techniques.

2.4.2. Egg break-out and germinal disc evaluation for fertilisation status

Egg break-out and evaluation of the germinal disc (GD) is an objective method for the evaluation of true egg fertility, since 17 to 20 % of discarded ostrich eggs at mid-incubation candling is related to early embryonic mortality and not infertility (Kosin, 1944, 1945; Romanoff and Romanoff, 1949; Wilson, 1991; Brown *et al.*, 1996; Bakst, 1998; Malecki and Martin, 2003; Brand *et al.*, 2007, 2014; Malecki *et al.*, 2008). The establishment of early fertility by means of alternative methods like candling is subjective since the shadowed area of the embryo is still poorly defined before 7 days of age (Deeming, 1995; Brand *et al.*, 2014). Egg fertilization status was observed by means of egg break-out and evaluation of the appearance of the GD, where the absence of the blastoderm would indicate infertility and the presence of the blastoderm fertility (Malecki *et al.*, 2008). The opening of the eggs was done by carefully breaking the eggshell in the region of the air cell and removing excess albumin and membranes. The opened egg was then placed on a pedestal with an extra lighting source (Olympus LG-PS2, Olympus Optical Co., Ltd, Tokyo, Japan) under the stereo microscope (Olympus SZ-61, Olympus Optical Co., Ltd, Tokyo, Japan) to obtain a clear digital image by means of a mounted colour view camera (ColorView IIIU, Soft Imaging System) and image analysis software (analysis FIVE, Olympus Optical Co., Ltd, Tokyo, Japan). The GD area was classified according to the description given by Malecki *et al.* (2008) and Brand *et al.* (2014) for the ostrich.

2.4.3. Quantifying of sperm trapped in the outer perivitelline layer

The counting of trapped sperm in the outer perivitelline layer (OPVL) near the ovum surface is a good predictor of fertility and the probability of fertilisation, indication of sperm storage tubule (SST) storage, transport, depletion, the functionality of the processed sperm and thus serves as an indication to adjust sperm processing methods and

insemination practices (Wishart, 1997; Wishart and Staines, 1999; Malecki and Martin, 2003). A 2 x 2 cm piece of perivitelline layer overlying the GD area was collected and cleaned from excess yolk with PBS, mounted on a glass slide and stained with 1 µg/mL Diamidino-2-phenylindole (DAPI)/PBS working solution whereafter it was left to dry at room temperature protected from light (Wishart, 1987). The sperm nuclei were visualised with a fluorescence microscope (Olympus BX40, Olympus Optical Co., Ltd, Tokyo, Japan) using a “U” filter cube with 372 nm excitation and 456 nm emission wavelengths. The number of sperm in the OPVL within the GD area was estimated by counting in a frame placed in the centre and moving it 5 frames, away from the centre.

2.5. Statistical analyses

Sperm traits as percentages, and with skewed distribution (as determined by the Shapiro-Wilk test: $P < 0.05$) were transformed using the arc sine of the percentage mean square-root ($\text{degree.arcsin } \sqrt{\%}$), while the sperm concentrations were transformed to natural logarithms. Analyses included a distribution analysis and summary statistics to obtain variance parameters and graphs, describing the sperm traits. The total number of records, means, standard deviation, minimum, maximum and coefficients of variation (CV) was determined for each sperm trait. The contribution of each fixed effect (FE) to a specific sperm trait was evaluated by expressing the sum of squares for such an effect as a percentage of the total corrected sum of squares (TCSS) (Leighton *et al.*, 1982; Smith, 2010). Least squares means, standard errors (S.E.) and CV's were calculated and subjected to Tukey's multiple range tests to investigate differences between least squares means. Correlations (Pearson) and regressions (linear and non-linear) were used to determine significant ($P < 0.05$) relationships between traits.

Separate general linear mixed models (GLMM) were performed during Experiment 1 for sperm traits motility (PMOT, MOT, VCL, VSL, VAP, ALH, LIN, STR, WOB and BCF) which included the fixed effects of semen processing stage (S, neat, diluted, cooled, after CP addition and equilibration, freezing without LN immersion at different end temperatures and freezing and thawing after different end temperatures with LN immersion), freezing rate (R), sperm concentration (C, as a linear covariate) and male (M) as a random effect. GLMM performed in Experiment 2 for sperm function traits namely motility (PMOT, MOT, VCL, VSL, VAP, ALH, LIN, STR, WOB and BCF) which included the fixed effects of semen processing stage (P, neat, diluted, cooled, CP addition and equilibration, after

freezing at different plate heights without LN immersion and freezing and thawing after LN immersion at different plate heights), and plate height (H, 3.5 cm, 7cm and 10cm), sperm concentration (C, as a linear covariate) and male (M) as a random effect.

GLMM performed in Experiment 3 for *in vivo* fertility assessment traits included fertilization status and number of sperm trapped in the OPVL with the fixed effects of semen treatment (fresh diluted and cryopreserved), female age (A, as a linear covariate), number of OPVL sperm (O, as a linear covariate) and the number of days after last AI (N, as a linear covariate) where appropriate and female (F) as a random effect to account for the repeated sampling of the same females. GLMM were applied to assess the specific effect of females on fertility assessment traits and its interaction with other fixed effects. All analyses were done using the Statistical Analysis System (SAS, version 9.3).

Example of the GLMM fitted with Y being the dependent sperm traits of motility in Experiment 1:

$$Y_{ijk} = \mu + M_i + S_j + R_k + b_0(C)_{ijk} + e_{ijk}$$

Where: Y_{ijk} = Sperm trait under assessment

μ = population mean

M_i = random effect of the i^{th} male ($i = 1, 2, 3, 4$)

S_j = fixed effect of the j^{th} processing stage ($j = \text{neat, diluted, cooled, CP addition and equilibration, freezing without LN immersion at different end temperatures, freezing and thawing after LN immersion at different end temperatures}$)

R_k = fixed effect of the k^{th} freezing rate ($k = 1 \text{ and } 10 \text{ }^\circ\text{C/minute}$)

C_{ijk} = sperm concentration fitted as a linear covariate

b_0 = regression coefficients of Y_{ijk} on sperm concentration (C)

e_{ijk} = random error

Example of the GLMM fitted with Y being the dependent sperm traits of motility in Experiment 2:

$$Y_{ijk} = \mu + M_i + P_j + H_k + b_0 (C)_{ijk} + e_{ijk}$$

Where: Y_{ijk} = Sperm trait under assessment

μ = population mean

M_i = random effect of the i^{th} male ($i = 1, 2, 3, 4$)

P_j = fixed effect of the j^{th} processing stage ($j = \text{neat, diluted, cooled, CP addition and equilibration, after freezing without LN immersion and freezing and thawing after LN immersion}$)

H_k = fixed effect of the k^{th} plate height ($k = 3.5, 7, 10 \text{ cm}$)

C_{ijk} = sperm concentration fitted as a linear covariate

b_0 = regression coefficients of Y_{ijk} on sperm concentration (C)

e_{ijk} = random error

Example of the GLMM fitted with Y being the *in vivo* fertility assessment traits that include fertilization status and number of OPVL sperm in Experiment 3:

$$Y_{ij} = \mu + F_i + T_j + b_1 (A)_{ij} + b_2 (N)_{ij} + b_3 (O)_{ij} + e_{ij}$$

Where: Y_{ij} = Fertilization status

μ = population mean

F_i = random effect of the i^{th} female ($i = 1, 2, 3, 4, \dots, 10$)

T_j = fixed effect of the j^{th} treatment ($j = \text{fresh diluted, cryopreserved}$)

A_{ij} = female age fitted as a linear covariate

b_1 = regression coefficients of Y_{ij} on female age (A)

N_{ij} = number of days after last insemination as a linear covariate

b_2 = regression coefficients of Y_{ij} on number of days after last insemination (N)

O_{ij} = number of OPVL sperm/mm² as a linear covariate

b_3 = regression coefficients of Y_{ij} on number of OPVL sperm/mm² (O)

e_{ij} = random error

4. Results

Experiment 1: The effect of controlled freezing rates in a programmable freezer on sperm function in vitro

3.1. Descriptive statistics

Across analyses the mean (\pm SE) sperm concentration was $3.23 \times 10^9 \pm 3.67 \times 10^7$ sperm cells / mL, with a CV of 21.11 % over 345 records. The summary statistics including the number of records, mean, standard deviation, minimum, maximum and coefficient of variation for the different sperm function traits are set out in Table 19.

Table 38: Description of sperm traits that included progressive motility (PMOT, %), motility (MOT, %), curve-linear velocity (VCL, μ m/s), straight-line velocity (VSL, μ m/s), average path velocity (VAP, μ m/s), amplitude of lateral head displacement (ALH, μ m), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat-cross frequency (BCF, Hz).

Sperm trait	Records	Mean	S.D	Minimum	Maximum	CV (%)
Progressive motile (%)	343	40.66	23.97	0.00	84.00	58.95
Motile (%)	343	56.71	29.35	0.00	96.40	51.76
Curve-linear velocity (μ m/s)	343	71.24	17.72	0.00	104.70	24.88
Straight-line velocity (μ m/s)	343	49.46	14.28	0.00	91.30	28.87
Average path velocity (μ m/s)	343	61.37	17.04	0.00	95.50	27.76
Amplitude of lateral head displacement (μ m)	343	2.18	0.57	0.00	3.50	26.31
Linearity (%)	343	67.78	13.10	0.00	91.20	19.32
Straight (%)	343	79.50	13.58	0.00	97.70	17.08
Wobble (%)	343	84.09	11.26	0.00	94.70	13.39
Beat-cross frequency (Hz)	343	8.87	2.27	0.00	13.30	25.58

S.D: Standard deviation; CV: Coefficient of variation

The contribution of each FE with its degrees of freedom (DF), R-square value and CV is shown in

Table 21 for motility (PMOT and MOT) and kinematic sperm traits (VCL, VSL, VCL, VAP, ALH, LIN, STR, WOB and BCF). The least squares means for the different sperm traits are set out in Table 23.

Table 39: The source of variation for the fixed effect of freezing rate, processing stage and the interaction of freezing rate with processing stage with their variation contribution (FE, %) with its degrees of freedom (DF) on sperm traits, progressive motility (PMOT, %), motility (MOT, %), curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat-cross frequency (BCF, Hz).

Variation source	DF	PMOT	MOT	VCL	VSL	VAP	ALH	LIN	STR	WOB	BCF
Freezing Rate	1	1.56***	1.55***	0.90	0.49	1.05*	0.95	0.02	0.05	0.42	0.45
Processing Stage	15	71.18***	76.65***	32.52***	28.67***	33.26***	21.66***	13.25***	8.61**	17.73***	21.57***
FR x PS	15	4.34***	5.66***	4.24	2.10	3.92	3.53	0.97	1.82	3.07	4.44
TCSS	343	91738.68	133790.34	107414.42	69736.79	99277.66	112.87	30835.46	37928.11	27054.51	1758.66
Error mean square	312	16474.83	16516.68	61466.40	44854.98	56436.71	81.10	25583.44	33438.31	20063.57	1199.37
R ²		0.82	0.88	0.43	0.36	0.43	0.28	0.17	0.12	0.26	0.32
CV (%)		19.04	14.84	19.73	24.28	21.95	23.39	16.37	16.29	12.02	22.15

FR: Freezing rate; PS: Processing stage; CV: Coefficient of variation; TCSS: Total corrected sum of squares; *P < 0.05; **P < 0.01; ***P < 0.001

Table 40: The least square means (\pm S.E) of freezing rate, processing stage and the interaction of freezing rate with processing stage on the progressive motility (PMOT, %), motility (MOT, %), curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat-cross frequency (BCF, Hz).

Variation source	PMOT	MOT	VCL	VSL	VAP	ALH	LIN	STR	WOB	BCF
Freezing Rate	***	***	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1 °C per minute	32.38 \pm 2.71 ^a	45.53 \pm 2.53 ^a	67.73 \pm 2.95	48.04 \pm 2.81	57.38 \pm 2.60	2.15 \pm 0.11	68.72 \pm 2.33	82.04 \pm 2.84	82.21 \pm 1.14	8.77 \pm 0.39
10 °C per minute	40.21 \pm 2.74 ^b	56.73 \pm 2.56 ^b	71.50 \pm 3.01	49.67 \pm 2.85	61.64 \pm 2.67	2.27 \pm 0.11	68.55 \pm 2.40	80.22 \pm 2.90	84.90 \pm 1.25	9.05 \pm 0.40
Processing Stage	***	***	***	***	***	***	***	**	***	***
Neat	55.02 \pm 3.01 ^a	84.97 \pm 2.84 ^a	68.01 \pm 3.50 ^a	45.68 \pm 3.23 ^a	56.92 \pm 3.18 ^a	2.27 \pm 0.13 ^a	66.65 \pm 2.91 ^a	80.16 \pm 3.37 ^a	83.30 \pm 1.86 ^a	9.67 \pm 0.47 ^a
Diluted (1:1)	68.81 \pm 3.01 ^b	86.16 \pm 2.84 ^a	85.21 \pm 3.50 ^b	60.51 \pm 3.23 ^b	75.34 \pm 3.18 ^b	2.39 \pm 0.13 ^a	71.35 \pm 2.91 ^a	80.94 \pm 3.37 ^a	88.38 \pm 1.86 ^b	8.82 \pm 0.47 ^a
Cooled	64.35 \pm 3.01 ^{cb}	83.33 \pm 2.84 ^a	82.91 \pm 3.50 ^b	59.56 \pm 3.23 ^b	73.36 \pm 3.18 ^b	2.34 \pm 0.13 ^a	72.17 \pm 2.91 ^a	81.81 \pm 3.37 ^a	88.36 \pm 1.86 ^b	8.74 \pm 0.47 ^a
CP addition	61.84 \pm 3.02 ^{dc}	82.79 \pm 2.8 ^a	80.61 \pm 3.52 ^b	56.79 \pm 3.24 ^b	70.34 \pm 3.19 ^b	2.44 \pm 0.13 ^a	70.45 \pm 2.91 ^a	80.89 \pm 3.38 ^a	87.31 \pm 1.88 ^b	9.29 \pm 0.48 ^a
-19 °C	58.29 \pm 3.16 ^{ed}	78.97 \pm 3.00 ^a	74.75 \pm 3.77 ^b	51.89 \pm 3.44 ^b	64.03 \pm 3.45 ^b	2.36 \pm 0.14 ^a	69.87 \pm 3.18 ^a	81.98 \pm 3.62 ^a	85.41 \pm 2.15 ^b	9.72 \pm 0.51 ^a
-22 °C	50.37 \pm 6.22 ^{fe}	68.74 \pm 6.08 ^a	73.59 \pm 8.58 ^b	50.77 \pm 7.36 ^b	61.41 \pm 8.22 ^b	2.45 \pm 0.31 ^a	68.88 \pm 7.79 ^a	82.58 \pm 8.25 ^a	83.25 \pm 6.32 ^b	9.26 \pm 1.20 ^a
-28 °C	35.74 \pm 5.30 ^g	52.17 \pm 5.16 ^b	72.95 \pm 7.19 ^b	53.99 \pm 6.21 ^b	62.78 \pm 6.86 ^b	2.53 \pm 0.26 ^a	74.58 \pm 6.49 ^a	86.59 \pm 6.92 ^a	86.55 \pm 5.16 ^b	9.36 \pm 1.01 ^a
-30 °C	34.34 \pm 3.05 ^{hg}	48.90 \pm 2.89 ^b	71.30 \pm 3.59 ^{cb}	50.40 \pm 3.30 ^b	61.48 \pm 3.27 ^{cb}	2.19 \pm 0.13 ^{ab}	68.87 \pm 3.00 ^a	80.72 \pm 3.45 ^a	84.15 \pm 1.96 ^b	9.29 \pm 0.49 ^a
-60 °C	20.19 \pm 3.17 ⁱ	31.30 \pm 3.01 ^c	70.07 \pm 3.79 ^c	49.25 \pm 3.45 ^b	60.29 \pm 3.48 ^c	2.27 \pm 0.14 ^b	70.24 \pm 3.20 ^a	82.43 \pm 3.65 ^a	85.52 \pm 2.18 ^b	9.46 \pm 0.52 ^a
-80 °C	19.25 \pm 3.16 ^j	29.45 \pm 3.00 ^c	65.53 \pm 3.77 ^c	47.15 \pm 3.43 ^b	55.98 \pm 3.45 ^c	2.00 \pm 0.14 ^b	71.33 \pm 3.18 ^a	84.50 \pm 3.62 ^a	84.36 \pm 2.15 ^b	9.54 \pm 0.51 ^a
THAW -19 °C	5.69 \pm 3.22 ^j	9.11 \pm 3.06 ^d	42.35 \pm 3.87 ^d	28.37 \pm 3.52 ^c	34.23 \pm 3.56 ^d	1.30 \pm 0.14 ^c	52.94 \pm 3.29 ^b	66.92 \pm 3.73 ^b	67.85 \pm 2.26 ^c	5.11 \pm 0.53 ^b
THAW -22 °C	19.69 \pm 6.22 ^j	29.16 \pm 6.08 ^c	58.96 \pm 8.58 ^c	40.59 \pm 7.36 ^b	48.59 \pm 8.22 ^c	1.95 \pm 0.31 ^b	67.13 \pm 7.79 ^a	83.76 \pm 8.25 ^a	79.98 \pm 6.32 ^b	8.26 \pm 1.20 ^a
THAW -28 °C	21.51 \pm 5.30 ⁱ	33.24 \pm 5.16 ^c	68.02 \pm 7.19 ^c	49.56 \pm 6.21 ^b	58.28 \pm 6.86 ^c	2.50 \pm 0.26 ^b	73.38 \pm 6.49 ^a	86.22 \pm 6.92 ^a	85.78 \pm 5.16 ^b	9.06 \pm 1.01 ^a
THAW -30 °C	28.54 \pm 3.05 ^j	41.19 \pm 2.89 ^b	70.57 \pm 3.79 ^c	50.40 \pm 3.29 ^b	60.05 \pm 3.27 ^c	2.22 \pm 0.13 ^b	65.36 \pm 3.00 ^a	77.64 \pm 3.45 ^a	83.31 \pm 1.96 ^b	8.74 \pm 0.49 ^a
THAW -60 °C	18.90 \pm 3.17 ⁱ	30.37 \pm 3.01 ^c	67.22 \pm 3.79 ^c	46.43 \pm 3.45 ^b	56.90 \pm 3.47 ^c	2.18 \pm 0.14 ^b	68.89 \pm 3.20 ^a	81.98 \pm 3.65 ^a	84.34 \pm 2.18 ^b	9.66 \pm 0.52 ^a
THAW -80 °C	18.25 \pm 3.16 ⁱ	28.23 \pm 3.00 ^c	61.81 \pm 3.77 ^c	43.45 \pm 3.43 ^b	52.16 \pm 3.45 ^c	1.93 \pm 0.14 ^b	66.07 \pm 3.18 ^a	78.96 \pm 3.62 ^a	79.02 \pm 2.15 ^b	8.55 \pm 0.51 ^a
FR x PS	***	***	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05

FR: Freezing rate; PS: Processing stage; *P < 0.05; **P < 0.01; ***P < 0.001

3.2. The effect of controlled freezing rates on sperm traits.

Freezing rate contributed to the variation associated with PMOT (FE = 1.56 %; $P < 0.0001$) and MOT (FE = 1.55 %; $P < 0.0001$). Kinematic traits were not influenced ($P > 0.05$) by freezing rate. PMOT and MOT were significantly compromised by a slower rate of 1 °C/min, compared to the faster rate of 10 °C/min. A 7 to 11 % reduction was recorded for these two traits when the freezing rate was reduced to 1 °C/min. Weak positive Pearson correlation coefficients confirmed the relationships of PMOT ($r = 0.17$; $P = 0.0028$), and MOT ($r = 0.16$; $P = 0.0028$) with freezing rate. Linear regressions ($Y = \alpha + \beta_1 X$; $P < 0.05$) depicting these relationships amounted to $Y = 34.38 + 0.594 X$; $P = 0.0028$; $R^2 = 0.03$ for PMOT to $Y = 44.48 + 0.717 X$; $P = 0.0028$; $R^2 = 0.03$ for MOT. Accordingly, an increase of 0.6 and 0.7 % for PMOT and MOT respectively, can be expected with every 1 °C/min increase in freezing rate.

3.3. The effect of processing stage on sperm traits.

Processing stage contributed to the variation associated with all sperm traits measured PMOT (FE = 71.18 %; $P < 0.0001$), MOT (FE = 76.65 %; $P = 0.0011$), VCL (FE = 32.52 %; $P < 0.0001$), VSL (FE = 28.67 %; $P < 0.0001$), VAP (FE = 33.26 %; $P < 0.0001$), ALH (FE = 21.66 %; $P < 0.0001$) LIN (FE = 13.25 %; $P < 0.0001$), STR (FE = 8.61 %; $P = 0.0069$), WOB (FE = 17.73 %; $P < 0.0001$) and BCF (FE = 21.57 %; $P < 0.0001$). Dilution of semen maintained sperm function (PMOT, VCL, VSL, VAP and WOB) at a higher level ($P < 0.05$), compared to undiluted (neat) semen where sperm function was compromised. The pre-processing stages of dilution, cooling and CP addition and equilibration reduced sperm function to a limited extent. PMOT was the only motility trait that was reduced with ~ 7 % when evaluated after CP was added and equilibrated. MOT, VCL, VSL, VAP, ALH, LIN, STR, WOB and BCF were maintained at the same level over the three pre-processing stages. Further cooling/freezing of semen to the different end temperatures ranging from -19 to -80 °C resulted in increased sperm function loss. PMOT and MOT evaluated ($P < 0.05$) at -19 °C and -22 °C was at the same level ($P > 0.05$) obtained after CP addition and equilibration. PMOT and MOT were significantly reduced when evaluated at lower end temperatures of -28 °C to -80 °C (Figure 57).

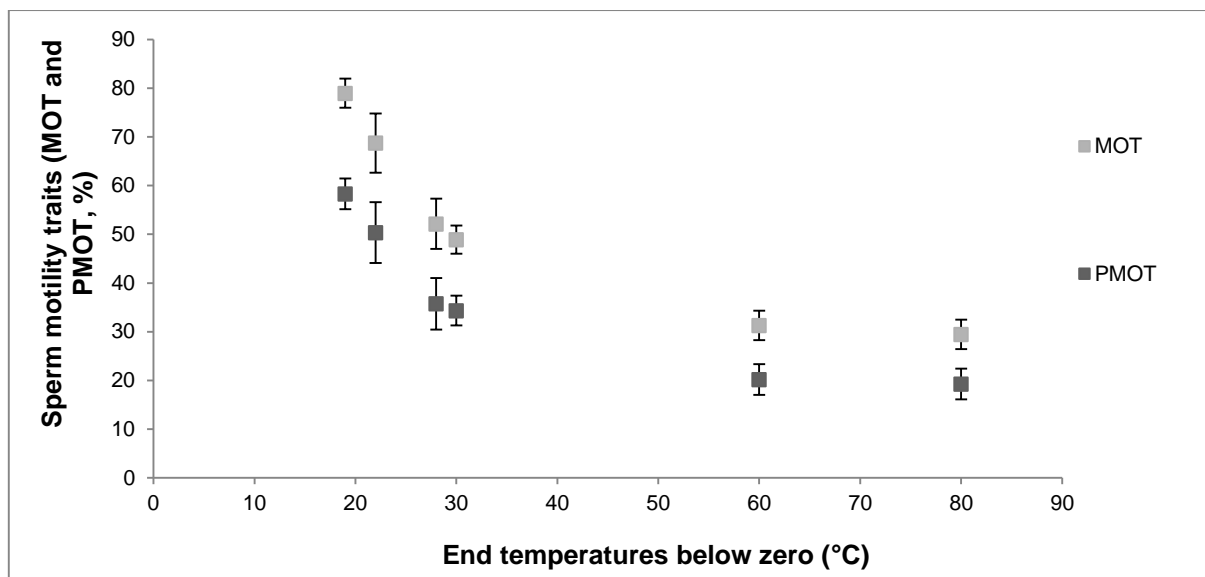


Figure 57: The effect of the cooling/freezing processing stage, across the different cooling/freezing rates, on sperm motility traits namely: percentage motile sperm (MOT, %) and percentage progressive motile sperm (PMOT, %) evaluated at different end temperatures (-19, -22, -28, -30, -60, -80 °C) before LN immersion. Standard error is indicated by vertical bars at the mean.

Kinematic traits of VSL, LIN, STR, WOB and BCF remained unchanged ($P > 0.05$) while VCL, VAP, ALH declined ($P < 0.05$) when evaluated at the different end temperatures over the range of -19 to -80 °C.

Thawing after deep freezing in liquid nitrogen (LN, -196 °C) at different end temperatures resulted in significant further reductions of sperm traits when immersed at a high end temperature of -19 °C. Freezing and thawing after immersion in LN at -19 °C was most detrimental in terms of PMOT, MOT, VCL, VSL, VAP, ALH, LIN, STR, WOB and BCF reduction. The lower temperature range of -22 °C to -30 °C freezing prior to immersion in LN maintained, PMOT and MOT at their highest levels with -30 °C being the best end temperature to immerse straws in LN. A slight reduction was observed at lower end temperatures of -60 to -80 °C (Figure 58). The degree of reduction observed in PMOT and MOT after freezing and thawing at -22, 28, -60 and -80 °C was not different ($P > 0.05$) from that observed during the cooling/freezing end temperatures of -60 and -80 °C that did not include LN-immersion.

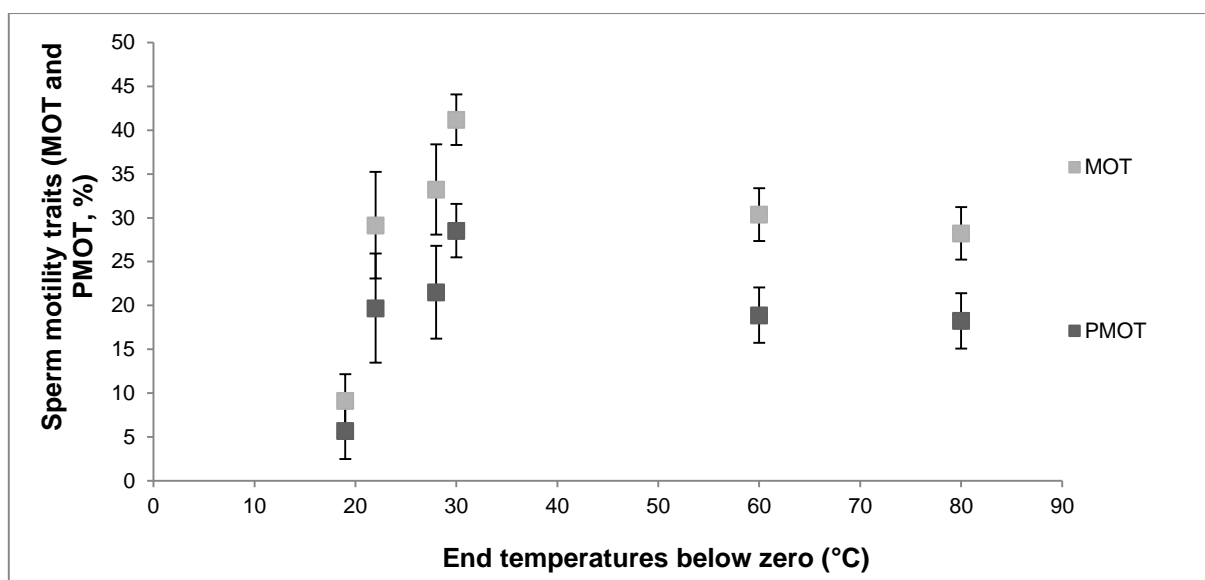


Figure 58: The effect of the freezing/thawing processing stage, across the different cooling/freezing rates, on sperm motility traits namely: percentage motile sperm (MOT, %) and percentage progressive motile sperm (PMOT, %) evaluated at different end temperatures (-19, -22, -28, -30, -60, -80 °C) after liquid nitrogen immersion (LN, -196 °C). Standard error is indicated by vertical bars at the mean.

3.4. *The interaction of controlled freezing rates with processing stage.*

The interaction of processing stage with freezing rate was highly significant for PMOT and MOT. It was evident that the degree of PMOT and MOT reduction was less at higher end temperatures (-19, -22, -28 °C), that were not significantly different from each other, compared to lower end temperatures of -30 to -80 °C evaluation. Freezing at 10 °C/min was better in reducing the damage exerted on the cells at the different end temperatures for PMOT and MOT, compared to 1 °C/min. This beneficial effect of faster freezing was more evident at lower end temperatures of -30 to -80 °C where most damage was exerted to the cells (Figure 59 and Figure 60).

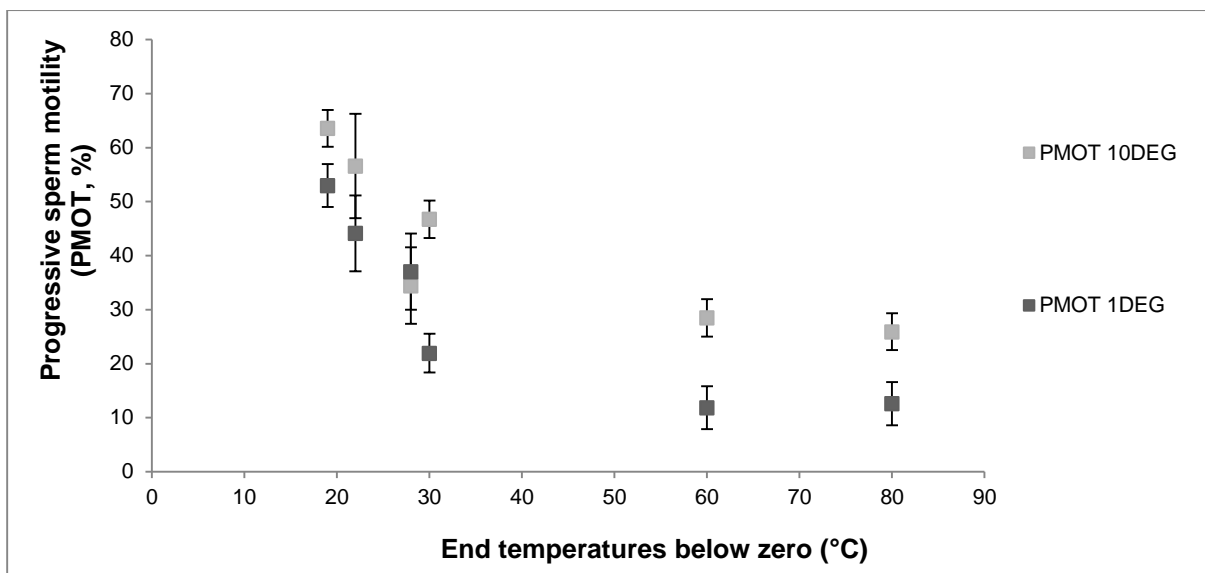


Figure 59: The effect of freezing rate during the cooling/freezing processing stage on sperm motility traits namely: percentage progressive motile sperm (PMOT, %) evaluated at different end temperatures (-19, -22, -28, -30, -60, -80 °C) without LN immersion (LN, -196 °C). Standard error is indicated by vertical bars at the mean.

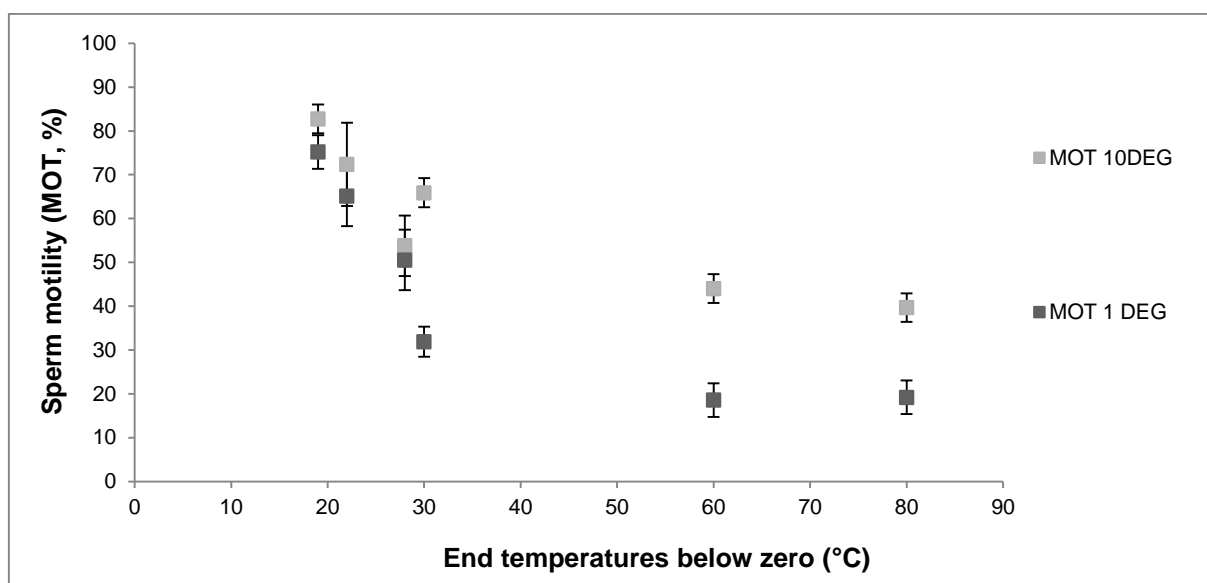


Figure 60: The effect of freezing rate during the cooling/freezing processing stage on sperm motility traits namely: percentage motile sperm (MOT, %) evaluated at different end temperatures (-19, -22, -28, -30, -60, -80 °C) without LN immersion (LN, -196 °C). Standard error is indicated by vertical bars at the mean.

Thawing at the different end temperatures of -30 to -80 after freezing and LN immersion demonstrated the beneficial effect of freezing at 10 °C/min, compared to 1 °C/min on PMOT (Figure 61) and MOT (Figure 62). PMOT and MOT were best ($P < 0.05$) maintained at respectively 39.26 ± 3.46 % and 55.82 ± 3.31 %, when frozen at 10 °C/min to an end temperature of -30 °C, and then immersed in LN and thawed. Compared to pre-

processing percentages of PMOT and MOT this protocol resulted in a ~ 29 % reduction in motility after processing for cryopreservation.

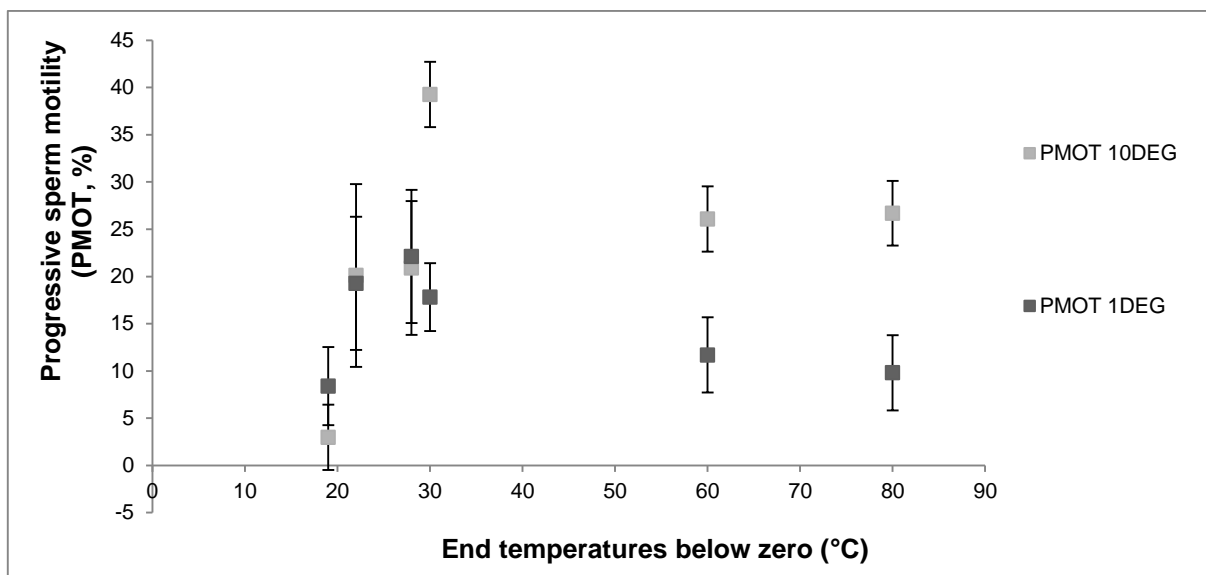


Figure 61: The effect of freezing rate during the freezing/thawing processing stage on sperm motility traits namely: percentage progressive motile sperm (PMOT, %) evaluated at different end temperatures (-19, -22, -28, -30, -60, -80 °C) after liquid nitrogen immersion (LN, -196 °C). Standard errors are indicated by vertical bars about means.

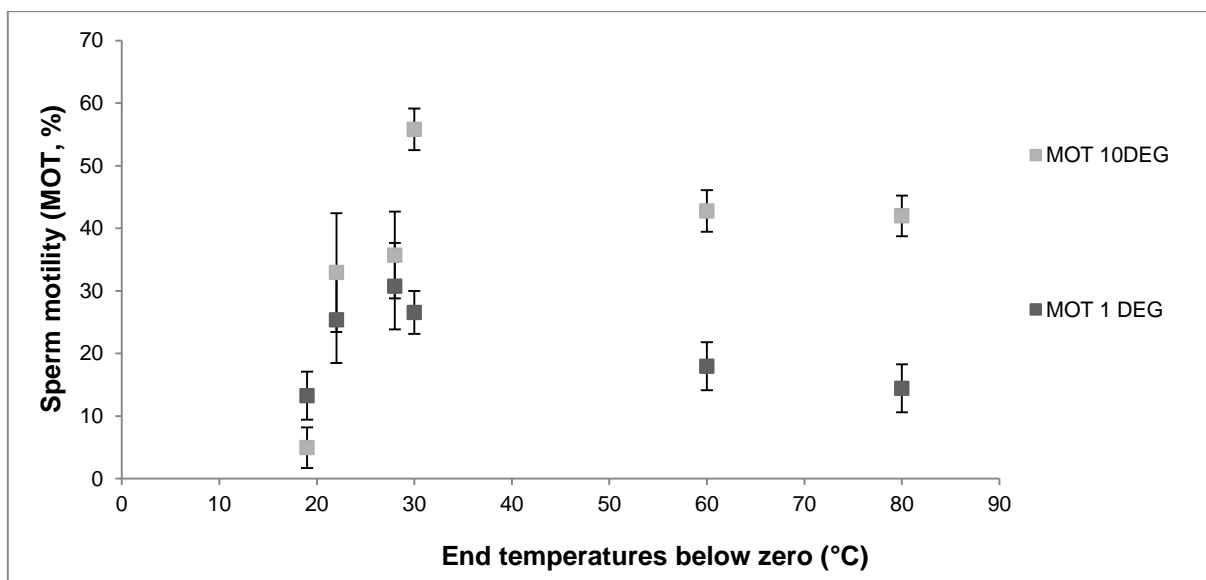


Figure 62: The effect of freezing rate during the freezing/thawing processing stage on sperm motility traits namely: percentage motile sperm (MOT, %) evaluated at different end temperatures (-19, -22, -28, -30, -60, -80 °C) after liquid nitrogen immersion (LN, -196 °C). Standard error is indicated by vertical bars at the mean.

Experiment 2: The effect of faster freezing rates by means of LN vapour on sperm function in vitro

3.5. Descriptive statistics

Across analyses the mean (\pm SE) sperm concentration was $3.68 \times 10^9 \pm 2.54 \times 10^7$ sperm cells / mL with a CV of 12.02 %, over 304 records. The summary statistics including the number of records, mean, standard deviation, minimum, maximum and coefficient of variation for the different sperm function traits are set out in Table 41.

Table 41: Description of sperm traits that included progressive motility (PMOT, %), motility (MOT, %), curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat-cross frequency (BCF, Hz).

Sperm trait	Records	Mean	S.D	Minimum	Maximum	CV (%)
Progressive motile (%)	303	54.57	20.40	12.00	95.40	37.38
Motile (%)	303	75.59	17.46	24.20	98.20	23.10
Curve-linear velocity ($\mu\text{m/s}$)	303	76.57	12.04	43.20	104.60	15.72
Straight-line velocity ($\mu\text{m/s}$)	303	50.81	12.82	15.80	84.70	25.22
Average path velocity ($\mu\text{m/s}$)	303	64.87	12.59	27.50	95.50	19.41
Amplitude of lateral head displacement (μm)	303	2.44	0.40	1.60	3.30	16.59
Linearity (%)	303	65.77	10.28	32.20	90.90	15.64
Straight (%)	303	77.94	10.25	54.00	96.60	13.15
Wobble (%)	303	84.29	5.91	56.00	94.70	7.02
Beat-cross frequency (Hz)	303	9.56	4.52	6.10	85.00	47.25

S.D: Standard deviation; CV: Coefficient of Variation

3.6. The effect of faster freezing rates achieved with LN vapour on sperm traits.

Sperm traits, namely PMOT and MOT varied significantly when treated with different uncontrolled freezing rates achieved by different plate heights (3.5, 7.0, 10.0 cm) in LN vapour before with LN immersion. The contribution of plate height to the variation in different sperm traits ranged from 10.71 % for PMOT to 11.99 % for MOT when evaluated after thawing. PMOT and MOT were best ($P < 0.05$) maintained at the faster freezing rate achieved by the lower plate height of 3.5 cm (Figure 63). The lower plate height of 3.5 cm performed significantly better than the slowest freezing rate achieved at the highest plate height of 10.0 cm, but did not differ from 7.0 cm. The linear regressions of PMOT and MOT on plate height were described by the respective equations $Y = 46.41 - 1.38 X$ ($P = 0.0243$; $R^2 = 0.1$) and $Y = 64.52 - 1.38 X$ ($P = 0.0138$; $R^2 = 0.1$), suggesting a 1.38 % reduction in PMOT and MOT with every 1 cm increase in plate height. Compared to pre-processing percentages of PMOT and MOT the optimal rate indicates a 20 % and 26 % reduction in motility respectively, after processing for cryopreservation.

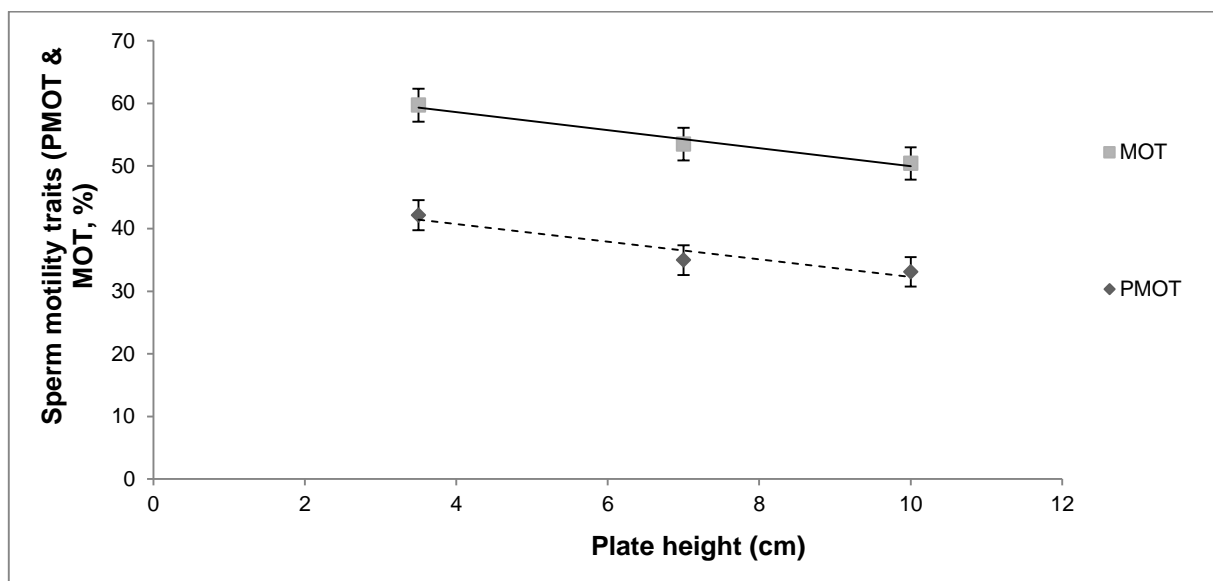


Figure 63: The effect of faster freezing rates achieved at different plate heights (3.5, 7.0, 10.0 cm) in LN vapour and LN immersion on sperm traits namely sperm motility (MOT, %) and progressive motility (PMOT, %). Standard error is indicated by vertical bars at the mean.

Experiment 3: *The effect of cryopreserved semen on in vivo fertility through perivitelline techniques.*

3.7. Descriptive statistics

Across the analyses traits for females used for *in vivo* (artificial insemination) testing included the mean (\pm SE) female age amounted to 6.80 ± 0.26 years with a CV of 27.77 % over 55 records. A minimum of 0 OPVL sperm/mm² and a maximum of 1115.61 OPVL sperm/mm² was obtained across the 55 records with a mean OPVL number of 33.86 ± 21.04 sperm/mm² and a CV of 460.79%. Across the analyses cryopreserved sperm traits for insemination included the mean (\pm SE) sperm concentration (mean \pm SE) was $3.45 \times 10^9 \pm 2.14 \times 10^7$ sperm cells / mL with a minimum of 2.42×10^9 and a maximum of 4.21×10^9 / mL. LIVE = 54.93 ± 3.72 %, HOS = 52.33 ± 2.35 %, PMOT = 43.20 ± 2.12 %, MOT = 59.07 ± 3.27 %, VCL = 78.87 ± 4.03 μ m/s, VSL = 56.17 ± 3.89 μ m/s, VAP = 69.10 ± 4.50 μ m/s, ALH = 2.31 ± 0.28 μ m, LIN = 70.87 ± 1.79 %, STR = 81.20 ± 1.97 %, WOB = 87.27 ± 1.27 % and BCF = 9.64 ± 0.36 Hz were obtained. Variation coefficients ranged from 3.86 to 17.92 %. Strong positive ($P < 0.0001$) Pearson correlation coefficients were obtained between the most important sperm functions (HOS, LIVE, PMOT and MOT), ranging from 0.5 to 0.7 The contribution of each fixed effect (FE, %) with its degrees of freedom (DF), R-square value and CV are shown in Table 42Table 32 for fertilization status and number of OPVL sperm

Table 21.

Table 42: The source of variation given by the fixed effect of semen treatment (fresh diluted, frozen stored), female age, OPVL sperm number and the number of days after the last insemination with their variation contribution (FE, %) with its degrees of freedom (DF) on fertility assessment traits that include fertilization status and OPVL sperm number.

Variation Source	DF	Fertilization status	DF	OPVL sperm
Semen treatment	1	0.63	1	5.96
Female age	1	1.54	1	0.10
OPVL sperm	1	14.79***	n/a	n/a
Number of days after last AI	1	0.49	1	2.13
TCSS	51	6.77	51	1284998.3
Error mean square	47	5.31	48	1193835.7
R ²		0.22		0.07
CV (%)		218.44		492.67

OPVL sperm: the number of sperm trapped in the outer perivitelline layer; CV: Coefficient of variation (%); TCSS: Total corrected sum of squares; n/a: fixed effect not applicable for the specific model *P < 0.05; **P < 0.01; ***P < 0.001

3.8. The effect of the number of OPVL sperm, semen treatment, female age, female identity and days after last AI on fertility assessment traits

The number of OPVL sperm was the only fixed effect that contributed ($P < 0.001$) to the variation (FE = 14.79 %) in fertilization status treated as a binary effect. Female age, semen treatment (fresh diluted; cryopreserved) and days since last AI did not affect fertilization status or the number of OPVL sperm of eggs obtained through AI. A strong positive Pearson correlation coefficient ($r = 0.45$; $P = 0.0006$) was obtained between OPVL sperm and fertilization status indicating that fertilization would improve with a higher number of OPVL sperm. Fertilised eggs had a mean number of 191.10 ± 120.57 OPVL sperm/mm², while non-fertilized eggs had a mean number of 3.09 ± 0.87 OPVL sperm/mm².

4. Discussion

4.1. *The effect of processing stage, programmable freezing rates and the interaction of freezing rate with processing stage on sperm function traits*

The maintenance of sperm function and thus the success of sperm cryopreservation can be characterized by the different processing stages involved. Optimization of the different pre-processing stages (dilution, cooling, CP addition and equilibration) for the ostrich have all been shown as vital in maintaining most of the *in vitro* sperm functions (MOT, VCL, VSL, VAP, ALH, LIN, STR, WOB and BCF). PMOT was the only function that showed a slight decrease of ~ 7 % during CP addition and equilibration. This result probably stems from sensitivity towards hyper-osmotic stress conditions inflicted on the cell by the CP that manifests itself in membrane changes and injury, oxidative stress as well as reduced mitochondrial activity and thus bioenergy metabolism while the cell is actually in a higher bio-energetic demand due to the of stimulated ion pump activity that will ultimately result in a decline of progressive sperm motility (Hammerstedt, 1990; Chatterjee and Gagnon, 2001; Park *et al.*, 2003; Guthrie *et al.*, 2008; Partyka *et al.*, 2012; Chapter VI). This result was in agreement with that of Ashrafi *et al.* (2011) reporting that PMOT, a comprehensive measurement of different aspects of cell physiology, was more sensitive to cryobiological damage and more likely to be affected by cryopreservation, than any other motility parameter. PMOT is a function that can be exploited for evaluation purposes of processing stress after cryopreservation as has also been shown by Blesbois *et al.* (2008).

The final stage of the cryopreservation process, the freezing-thawing stage, was most detrimental to sperm function, compromising PMOT and MOT by as much as 59 % and 77 %, respectively. The deterioration of sperm function after thawing is partially a result of the adverse effects of the pre-processing stages since cellular damage is irreversible while the majority of function loss was a direct cause of the freezing-thawing stage. The controlled slower freezing rate of 1 °C/min deteriorated PMOT and MOT significantly, compared to a faster rate of 10 °C/min. A 7 to 11 % difference was observed in PMOT and MOT between the slower and faster rates of 1 and 10 °C/min. Sperm function evaluated at different end temperatures, independently of freezing rate without LN immersion, indicated that damage to the sperm function occurred after -22 °C, with a steady decline in PMOT and MOT from -28 to -80 °C. This is a lower starting temperature range than that, -15 to -60 °C, pointed out

by Mazur (1965) or the -10 to -25 °C by Salamon and Maxwell (2000). The results from this study indicates that a critical temperature zone for ostrich sperm is most probably around -22 °C likely because of the CP (DMA) influence by lowering this critical point for the cell or due to sperm unique molecular composition and thermal properties. DMA has a freezing point of -20 °C and it is a known fact that CP's has the potential to reduce the freezing point of the medium that are used in (Gao *et al.*, 1993; Holt, 2000; Purdy, 2006). This critical temperature zone is most possibly the initiation point for phase transition or separation, since the sperm membrane of a specie is unique and the mixture of different molecular species of lipids (saturated versus unsaturated), as well as other constituents such as sterols and intrinsic membrane proteins that have an influence on thermal behaviour (Watson and Morris, 1987; Parks and Lynch, 1992; White, 1993; Blesbois *et al.*, 2005). The ability to phase separate upon cooling/freezing depends on the difference in phase transition temperatures of the individual molecular components, the rate of cooling, and interaction between the polar groups (Quinn, 1985). Quinn (1985) specifically pointed out the phase behaviour of lipids around -20 °C, with unsaturated lipids remaining in the fluid phase, while the saturated lipid phase changed to the crystalline state. Cooling/freezing beyond -20 °C initializes the phase change of the unsaturated lipid through crystallization where a complete cell phase transition will occur. The latter representing the possibility that ostrich sperm might have a higher proportion of unsaturated fatty acids due to the observed lowered critical temperature zone for phase transition. Species with a higher proportion of polyunsaturated fatty acids (PUFA) will maintain functionality of the protective layer at lower temperatures better, since polyunsaturated fatty acids are more fluid and better equipped to lower the phase transition temperature (Drobnis *et al.*, 1993; Saragusty *et al.*, 2005). However the declines in PMOT and MOT from -28 to -80 °C end temperatures were less when a faster rate of 10 °C/min was implemented, compared to 1 °C/min. A faster rate of 10 °C/min is better in reducing the hazardous effects of the critical temperature zone possibly by limiting the exposure time of the cell to transition effects.

After LN immersion at the different end temperatures and thawing it was evident that the end temperature of -30 °C was best, with only a ~ 29 % reduction from the initial PMOT and MOT when a higher freezing rate of 10 °C/min was applied. This is in contrast with results of Sexton (1980) who reported -80 °C as the most appropriate end temperature before LN immersion to maintain fertilising ability in chicken semen. The beneficial effect obtained

through the methodology applied in this experiment demonstrated that the cell was closest to remaining in equilibrium with its extracellular environment that guaranteed good post-thaw sperm function. Freezing/cooling the cell from 5 to -30 °C at 10 °C/min assured a sufficient degree of cell dehydration, which limited damaging internal ice formation caused by excess internal water, without exposing the cell to critical volumetric and osmotic changes associated with water leaving the cell. Liquid nitrogen immersion of semen at -30 °C, which ultimately stopped further cooling/freezing of the cells, prevented an extreme degree of cell dehydration and limited the exposure time of the cell to precarious hyper-osmotic and diminishing volumetric conditions associated with the dehydration effect. Although faster freezing rates require increased thawing rates to avoid any deleterious water neocrystallization during the thawing process, the rehydration and limited deterioration observed in cell function post-thaw confirms the satisfactory performance of the present protocol.

4.2. The effect of faster freezing rates achieved with LN vapour on sperm function traits

The fastest rate achievable by the programmable freezer was 10 °C/min and it has been shown in different species namely, ovine (Towhidi *et al.*, 2013), equine (Garcia *et al.*, 2011), bovine (Towhidi and Parks, 2012) and avian (Tselutin *et al.*, 1999; Barna *et al.*, 2010; Váradi *et al.*, 2013) that faster rates could be achieved by placing the semen in LN vapour at a certain height above the LN, followed by LN immersion. The post-thaw success in terms of *in vitro* and *in vivo* evaluation was, however, variable between authors. Freezing in LN vapour was done for the ostrich and it was found that the lowest plate height (3.5 cm above the LN and also the fastest rate tested), was best for PMOT and MOT maintenance post thaw. Only a 20 to 26 % reduction was observed between pre-processing PMOT and MOT percentages. This is much higher than the suggested one third to one half cryopreservation survival rate of the initial sperm population for chickens specifically in terms of LIVE that has been highly correlated with the % motile sperm in some studies (Seigneurin and Blesbois, 1995; Chalah *et al.*, 1999). Blach *et al.* (1989) reported a motile range of 5 – 55 % of motile sperm surviving cryopreservation in the equine, a cryopreservation-sensitive species. Wishart and Palmer (1986) reported respective motility of 24 and 22 % for chicken and turkey sperm after cryopreservation. Tselutin *et al.* (1999) suggested that the success of rapid freezing rates is possibly due to the state (vitrified and not crystallized) of the cell after

freezing with specific reference to DMA as CP. This contention was supported by results of Woelders *et al.* (2006) for chicken semen and by results of Phuong (2014) for Guinea fowl semen. In this study a vitrified state could have been possible, as a high CP concentration was used. One of the determinants of successful vitrification, suggested by Rall (1987), is very small straws (250 μ l) that would guarantee a small volume, together with a very fast freezing rate that promotes super-cooling of the semen and a vitrified state, as described by Fahy *et al.* (1984). The morphological shape of the ostrich sperm cell can also be a contributory factor, as pointed out by Seigneurin *et al.* (2013) for Guinea fowl sperm. A small amount of cytoplasm and presumably water allowed by the morphological cell structure would permit very rapid freezing rates with a fairly low risk of intracellular water crystal formation that would destroy the cell.

4.3. The effect of the number of OPVL sperm, semen treatment, female age, egg weight, days after last AI and female identity on fertility assessment traits

The number of sperm trapped in the outer perivitelline membrane was the only fixed effect affecting the fertilization status of the eggs collected after AI with either fresh diluted or cryopreserved semen. The strong correlation between fertilization probability and trapped OPVL sperm is not a new finding and has been supported by research on poultry by Wishart (1987), Brillard and Bakst (1990), Brillard (1993) and for the emu (Malecki and Martin, 2002, 2003, 2004) as well as the ostrich (Malecki and Martin, 2003; Malecki *et al.*, 2004; Chapter V).

Insemination of ostrich females resulted in a wide range of OPVL sperm numbers (0 to 1115.61 sperm/mm²) which is in agreement with Malecki and Martin (2003) whom reported a range of 0 to 1330 sperm/mm² in the eggs of females during natural mating, however not with that reported by Smith (Chapter V). Smith (Chapter V) reported a range of 0 to 21.58 OPVL sperm/mm² in eggs of females inseminated with chilled and freshly diluted semen was reported. The difference in OPVL sperm numbers can be attributed to the large member of between-animal variation reported for the resource flock (Chapter V). However, it has been reported in other avian studies that dramatic losses in sperm number occur during insemination, with less than 1 % of the inseminated sperm reaching the SST (Allen and Grigg, 1957; Howarth, 1971; Brillard, 1993). The large amount of variation (CV = 460.79 %) associated with OPVL sperm numbers observed in this study, with very low OPVL numbers

in some. Thus no reason for apprehension since it has been shown that avian eggs can be fertilized even when only a few sperm is present or even when no sperm can be found in the OPVL of the GD region (Malecki and Martin, 2002), or even when only one sperm has entered the egg (Bramwell *et al.*, 1995; Wishart, 1997). Even though the process of ostrich semen cryopreservation have been shown to reduce ostrich sperm function by 20 to 26 %, no significant difference was observed in this study for OPVL sperm numbers or the fertilization status of eggs produced after fresh, diluted or cryopreserved semen inseminations. The latter indicates that processing stress experienced by the sperm, associated with the current protocol for cryopreserved semen was limited and that other factors influencing the OPVL sperm numbers should possibly be explored to increase fertilization success. Female factors influencing OPVL sperm numbers reported in the literature include specific selection requirements of sperm, female age, oviposition and timing between inseminations, as well as the onset of egg production (Brillard and Bakst, 1990; Brillard, 1993). These female factors are mainly attributed to the specialized functionality of the SST, which is a unique structure with specific capability to efficiently store high sperm numbers in females of different species. The number of OPVL sperm obtained in other avian females like the turkey correlated strongly ($r = 0.85$) with the number of sperm stored in the SST (Brillard and Bakst, 1990). The SST of the ostrich has been described by Bezuidenhout *et al.* (1995). It is noted that this species has a fertile period of up to 28 days post-coitus (Bezuidenhout *et al.*, 1995; Swan and Sicouri, 1999; Malecki *et al.*, 2004; Dzoma, 2010). By incorporation of these female factors during AI one can potentially increase OPVL sperm numbers and thus fertility rates with the potential to gain more knowledge about semen processing treatments for optimization purposes. For instance, inseminating prior to the onset of egg production has been found to increase the number of SST stored sperm and thus OPVL sperm numbers in chickens and turkeys (Brillard and Bakst, 1990; Brillard, 1993). Females in this study laid fertilised eggs for up to 9 days after the last insemination with cryopreserved semen and 6 days after the last insemination with freshly diluted semen while sperm were still visible in the perivitelline membrane after a maximum of 13 and 15 days after the last insemination with fresh, diluted and cryopreserved semen in this study. These results indicate a shorter availability of sperm compared to a previous study by Smith (Chapter V) who reported fertilised eggs up to 11 days after the last insemination with freshly diluted or chilled semen and visible OPVL sperm up to 13 to 24 days after the last insemination. The shorter availability of sperm is most possibly because

of depleted SSTs that is a direct result of the lower insemination dosages used in this study compared to the study done by Smith (Chapter V). The present results, together with the results of other ostrich studies, including a fertile period of 9 days for females bred artificially with 3 billion sperm on five consecutive days (Bonato and Cloete, 2013) and a fertile period of 28 days when bred naturally (Malecki *et al.*, 2004), suggest that insemination less frequently, but with higher initial dosages of highly functional sperm should boost SST numbers and enable a consistent sperm release, without the stress associated with weekly inseminations and increased male collection frequency. This is specifically important for a recently domesticated species like the ostrich female that often displays a high flight reaction that may have an indirect impact on OPVL numbers and thus fertility after insemination. The AI technique should also be considered as a factor that could influence OPVL numbers and with possible optimization specifically for the ostrich female.

5. Conclusion

Cryopreservation of ostrich semen is now for the first time a viable option in terms of high quality *in vitro* sperm function and fertile eggs after *in vivo* testing through artificial insemination. Cryopreserved semen would allow genetic resource management of superior individuals *ex situ* by means of bio-banking and increase productivity of the ostrich industry on a commercial level. The success rate of the ostrich cryopreservation protocol is, however, dependent on specific pre-processing factors (1:1 dilution at 24 °C with OS7, slow cooling at 1 °C /min to 5 °C, 1:1 dilution of pre-cooled DMA with semen to a final concentration of 16 %), that should be implemented together with controlled fast freezing at 10 °C /min in a programmable freezer until -30 °C whereafter LN immersion should be applied. Further studies are necessary to investigate possible phase separation and transition at this temperature range with specific exploration of membrane lipid compositions. Placing semen at a plate height of 3.5 cm in LN vapour for 5 minutes was shown to be an even more viable method to cryopreserve ostrich semen. This is of specific benefit since the equipment for this method, namely LN and a Styrofoam box, is highly cost effective for an on-farm approach. The latter result pointed to the suitability of ostrich semen for vitrification. *In vivo* testing should combine less frequent inseminations with higher initial dosages to support a consistent sperm release from the SSTs. Special attention should be given to fertile female selection to fully exploit *in vitro* storage protocols.

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CHAPTER VIII

GENERAL CONCLUSION AND RECOMMENDATIONS

1. To conclude

Individual chapters enclosed in this dissertation already contain individual abstracts and conclusions, therefore only the most important general conclusions are commented on here. The outcomes of this dissertation confirmed that fundamental processing steps sourced from the literature were needed to develop short and long term storage protocols for ostrich semen. Sequentially, these processing steps included dilution rate and temperature (Sexton, 1977), storage medium composition and associated osmotic and pH properties (Sexton and Fewlass, 1978, Lake and Wishart, 1984; Wishart, 1989; Blanco *et al.*, 2000, 2008), storage time and temperature (Clarke *et al.*, 1982), type of cryoprotectant (CP) and its concentration (Tselutin *et al.*, 1999), cooling/freezing rates (Sexton, 1980; Seigneurin and Blesbois, 1995) and thawing rates (Tselutin *et al.*, 1995; Blesbois and Brillard, 2007). This study has confirmed results of other avian (Wilcox *et al.*, 1961; Blanco *et al.*, 2000; Blesbois *et al.*, 2005) and mammalian (Holt, 2000; Waberski *et al.*, 2011) studies that requirements for acceptable results after storage differ among species, and that ostrich sperm have their own unique set of storage processing requirements. These requirements may be due to the distinctive properties of ostrich sperm cells that determine their sensitivity towards the different processing stressors experienced during the steps of the preservation protocol. Different objective tests for assessing sperm motility (MOT, %), progressive motility (PMOT, %), curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), beat-cross frequency (BCF, Hz), sperm viability (LIVE, %), membrane integrity (HOS, %) and sperm morphology (NORM, %) have been developed for the ostrich prior to storage and been demonstrated as useful to indicate the sensitivity of the cell towards the different processing steps and accompanied stressors. These tests have also proven handy when setting up an informative baseline for evaluation and classification of the initial ejaculate or for the assessment of specific males. Although these functional tests were correlated with one another processing stress may vary between the different processing steps, with processing damage affecting diverse cell functions. This principle has been demonstrated when a set of comprehensive tests are used during the

different processing steps. Specific functional tests were more informative than others during some processing steps.

From the first research chapter (Chapter II, Classification of ostrich sperm characteristics), it can be concluded that males differ in semen quality when evaluated by objective comprehensive *in vitro* sperm function tests. Although comprehensive evaluation is optimal, certain motility functions namely PMOT, MOT, VCL, VSL, VAP and LIN can be used for an initial time efficient evaluation. By implementing *in vitro* function tests, evaluation of males can commence prior to breeding and to predict the suitability of males and ejaculates for future breeding as well as for processing for storage. Above average semen quality (PMOT > 50 %, MOT > 80 %, VCL > 70 $\mu\text{m/s}$, VSL > 40 $\mu\text{m/s}$, VAP > 50 $\mu\text{m/s}$ and LIN > 60 %) can be considered for long-term (cryopreservation) storage purposes. Ejaculates with above average and average semen quality (PMOT 40 – 50 %, MOT 70 – 80 %, VCL 60 - 70 $\mu\text{m/s}$, VSL 30 - 40 $\mu\text{m/s}$, VAP 40 - 50 $\mu\text{m/s}$ and LIN 50 - 60 %) can be considered for short-term (chilled) storage purposes. Males with below average quality should be monitored over a few collections before being culled, while ejaculates of poor quality should be rejected immediately after evaluation and not considered for storage processing also considering the fixed effect of season. Objective evaluation and collection for storage processing should only be considered in late spring and early summer when semen quality is maximised as demonstrated by comprehensive *in vitro* sperm function and sperm concentration in this study. Dilution has shown to be a very important initial processing step of ostrich semen to maintain these sperm functions for evaluation upon collection and to progress to storage processing.

From the second research chapter (Chapter III, Development of an ostrich specific storage extender), it is evident that a biochemical analyses of ostrich seminal plasma was effective to determine the most important constituents to formulate a medium suitable for ostrich sperm dilution that could maintain sperm function when processed for storage. Although a medium (OS1), containing similar macro minerals (sodium, potassium, phosphorus, calcium, magnesium) in equal concentrations than in ostrich seminal plasma has proven to be sufficient for ostrich semen processing. A medium (OS2) containing macro and micro minerals (selenium and zinc) has been found to maintain swim quality (LIN, STR and WOB), better when exposed to prolonged short-term storage. The latter results indicated that the

loss of sperm function during short-term storage processing could be a result of oxidative stress and proneness of ostrich sperm towards reactive oxygen species since the combating actions of Se and Zn in antioxidant cell actions are well known in the literature.

From the third research chapter (Chapter IV, Prolonged storage of ostrich semen at 5 °C through intermediate dilution), the importance of dilution rate has been demonstrated and it can be concluded that initial dilution immediately upon collection at an interpolated 1:6 (semen: ostrich specific diluent) rate, is needed to maintain sperm quality for evaluation and further storage processing. Dilution at temperatures of ≥ 21 °C have been found beneficial to maintain PMOT compared to dilution with a pre-cooled extender set at 5 °C. Higher dilution temperatures are convenient for field processing, since it avoids pre-cooling of extender as well as temperature regulation. However this does suggest sensitivity of ostrich sperm towards fast cooling. A 24-hour short-term storage period for chilled semen is optimal with no deterioration in sperm viability or morphology and minimum deterioration on other sperm functions during this period. Males differed in their ability to maintain sperm function after processing for short-term storage, confirming that not all males are suitable for storage processing. The fourth research chapter (Chapter V, The response of ostrich sperm to cooling at 5 °C storage and *in vivo* application through artificial insemination) reported the short-term storage protocol for chilled semen. It was evident that ostrich sperm are indeed sensitive to fast (> 1 °C/min) cooling. A slower cooling rate of 1 °C/min is more beneficial from collection (~ 24 °C), to a suitable temperature of 5 °C for chilled semen storage. *In vivo* assessment of the chilled semen storage protocol confirmed viability of the protocol with fertile eggs obtained after artificial insemination. A schematic diagram that summarizes the short-term storage protocol for ostriches is demonstrated in Figure 64.

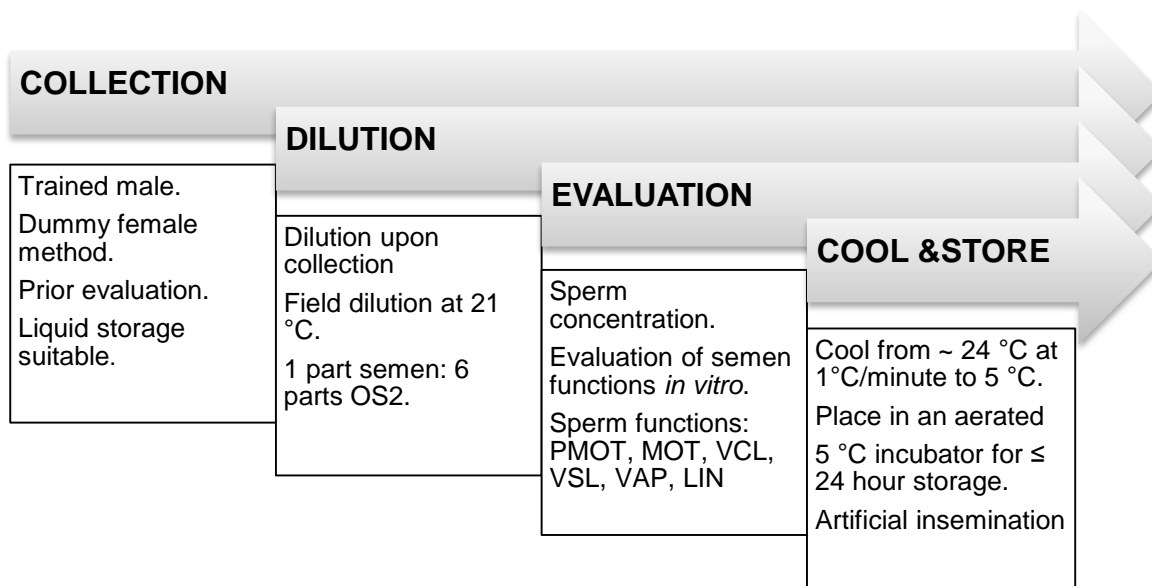


Figure 64: Flow diagram depicting the actions needed for short-term (chilled/liquid) storage protocol of ostrich semen.

From the fifth research chapter (Chapter VI, Sensitivity of ostrich sperm towards cryoprotectant toxicity) it can be concluded that addition and equilibration of ostrich sperm with different cryoprotectants (CP's) may induce severe sperm function loss. However, the cryoprotectant (CP) dimethylacetamide (DMA) at a level of 16 % did not induce any deleterious effects on sperm function and can be used to cryopreserve semen in combination with an appropriate freezing rate. The most important sperm functions namely NORM, LIVE, HOS, PMOT, MOT, VCL, VSL, and VAP were not reduced after DMA addition and equilibration, compared to the other CPs tested. The sixth research study, (Chapter VII, Cryopreservation of ostrich sperm through fast freezing and *in vivo* application through artificial insemination) set out the cryopreservation protocol. During controlled freezing in a programmable freezer, a fast freezing rate of 10 °C/min was most suitable to cryopreserve sperm than a slow freezing rate of 1 °C/min. Fast freezing, in combination with DMA 16 %, should be applied once the cell has been slow cooled (1 °C/min) to 5 °C where after fast freezing (10 °C/min) should commence up to -30 °C, followed by immediate liquid nitrogen (LN) immersion at -196 °C. Only a ~ 29 % reduction from the initial PMOT and MOT was observed after fast thawing for 12 seconds at 5 °C. Uncontrolled freezing in LN vapour was just as effective as controlled freezing. This method was more suitable for the on-farm approach since a low cost Styrofoam box filled with LN can be used without the supervision of technical personnel. A plate set at 3.5 cm above the LN in the Styrofoam box can be set up to achieve the best fast freezing rate of semen, followed by the immersion of straws in

LN. Uncontrolled fast freezing together with fast thawing of 12 seconds at 5 °C has been shown to revive sperm with only a 20 to 26 % reduction between pre-processing PMOT and MOT percentages. *In vivo* assessment of the cryopreserved semen storage protocol confirmed the viability of this protocol with fertile eggs obtained after artificial insemination. A schematic diagram that summarizes the long-term storage protocol for ostriches is set out in Figure 65.

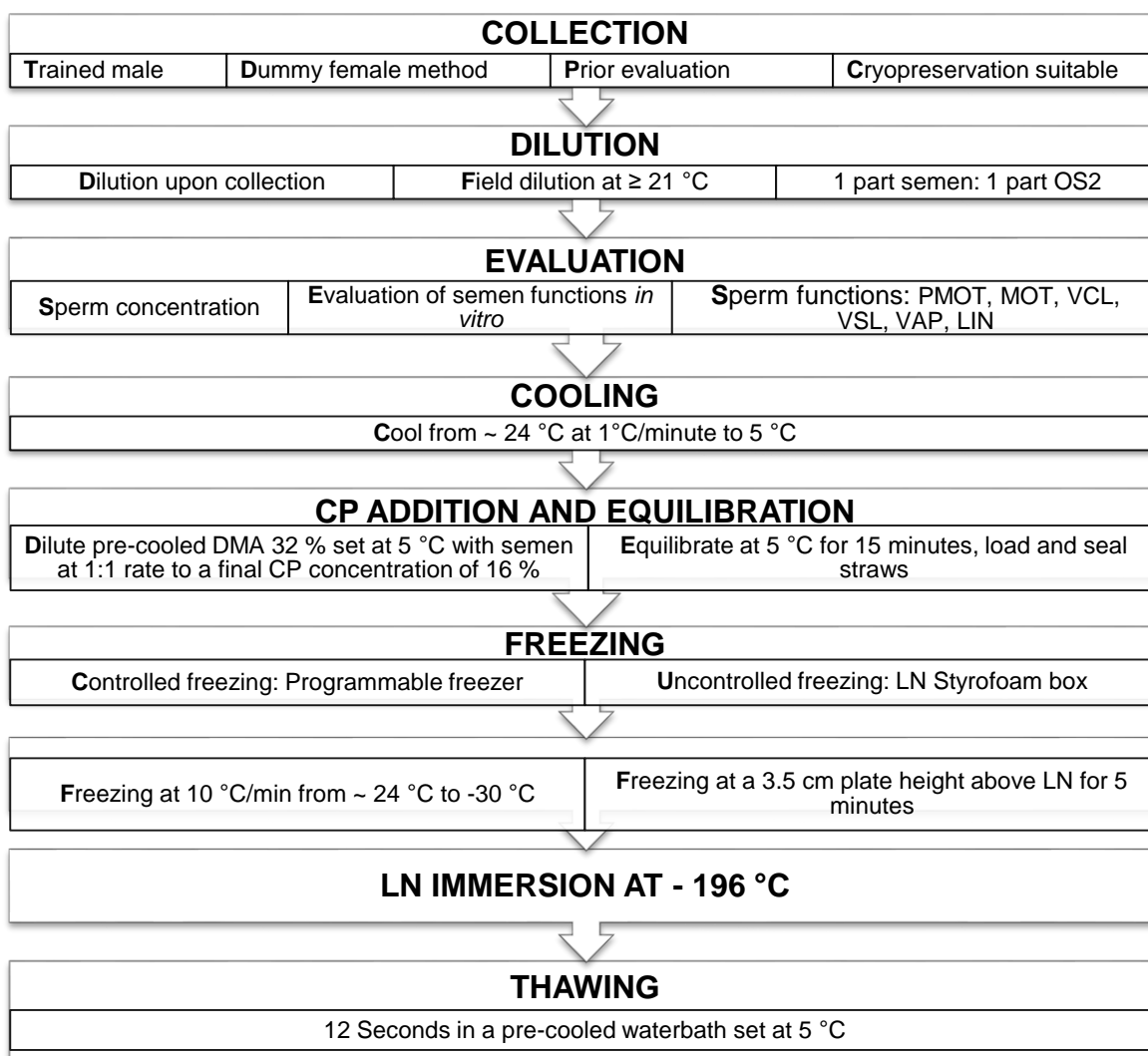


Figure 65: Flow diagram listing the actions needed for the long-term (frozen/cryopreserved) storage protocol of ostrich semen.

2. Future recommendations

2.1. *Optimization of the ostrich specific diluents and storage protocols for ostrich sperm*

Firstly, a further optimization of the current OS1 and OS2 diluents could possibly prolong short-term storage protocols for periods ≥ 48 hours. Since viability (LIVE) and membrane integrity (HOS) was not affected after 24 hours, one would expect energy depletion since motility functions were compromised. Furthermore, even though sperm motility was reduced after storage the micro minerals of Se and Zn, known for their involvement in anti-oxidation pathways and improved sperm swim quality. This result indicates that ostrich sperm is sensitive to the detrimental effects of oxidative stress associated with prolonged (48 hours) short-term storage. With the inclusion of 2.1.1. Exogenous energy substrates and 2.1.2.

Anti-oxidants and anti-oxidant enzymes short-term storage for ostrich sperm may be prolonged further. Ostrich sperm has also been found to be cooling sensitive, especially when exposed to higher cooling rates after collection to a 5 °C storage temperature. By enhancing the diluent with 2.1.3. Exogenous membrane components, the sensitivity of ostrich sperm to cooling might be further reduced and sperm function maintained at a higher level. Although 1 °C/minute has been found sufficient to sustain sperm function after cooling and after storage it would be beneficial to test lower rates of < 1 °C/minute that are possible by means of a programmable freezer. Although DMA has been identified as the CP of choice, it may be possible that other CPs like MA would also be suitable when tested at a suitable concentration with an adapted protocol. However, at this stage this would be an unnecessary task, since the previously listed research has a higher priority.

2.1.1. *Exogenous energy substrates*

It has been shown that it is important that metabolic processes and energy values need to be maintained during storage to sustain sperm survival (Wishart, 1989). A higher level of extra-cellular energy source or a combination of energy sources supplied *in vitro*, by means of an adapted diluent, may be utilized by sperm to maintain basal metabolism and motility to compensate for the low inter-cellular energy reserves found at the time of ejaculation (MacIndoe and Lake, 1973; Sexton, 1974; Christensen, 1995; Thurston, 1995; Blesbois and Brillard, 2007). Species substrate preference is an important factor when formulating a suitable storage medium with exogenous energy sources. The energy metabolites available

to sperm *in vivo* vary between species, but consist mainly of sugars and fatty acids, although chicken sperm have been shown to utilize limited amounts of glutamic acid, as well (MacIndoe and Lake, 1973; Sexton, 1974; Blesbois and Brillard, 2007). Furthermore research on energy substrates for the maintenance of ostrich sperm function is thus indicated.

2.1.2. Anti-oxidants and anti-oxidant enzymes

The inclusion of exogenous anti-oxidants like synthetic tocopherol (Vitamin E), ascorbic acid (Vitamin C), carotenoids, butylated hydroxytoluene (BHT), Tempo and selenium (Se) has been suggested by numerous authors to counteract the action of free radicals formed during lipid peroxidation (Aitken and Clarkson *et al.*, 1988; Donoghue and Donoghue, 1997; Farshad *et al.*, 2010). The addition of phospholipase inhibitors have also been suggested by Blesbois and Brillard (2007). Donoghue and Donoghue (1997) showed improved sperm survival with the addition of Vitamin E, Tempo and BHT on membrane integrity as well as better maintenance of motility after 48 hours of liquid storage at 5 °C for the turkey. BHT is a lipophilic organic compound that readily penetrates the sperm membrane with high antioxidant properties. BHT has been suggested to protect the sperm plasma membrane (Watson and Anderson, 1983; Graham and Hammerstedt, 1992; Farshad *et al.*, 2010). BHT is incorporated into the sperm membrane, thereby decreasing viscosity of membrane lipids. The latter may increase lipid fluidity at lower temperatures that may prevent permeability changes of the sperm plasma membrane when the cells are stressed (Hammerstedt *et al.*, 1976). BHT may also act as a scavenger of harmful oxygen free radicals (Aitken and Clarkson, 1988; Killian *et al.*, 1989; Aitken, 1995; Farshad *et al.*, 2010). Vitamin E is a less active antioxidant compared to BHT, but enhances sperm-oocyte fusion at high concentrations. The latter implies that more than one compound may be necessary to maintain full membrane protection during processing (Parks and Graham, 1992). Further studies on these topics are clearly needed.

2.1.3. Exogenous membrane components

It has been demonstrated that the addition of purified lipid preparations (like phosphatidylcholine), to sperm may assist in elevated chilling resistance as well as freeze-thaw damage (Parks *et al.*, 1981; Graham and Foote, 1987; Parks and Graham, 1992). The success of such exogenous added lipids may depend on the molecular species and its ability

to interact with the sperm membrane keeping in mind that the membrane is compartmental to some extent (Watson *et al.*, 1987; Steponkus *et al.*, 1988; Parks and Graham, 1992). Chilling injury and lipid peroxidation sensitive species, like the Asian elephant and turkey that has a high proportion of polyunsaturated fatty acids, responds positively in terms of membrane integrity and fertility, when supplemented with egg-phosphatidylcholine (Saragusty *et al.*, 2005; Long and Bakst, 2008). Cholesterol contributes to membrane fluidity, as well as the stability of the membrane and can also potentially assist to maintain membrane function when exposed to cooling. Endogenous cholesterol molecules are packed in between the phospholipid molecules where they prevent fatty acid chains from packing together and crystallizing, a process that drastically reduces membrane fluidity (Sherwood, 2004). Such studies are also needed for the further development of a storage protocol for ostrich semen.

2.2. *In vitro* sperm functional tests

A sperm cell requires many attributes to fertilize an oocyte, which make comprehensive *in vitro* testing necessary (Graham and Moce, 2005). The need to expand on the current set of *in vitro* functional tests to evaluate different functions of the cell before and after processing to indicate the type of damage or constraints experienced by the cell and the remaining fertilizing potential was highlighted in this study. *In vitro* testing is time efficient and cost effective, compared to *in vivo* fertility trials. It correlates well with fertilising ability after artificial insemination, although true fertility may be over-estimated to a certain degree due to the loss associated with the female tract and some subtle losses that are accounted for. Such tests would enable further optimization of the processing steps, and thus the protocols, to alleviate constraints that may still exist.

2.2.1. Acrosomal membrane integrity and reaction

The most important *in vitro* function evaluation would be that of the acrosomal status. Fertilization ability of the sperm cell depends on the sperm cell to undergo the acrosome reaction, which means the release of the acrosomal enzymes permitting the sperm to digest a hole through the zona pellucida (mammals) or egg envelope (avian) and allow access to the oolemma. The acrosome must stay intact up to the time the sperm cell binds to the zona pellucida/egg envelope of the oocyte (Esteves *et al.*, 2007; Partyka *et al.*, 2010). During the

acrosome reaction the contents of the anterior acrosome are released, a large part of the anterior head membranes are lost as hybrid vesicles of the plasma membrane and the outer acrosomal membrane and a proportion of the acrosomal membrane becomes continuous with the plasma membrane (Cross and Meizel, 1989). The acrosome reaction is independent of the capacitation process, a common occurrence associated with the acrosome reaction in mammals (Lemoine *et al.*, 2008, 2011; Moce *et al.*, 2010). Processing, more specifically cryopreservation and the effect of cryoprotectant addition, can cause a premature acrosome reaction by means of acrosomal matrix content loss before the sperm has reached its final destination (Esteves *et al.*, 2007; Moce *et al.*, 2010; Partyka *et al.*, 2010). Xia *et al.* (1988) reported that frozen-thawed fowl sperm were acrosome absent, or had a bulbous appearance. Partyka *et al.* (2010) reported 71% dead cells with ruptured acrosomes in frozen-thawed fowl semen, indicating that cryopreservation-related destruction of the acrosome may be occurring after cell death. Nagy *et al.* (2003) reported populations of acrosome damaged dead cells to consist out of a mixture of cells that died after the acrosome reaction and cells that lost their acrosomes at cell death. There are two classes of fluorescent probes that can be used to evaluate acrosome status that can be manually counted by fluorescent microscopy or automatically counted by means of a flow cytometer. The two classes can be distinguished from each other in terms of cell permeability to detect and label intracellular acrosome associated material. These include lectins and antibodies to intracellular acrosomal antigens. The second category is those probes that can be used on living, non-permeabilized cells that can include chlortetracycline (CTC) and antibodies to externally exposed acrosomal antigens (Cross and Meizel, 1989). The antibiotic, CTC, is a very fast assay and produces fluorescent patterns that reveal important information about the progress of capacitation and the acrosomal status with minimal sperm loss). Lectins, the most accessible reagents, bind to glycoconjugates of the acrosomal matrix or outer acrosomal membrane and are being used with great success in many species (Cross and Meizel, 1989).

Arachis hypogea (peanut) agglutinin with fluorescein (FITC-PNA) and *Pisum sativum* (edible pea) agglutinin with fluorescein (FITC-PSA) or PNA conjugated to phycoerythrin (PE) are commonly used lectins that renders acrosome-intact cells brightly fluorescent over the anterior sperm head and, for many of these probes, as well the equatorial region (Graham and Moce, 2005; Partyka *et al.*, 2010). FITC-PNA has been reported to bind to sperm which

still have an outer acrosomal membrane present, while FITC-PSA binds to the acrosome reacted sperm since it localizes within acrosomal contents (Mortimer *et al.*, 1987). Hoechst 33258 (H258) can also be combined with the latter as a supravital stain on the FITC-PSA, or PNA labeling procedure. The two fluorochromes can be visualized simultaneously as blue (H258) and yellow-green (FITC-PSA). SYBR 14 and PI have also been found very effective in combination with PNA-PE in order to establish acrosomal membrane integrity (Nagy *et al.*, 2003). LysoTracker, an acidotropic probe that identifies living sperm with intact acrosomes and stains the acrosome green, was most effective to evaluate acrosome integrity (Thomas *et al.*, 1998). LysoTracker can also be used in combination with PI. This may result in a cell (red or unstained depending if cell is dead or not) with a dull green cap (if acrosome is intact). These applications need to be tested and optimized for ostrich sperm, if applicable.

2.2.2. Mitochondrial function

Mitochondrial status plays an important role in determining sperm cell competence in terms of energetic value providing a majority of the ATP necessary for metabolism and motility essential for fertilization to be successful (Graham and Moce, 2005; Blesbois *et al.*, 2008; Giannoccaro *et al.*, 2010; Martinez-Pastor *et al.*, 2010; Partyka *et al.*, 2010). Xia *et al.* (1988) reported a higher occurrence of ultrastructure abnormalities in the mitochondria of frozen-thawed avian sperm that may lead to energy loss, motility reduction and even cell death. There are several fluorescent probes (Mito Tracker, Rhodamine 123, JC-1) available for sperm of other species to detect the mitochondrial status (Partyka *et al.*, 2010). The probe gets transported into the active respiratory mitochondria with more active mitochondria accumulating more of the probe (Graham and Moce, 2005). Mito Tracker and Rhodamine 123, can only differentiate between respiring and non-respiring mitochondria while JC-1 can differentiate between respiring and non-respiring mitochondria as well as the level of respiring function by measuring the membrane potential. Like with membrane integrity, motility evaluation can act as a secondary evaluation of the mitochondrial status since the percentage of sperm with functioning mitochondria is highly related ($r = 0.97$) to the % of motile sperm (Auger *et al.*, 1989; Graham and Moce, 2005). However, some mammalian studies reported the absence of meaningful relations between mitochondrial status and sperm kinematic traits (Giannoccaro *et al.*, 2010; Martinez-Pastor *et al.*, 2010). No such studies have been conducted in ostriches yet.

2.3. *In vivo* fertility assessment

Fertility assessment *in vivo* at this stage is problematic since results are highly variable. A good correlation between *in vitro* and *in vivo* fertility traits needs to be established to allow extrapolation from *in vitro* results to the live female. *In vivo* fertility largely depends on the artificial insemination technique, the delivery of sufficient numbers of sperm to a site where uptake of sperm by the female oviduct will be most efficient. In the ostrich, the AI technique may be problematic because females do not crouch consistently, or do not remain in that position sufficiently long or are not relaxed to allow easy access to the oviduct. This technique relies on the full cooperation and may not be fully stress-free. Only a small number of females would exhibit voluntary crouch behaviour and also continue laying after the first and subsequent inseminations. To establish a flock of suitable females is of paramount importance to further protocol development for ostrich AI. However, little is still known about ostrich female sexual behaviour and its relationship to the imprinting process and eventual usage in an AI programme. Females are selected on voluntary crouch behaviour, but it appears that some females crouching spontaneously are not ready for AI. Studies to identify factors associated with receptivity of females to being inseminated is important, as this would allow dose delivery to a correct site and the retention of higher sperm numbers by the female on regular basis. Once the performance of females after AI is predictable, as they are after natural mating, and the optimal dose and frequency is established for fresh semen then such flocks will be ready for *in vivo* assessment of preserved semen.

2.4. *Biochemical and physiological analyses of the ostrich sperm cell and its seminal plasma*

The cell and its natural medium (seminal plasma) should be evaluated for its unique physiological and biochemical composition and for properties that are deemed necessary for future success in protocol development. An understanding of the molecular cellular components would allow prediction of the requirements and explain the behaviour of certain cell functions when processed and exposed to different stressors. The biochemical analyses for lipids and proteins should be prioritised, since these are the main constituents of the sperm membrane and they are closely associated with each other in a fluid bilayer. The latter is responsible for the proportion of lipids, specifically phospholipids and cholesterol, found in the seminal plasma together with that originating from the epididymides (Pickett and Komarek, 1966). The seminal lipids are a vital component since they relate to several

sperm functions that include structural support, metabolism, sperm capacitation and fertilization (Cross, 1998). Lipids consist mainly out of phospholipids, glycolipids and sterols. The lipid bilayer is dominantly composed out of phospholipids consisting of choline, ethanolamine phosphoglycerides and sphingomyelin. Phospholipids containing serine and inositol are less common in the bilayer. Sterol composition and the ratio of sterols to membrane phospholipids have been studied extensively as they contribute to processing stress resistance. The latter is due to their steric interaction with one another in modulating bilayer fluidity, stability and permeability (Parks *et al.*, 1981). It has been shown that the fatty acid composition of sperm, unsaturated and saturated fatty acids, greatly affects the sensitivity of the species to processing stress, as well as the ultimate success after storage (short- and long-term) for sperm function and fertilizing ability (Drobnis *et al.*, 1993; Blesbois *et al.*, 2005; Saragusty *et al.*, 2005;). Clearly there are further biochemical studies that need to be undertaken to assist in the further refinement of a protocol for the storage of ostrich semen.

3. Outlook

Great strides have clearly been made in the development of a feasible AI protocol specifically for ostriches. However, the existing protocol still needs to be refined further before being extended to the industry. This study proposes further refinements to the protocol for the short- and long-term storage of ostrich semen. However, concurrent studies on reproductive behaviour and physiology, imprinting and endocrinology of ostriches partaking in the development of a viable AI protocol are also needed. The option to substitute reproducing ostriches in pairs, trios and colonies would only become viable once all the boxes needed for the industry's acceptance are ticked. It will be prudent to extend this technology to the broader industry only at that stage.

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