A meta-analysis of the role of subpopulation analysis in the qualification and quantification of sperm sample quality, and the relationship with male fertility

by

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Declaration

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Summary

Given the predicted increase in the world population by 2050, the expected demand for animal products places increasing pressure on livestock farmers to farm as sustainable and cost-efficient as possible, taking environmental challenges such as global warming into account. The identification and selection of animals that have a superior ability to cope with and produce under conditions that are challenging, will assist livestock producers to contribute directly to food security. The development and application of assisted reproductive technologies (ART's) such as artificial insemination (AI) and *in vitro* embryo production (IVEP) and transfer, offers livestock producers the opportunity to overcome limitations such as the seasonal nature of reproduction in certain species, and long generation intervals. The successful application of ART's, however, requires the identification of quality spermatozoa that will be able to participate in fertilization. One of the limitations of current sperm sample evaluation protocols, is the inherent subjective nature of the evaluation protocols, which is largely due to the use of trained technicians, which in turn dictates that a certain level of experience is also required. This lead to the development of computer-assisted sperm analysis (CASA) systems, with the aim to overcome the limitation of the subjective nature of sperm sample evaluation protocols, and thus biased values obtained.

Sperm samples, whether ejaculate or epididymal in origin, naturally are characterized by a degree of heterogeneity, which contributed to the development of a sperm subpopulation approach to quantify sperm sample quality, and the fertilizing ability of spermatozoa in said samples. Knowledge of sperm fertilizing ability is important in determining male fertility under natural mating conditions (field fertility) or for use in ART's such as IVEP.

A meta-analysis was conducted to establish and quantify the use of sperm subpopulations to quantify male fertility by doing an in-depth analysis of existing peer-reviewed publications using specific criteria. All available web-based databases were used for this search, and publications that met all the selection criteria were thoroughly explored. Datasets for CASA-generated motility- and sperm head morphometry parameters were generated, and subjected to statistical analyses using a mode approach, where species, sperm subpopulation, type of CASA system used, and type of medium used, were considered as fixed effects.

Findings from this study highlighted the inconsistency between studies, as well as lack of an identified link between sperm subpopulations and male fertility, whether under field conditions or for use of spermatozoa in ART's. A lack of consensus amongst authors regarding subpopulation classification, especially in terms of the pre-determined cut-off values and thus the descriptive categories that are used to classify and describe sperm clusters, whether it be for sperm motility (i.e. fast, medium, and slow) or sperm head morphometry (i.e. large, short, round, elliptical, etc.), was reported. Given the multi-factorial nature of fertilization, standardization between research group and laboratories in terms of cut-off values for sperm subpopulations is warranted to allow for the establishment of whether a sperm subpopulation approach in quantifying sperm fertilising ability is firstly feasible, and secondly whether CASA can be incorporated in livestock and wildlife management programs as a management tool. It is recommended that future studies include both sperm motility and sperm head morphometry in the study designs, as this will provide a more accurate indication of sperm fertilising potential, and thus a male´s fertility. Additionally, studies should report more detail on

sample preparation, processing, and imaging, which will allow improved quantification of results and standardization between laboratories. Furthermore, there is under-representation for various species, and more studies are warranted to allow for the development of standardized species-specific protocols, which in turn can lead to the establishment of reproductive indices for animals, under field conditions and/or for use of their gametes in ART's, which in turn will assist livestock and wildlife managers to select species with an ability to cope under certain production conditions. Selection of wildlife species that can cope with challenging environmental conditions, will assist in maintaining of ecosystem stability, and also allow for the use of spermatozoa obtained from such animals, to be used in ART's in the establishment of genome resource banks for said species.

Opsomming

Gegewe die voorspelde toename in die wêreldbevolking teen 2050, plaas die verwagte vraag na diereprodukte toenemende druk op veeboere om so volhoubaar en kostedoeltreffend as moontlik te boer, met inagneming van omgewingsuitdagings soos aardverwarming. Die identifisering en seleksie van diere wat die beste vermoë het om onder moeilike omstandighede te produseer, sal veeprodusente help om direk tot voedselsekerheid by te dra. Die ontwikkeling en toepassing van ondersteunende reproduksietegnieke (ORT's), soos kunsmatige inseminasie (KI) en *in vitro* embrioproduksie (IVEP) en oordrag, bied veeprodusente die geleentheid om beperkings soos die seisoenale aard van voortplanting by sekere spesies en lang generasie-intervalle te oorkom. Die suksesvolle toepassing van ORT's vereis die identifisering van spermatozoë van gehalte wat aan vrugbaarheid kan deelneem. Een van die beperkings van die huidige spermmonster-evalueringsprotokolle is die inherente subjektiewe aard van die evalueringsprotokolle, wat grotendeels te wyte is aan die gebruik van opgeleide tegnici, wat weer bepaal dat 'n sekere vlak van ervaring ook nodig is. Dit het gelei na die ontwikkeling van rekenaargesteunde spermanalise (RGSA) stelsels, met die doel om die beperking van die subjektiewe aard van spermmonster-evalueringsprotokolle, en sodoende bevooroordeelde waardes, te oorkom.

Spermmonsters, hetsy ejakulaat of epididimaal van oorsprong, word natuurlik gekenmerk deur 'n mate van heterogeniteit, wat bygedra het tot die ontwikkeling van 'n spermsubpopulasie-benadering om die kwaliteit van die spermmonsters te kwantifiseer, en die bevrugtingsvermoë van spermatozoë in genoemde monsters te kwantifiseer. Kennis van spermvrugbaarheidvermoë is belangrik om manlike vrugbaarheid te bepaal onder natuurlike paringstoestande (veldvrugbaarheid) of vir gebruik in ORT's soos IVEP.

'n Meta-analise is uitgevoer om die gebruik van spermsubpopulasies om die vrugbaarheid van manlike diere te bepaal, vas te stel en te kwantifiseer deur 'n diepgaande analise te doen van bestaande portuurbeoordeelde publikasies volgens spesifieke kriteria. Alle beskikbare webgebaseerde databasisse is vir hierdie soektog gebruik en publikasies wat aan al die seleksiekriteria voldoen, is deeglik ondersoek. Datastelle vir RGSA-gegenereerde motiliteits- en spermkop morfometrie-parameters is gegenereer en onderwerp aan statistiese ontledings met behulp van 'n modusbenadering, waar spesies, subpopulasie van die sperma, tipe RGSA-stelsel en die gebruikte medium, as vaste effekte beskou word.

Bevindinge uit hierdie studie het die teenstrydigheid tussen studies beklemtoon, sowel as die gebrek aan 'n geïdentifiseerde verband tussen spermsubpopulasies en manlike vrugbaarheid, hetsy onder veldtoestande of vir die gebruik van spermatozoë in ORT's. 'n Gebrek aan konsensus tussen outeurs rakende subpopulasie-klassifikasie, veral in terme van die voorafbepaalde afsnywaardes en dus die beskrywende kategorieë wat gebruik word om spermklusters te klassifiseer en te beskryf, hetsy vir spermmotiliteit (d.w.s. vinnig, medium en stadige) of spermkopmorfometrie (d.w.s. groot, kort, rond, ellipties, ens.), is gerapporteer. Gegewe die multifaktoriese aard van vrugbaarheid, is standaardisering tussen navorsingsgroep en laboratoriums in terme van afsnywaardes vir spermsubpopulasies, geregverdig om vas

te stel of 'n spermsubpopulasiebenadering in die kwantifisering van die bevrugtingsvermoë van die sperm eerstens haalbaar is, en tweedens of RGSA in vee- en wildbestuur opgeneem kan word programme as 'n bestuursinstrument. Dit word aanbeveel dat toekomstige studies sowel spermmotiliteit as spermkopmorfometrie in die studieontwerpe insluit, aangesien dit 'n akkurater aanduiding van die bevrugtingspotensiaal vir sperms en dus die vrugbaarheid van 'n manlike dier sal gee. Daarbenewens moet studies meer besonderhede oor voorbereiding, verwerking en beelding van monsters rapporteer, wat verbeterde kwantifisering van resultate en standaardisering tussen laboratoriums moontlik maak. Verder is daar onderverteenwoordiging vir verskillende spesies, en meer studies is geregverdig om die ontwikkeling van gestandaardiseerde spesiespesifieke protokolle moontlik te maak, wat weer kan lei tot die daarstelling van reproduksie-indekse vir diere, onder veldtoestande en/of vir die gebruik van hul gamete in ORTs, wat weer vee- en wildbestuurders sal help om spesies te selekteer wat die vermoë het om sekere produksietoestande die hoof te kan bied. Die seleksie van wildspesies wat die uitdagende omgewingstoestande die hoof kan bied, sal help om die stabiliteit van die ekosisteem te handhaaf, en ook die gebruik van spermatozoë wat van sulke diere verkry word, te gebruik in ORT's vir die instelling van genoombronne vir genoemde spesies.

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Preface

This thesis is presented as a compilation of 6 chapters:

Chapter 1 General Introduction

Chapter 2 Literature Review

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Chapter 5 The relationship between sperm subpopulation classification parameters, sperm morphometric traits, and sperm fertilizing potential

Chapter 6 Discussion, General Conclusion and Recommendations

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Alphabetical List of Abbreviations

°C	Degrees Celsius
µL	Microliter
$\mu.ms^{-1}$	Micrometre per second
μm^2	Micrometre squared
%	Percentage
AI	Artificial Insemination
ART	Assisted Reproductive Technology
BCF	Beat Cross Frequency
CASA	Computer-Aided Sperm Analysis
DEA	Department of Environmental Affairs
DNA	Deoxyribonucleic Acid
fps	Frames per Second
FT	Frozen-thawed
Hz	Hertz
IVEP	*In Vitro* Embryo Production
LIN	Linearity coefficient
ml	Milliliter
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
SD	Standard Deviation
SDD	Study Standard Deviation
sec	Seconds
SEM	Standard Error of the Mean
spz	Spermatozoa
STR	Straightness of track
UN	United Nations
VAP	Average Path Velocity
VCL	Curvilinear Velocity
VSL	Straight-Line Velocity
WHO	World Health Organization
WOB	Wobble coefficient

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Chapter 1

General Introduction

The expected increase in the global population by 2050 (UN, 2017; Godfray, 2018) necessitates that livestock and game producers farm as cost-effectively as possible to ensure sustainable use of available space and resources to ensure the viability of their production systems, and ultimately food security. This becomes even more important against the background of global warming, which places increasingly more pressure on livestock producers to produce enough and safe food under the abovementioned circumstances (Root *et al.*, 2003; Xu & Victor, 2018). Southern Africa is expected to experience an increase in ambient temperature of more than 4°C by the end of 2099 due to global warming (DEA, 2019). It is therefore essential to be able to identify animal breeds that have the potential to adapt and cope, in terms of production, with the expected increase in environmental temperature. When the conservation of indigenous wildlife species is considered and seen against the background of the income that is generated by this sector in terms of ecotourism, hunting and sale of live animals, it becomes imperative to manage the conservation risks that factors such as global warming may pose to the survival of wildlife species, which in turn is important for ecosystem stability.

The development and use of assisted reproductive techniques (ART's) such as artificial insemination (AI) and *in vitro* embryo production (IVEP) and transfer, can assist livestock producers and game ranch managers, in conjunction with the genetic selection of superior animals, to overcome the challenges of modern livestock production and wildlife conservation. Genetic selection will assist with the identification of animals that carry desired traits to perform under certain production conditions and may involve among others, the use of quantitative trait loci (QTL's) or single nucleotide polymorphisms (SNP's) (Miyata *et al.*, 2007; De Koning, 2008; Dekkers, 2012). The identification and preservation of the genetic material of such animals can contribute to the establishment of genome-resource banks (GRB's) which can act as repositories should species be threatened by extinction or disease. The selection of breeds that are superior in terms of production and reproduction ability and resistance to diseases, will thus allow for herd or flock sizes to be decreased, which in turn will potentially alleviate the pressure on natural resources and decrease the space required for animal production activities.

The successful application of ART's requires the identification and use of sperm samples of acceptable quality. Traditionally the reproductive contribution of a male animal to its offspring sired is quantified by a reproductive soundness examination, and conventional macroscopic and microscopic evaluation of the

male's semen sample, according to specific criteria (Oehninger *et al.*, 2014). Microscopic criteria include the parameters viability, morphology, acrosome integrity and motility (Sudano *et al*., 2011). Before the development of computer-assisted software programs such as AndroVision® (Minitube, Spain) or the Sperm Class Analyzer (SCA®; Microptic, Spain) package, sperm sample evaluation was performed by using a direct microscope approach, specific staining materials or dyes, and trained technicians. The biggest consequence of this approach, however, is a variation of up to 60% in results reporting on sample quality parameters (Verstegen *et al.*, 2002; Brito, 2010).

This subjective nature of semen sample evaluation prompted the development of technologies such as computer-aided sperm analysis (CASA), in an effort to overcome this limitation and allow for the objective analysis of sperm motility and morphometry (Verstegen *et al.*, 2002; Amann & Waberski, 2014; Yániz *et al.*, 2015b). Typically CASA systems will consist of hardware and software components that enable researchers to digitize and quantify sperm parameters in order to provide meaningful information on spermatozoa and sperm populations (Amann & Katz, 2004). Early studies demonstrated the potential value of CASA in male fertility prediction through the correlation of sperm velocity with hamster oocyte fertilization (De Geyter *et al.*, 1998). Several improvements and refinements of the CASA approach resulted in the incorporation of more parameters to potentially allow for a more accurate prediction of sperm sample quality. Apart from motility and morphometry, additional parameters such sperm viability, DNA fragmentation, acrosome integrity, and sperm concentration can be automatically determined (Van der Horst, 2020).

Sperm motility is considered to be essential for fertilization success, as it ensures that a sperm cell is able to transit through the relatively hostile environment of the female reproductive tract in order to reach the oocyte to ultimately achieve fertilization (Maroto-Morales *et al.*, 2016; Hook & Fisher, 2020). Motility has thus been used as a reliable measure of sperm fertilizing ability and much emphasis has been placed on continued motility research. According to Bompart *et al.* (2019), motility can be considered as a relatively accurate indicator of fertility, however, the quantification of motility can be influenced by the level of experience of the evaluator and the approach or classification of motility classes used.

Morphometry, which is considered to be a sperm trait that is genetically determined, is considered by various authors to be a better predictor of the fertilizing ability of spermatozoa (Soler *et al.*, 2000; Mossman *et al*., 2009; Valverde *et al.*, 2016). The proportion of spermatozoa with heads that are characteristically large and long have been consistently found to achieve higher rates of fertility (Yániz, 2015a). In addition to sperm morphology, evaluation of sperm morphometry is considered crucial in assisting in the determination of the fertilizing ability of spermatozoa. Samples containing a high percentage of abnormal sperm have been shown to indicate a lower level of fertility in both humans and a variety of domestic species (Gravance *et al.*, 1997; Boersma *et al.*, 1999; Verstegen *et al.*, 2002; Yániz *et al.*,

2015a). Sperm morphometric parameters have also been linked to sperm cryo-resistance (Maroto-Morales *et al.*, 2016), and have been reported to relate to sperm kinematic parameters (Ramón, 2013). Despite the potential positive association between sperm motility, morphometry and fertility, it needs to be recognised that these parameters have not proven effective in predicting fertility, which potentially is a consequence of the evaluation of spermatozoa that, in the female reproductive tract, may not participate in fertilization at all (Holt & Van Look, 2004).

With the use of CASA, it is important to recognise that any biological sample such as a sperm sample, is characterized by a degree of heterogeneity, which in turn is determined by the existence of subpopulations within the sample (Rodríguez-Martínez, 2003; Holt & Van Look, 2004). There is increasing evidence that, under natural mating conditions, the occurrence of heterogeneity within a sperm sample has potential implications for a sperm's fertilizing ability and thus has functional relevance. Theoretically, the degree of heterogeneity present in a sperm sample would ensure a greater potential for an oocyte to be fertilized (Yániz *et al.*, 2015a). During CASA analysis, subpopulations are classified by using pre-determined cut-off values. In several studies that involved the use of subpopulations to quantify sample quality, no clear indication was provided to justify the selection of the cut-off values that were used to determine the subpopulations in terms of motility and morphometry. The absence of linking sperm subpopulations to fertilization success, complicates the standardization of evaluation protocols between laboratories as well as between species, which in turn complicates the standardization of the selection of samples for use in ART's or the characterization of a breed in terms of its prolificacy under certain production conditions.

To the best of our knowledge, no meta-analysis study has been performed to date on the use of sperm subpopulation analyses to quantify fertility and sperm fertilizing ability in animals i.e. terrestrial and aquatic animals. With existing studies, interesting relationships between sperm subpopulation structure and certain parameters emerged, however, no clear relationship between sperm subpopulation structure and fertilization success could be determined. The aim of this meta-analysis study is therefore to, by means of an in-depth analysis of existing studies that involved the use of sperm subpopulations to study sperm motility and morphometry, to determine the degree of standardization of subpopulation analysis between species and within species. A basic model approach was used for motility and morphometry and included species, sperm subpopulation, type of CASA system and medium used, respectively for motility; and breed, sperm subpopulation, and type of CASA system used for morphometry.

The study will assist in improving our understanding of the importance of the identification of objective sperm criteria that can potentially be included in a selection index that will allow for the identification of individuals that produce sperm with a superior fertilizing ability, and potentially will allow the species to produce optimally under challenging production conditions.

1.1 References

Amann, R.P., & Katz, D.F. 2004. Reflections on CASA after 25 years. J. Androl. 25,317–325.

Amann, R.P., & Waberski, D. 2014. Computer-assisted sperm analysis (CASA): Capabilities and potential developments. Theriogenology 81, 5-17.

Boersma, A.A., Braun, J., & Stolla, R. 1999. Influence of random factors and two different staining procedures on computer-assisted sperm head morphometry in bulls. Reprod. Domest. Anim. 34, 77–82.

Bompart, D., Vázquez, R.F., Gómez, R., & Valverde, A. 2019. Combined effects of type and depth of counting chamber, and rate of image frame capture, on bull sperm motility and kinematics. Anim. Reprod. Sci. 209, 106-169.

Brito, L.F.C. 2010. Variations in laboratory semen evaluation procedures and testing. Proc. 23rd Tech. Conf. Artif. Insemin. Reprod. 1–7.

Dekkers, J.C.M. 2012. Application of genomics tools to animal breeding. Curr. Genomics 13, 207–212.

De Geyter, C., De Geyter, M., Koppers, B., & Nieschlag, E. 1998. Diagnostic accuracy of computer-assisted sperm motion analysis. Hum. Reprod. 13, 2512–2520.

De Koning, D.J. 2008. Application of molecular information in sustainable animal breeding. Rev. Bras. Zootec. 37, 122–126.

Department of Environmental Affairs (DEA). 2016. South Africa's first annual climate change report. [online] Available at: https://www.environment.gov.za/otherdocuments/reports/monitoring_climatechange_responses [Accessed 15 Nov. 2020].

Godfray, H.C.J., Beddington, J.R., Crute, I.R., Haddad, L., Lawrence, D., Muir, J.F., Pretty, J., Robinson, S., Thomas, S.M., & Toulmin, C. 2010. Food security: The challenge of feeding 9 billion people. Science 327, 812-818.

Gravance, C.G., Champion, Z., Liu, I.K.M., & Casey, P.J. 1997. Sperm head morphometry analysis of ejaculate and dismount stallion semen samples. Anim. Reprod. Sci. 47, 149–155.

Holt, W.V., & van Look, K.J.W. 2004. Concepts in sperm heterogeneity, sperm selection and sperm competition as biological foundations for laboratory test of semen quality. Reproduction 127, 527–535.

Hook, K.A., & Fisher, H.S. 2020. Methodological considerations for examining the relationship between sperm morphology and motility. Mol. Reprod. Dev. 87, 633–649.

Van der Horst, G. 2020. Computer Aided Sperm Analysis (CASA) in domestic animals: Current status, three D tracking and flagellar analysis. Anim. Reprod. Sci. Article in Press.

Maroto-Morales, A., García-Álvarez, O., Ramón, M., Martínez-Pastor, F., Fernández-Santos, M., Soler, A., & Garde, J. 2016. Current status and potential of morphometric sperm analysis. Asian J. Androl. 18, 863–870.

Miyata, M., Gasparin, G., Coutinho, L.L., Martinez, M.L., Machado, M.A., Barbosa da Silva, M.V.G., Campos, A.L., Sonstegard, T.S., Fernandes do Rosário, M., & de Almeida Regitano, L. C. 2007. Quantitative trait loci (QTL) mapping for growth traits on bovine chromosome 14. Genet. Mol. Biol. 30, 364–369.

Mossman, J., Slate, J., Humphries, S., & Birkhead, T. 2009. Sperm morphology and velocity are genetically codetermined in the zebra finch. Evolution 63, 2730–2737.

Oehninger, S., Franken, D.R., & Ombelet, W. 2014. Sperm functional tests. Fertil. Steril. 102, 1528–1533.

Ramón, M., Soler, A.J., Ortiz, J.A., García-Alvarez, O., Maroto-Morales, A., Roldan, E.R.S., & Garde, J.J. 2013. Sperm population structure and male fertility: An intraspecific study of sperm design and velocity in Red Deer. Biol. Reprod. 89, 1–7.

Rodríguez-Martínez, H. 2003. Laboratory semen assessment and prediction of fertility: Still utopia? Reprod. Domest. Anim. 38, 312–318.

Root, T.L., Price, J.T., Hall, K.R., Schneider, S.H., Rosenzweig, C., & Pounds, J.A. 2003. Fingerprints of global warming on wild animals and plants. Nature 421, 57–60.

Soler, C., Pérez-Sánchez, F., Schulze, H., Bergmann, M., Oberpenning, F., Yeung, C.H., & Cooper, T.G. 2000. Objective evaluation of the morphology of human epididymal sperm heads. Int. J. Androl. 23, 77–84.

Sudano, M. J., Crespilho, A. M. I., Fernandes, C. B., Junior, A. M., Papa, F. O., Rodrigues, J., MacHado, R., & Landim-Alvarenga, F. D. C. 2011. Use of bayesian inference to correlate in vitro embryo production and *in vivo* fertility in Zebu bulls. Vet. Med. Int. 2011, 10–12.

United Nations (UN). World population projected to reach 9.8 billion in 2050, and 11.2 billion in 2100. 2017 [online] Available at: https://www.un.org/development/desa/en/news/population/world-population-prospects-2017.html#:~:text=News-,World%20population%20projected%20to%20reach%209.8%20billion%20in,and%2011.2%20billion%20in%202100&text=The%20current%20world%20population%20of,Nations%20report%20being%20launched%20today. [Accessed 15 Dec. 2020].

Valverde, A., Arenán, H., Sancho, M., Contell, J., Yániz, J., Fernández, A., & Soler, C. 2016. Morphometry and subpopulation structure of Holstein bull spermatozoa: Variations in ejaculates and cryopreservation straws. Asian J. Androl. 18, 851–857.

Verstegen, J., Iguer-Ouada, M., & Onclin, K. 2002. Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology 57, 149-179.

Xu, Y., Ramanathan, V. & Victor, D.G. 2018. Global warming will happen faster than we think. [online] Nature. Available at: https://www.nature.com/articles/d41586-018-07586-5 [Accessed 16 Nov. 2020].

Yániz, J.L., Palacín, I., Vicente-Fiel, S., Sánchez-Nadal, J.A., & Santolaria, P. 2015a. Sperm population structure in high and low field fertility rams. Anim. Reprod. Sci. 156, 128–134.

Yániz, J.L., Soler, C., & Santolaria, P. 2015b. Computer assisted sperm morphometry in mammals: A review. Anim. Reprod. Sci. 156, 1–12.

Chapter 2

Literature Review

According to United Nations (UN) sources, the world population is expected to grow to reach almost 10 billion by the year 2050, which implies that the production of safe and sufficient food becomes imperative (UN, 2017). About two thirds of the growth in the human population is expected in Africa (UN, 2019). Linked to the increase in the human population, the demands for meat, milk and cereal products are projected to increase substantially (FAO, 2020). To meet the demand for meat and milk, it requires that livestock producers ensure that they produce these products as optimally and cost-efficiently as possible, to thus ensure the viability of their enterprises as well as sustainable use of resources. The genetic selection of animals that are prolific is thus of utmost importance to ensure optimal animal production against the background of a decrease in available surface area to carry out animal production activities. Modern livestock production systems also need to accommodate environmental challenges such as global warming, with the expected increase in ambient temperature posing an additional physiological stress on maintenance of homeostasis in animals. Southern Africa is particularly susceptible to the effect of global warming, and livestock producers thus needs to be able to mitigate this by making use of genetic selection and improved animal production practices.

When the management of the production and reproduction ability of livestock and wildlife species is considered, it is evident that a firm understanding of reproductive physiology is fundamental to allow for the optimal management to ensure that the wellbeing of the animals can be maintained under natural as well as production conditions. A proper knowledge of the reproductive physiology of an animal is also required when ART's form part of the management "toolbox" of livestock and wildlife producers. The development of ART's was required by a need to overcome inherent reproductive problems such as the seasonality of reproduction in e.g. sheep in temperate zones, and mating difficulties, and to decrease generation intervals and thus accelerate genetic progress *(*Morrow *et al*., 2009; Amiridis & Cseh, 2012; Warriach *et al*., 2015; Comizzoli *et al*., 2018). Some of the most used ART's include AI, IVEP, embryo transfer (ET) and transvaginal ultrasound-guided ovum pickup (OPU).

Assisted reproductive techniques are considered a powerful tool for the production of animals for biomedical research, rapid propagation of the germplasm of genetically superior animals, as well as the conservation of endangered livestock and wildlife species (Morrow *et al*., 2009; Amiridis & Cseh, 2012; Warriach *et al*., 2015). Animal ART's have been clearly demonstrated to impact positively on animal health and -production, and allows for the international exchange of genetic material through frozen semen and embryos. The use of ART's also allows for the research of factors that can determine the quality and

viability of spermatozoa and oocytes, and ultimately influence the developmental competence and implantation potential of embryos (Purohit *et al*., 2000; Loutradi *et al*., 2006).

Central to the successful application of ART's is the identification of gametes that are suitable for use and that will allow for successful fertilization, and ultimately embryo development and implantation (Benkhalifa & Menezo, 2019). The importance of the identification of viable spermatozoa and their contribution to the success of ART application, is often underestimated. The development of standardized sperm evaluation criteria for animal species will allow for the standardization between laboratories and breeding centres, which in turn will allow for comparison of species between regions, producers, and countries. To the best of our knowledge, when the livestock and wildlife industries are considered, no set of standardized criteria, such as what has been developed and adopted in human reproduction centres, exists. Standardization of sperm evaluation criteria will assist in the development of management and breeding programs that will contribute to the genetic progress when selection is carried out for specific traits, as well as selection of animals that produce sperm of optimal quality.

When the evaluation of sperm samples is considered, it is important to recognize the purpose of the evaluation, and the fate of the spermatozoa evaluated. The evaluation of sperm quality and viability is required for the breeding soundness exams of male animals, as well as the identification of samples for use in ART's. Sperm samples can be collected by means of the artificial vagina method, by using electro-ejaculation or epididymal aspiration, or testicular biopsy. The latter method is seldom used in livestock and wildlife species, with the epididymal method used when high genetic merit animals die, and the germplasm of this animal needs to be preserved for propagation in future populations or for conservation purposes.

When the evaluation of sperm samples is considered, evaluation is carried out using a macroscopic and microscopic approach. Macroscopic criteria include volume, colour, pH, mass motility, and viscosity (WHO, 2010). Macroscopic parameters provide a relative indication of the quality and viability of the sample and is normally used when a sample needs to be evaluated in the field for immediate use or disqualification. Microscopic parameters are more important in terms of the qualification and quantification of sperm sample quality, and include amongst others, parameters such as motility, morphology, morphometry, DNA integrity, and acrosome and membrane integrity (WHO, 2010). Both acrosome and membrane integrity play an important role in the processes that lead up to and culminate in successful fertilization (Flesch & Gadella, 2000; Srivastava *et al.*, 2013; Eskandari & Momeni, 2016). Equipment employed in the microscopic evaluation of sperm samples include most commonly microscopes (e.g. brightfield or fluorescent), different dyes (e.g. eosin-nigrosine or fluorescent probes, depending on the parameter studied) to computers and different software programs (e.g. AndroVision® or SCA® programs) (Larsson & Rodríguez-Martínez, 2000; Holt & Van Look, 2004; Sellem *et al*., 2015). In certain industries such as the dairy and beef industries, flow

cytometry may also be used to sex sperm samples for the use in ART's to produce heifers for the dairy industry, and bull calves for the beef industry (Sellem *et al.*, 2015). The use of flow cytometry allows for a much more sensitive analysis of sperm samples, with a higher proportion of cells that can be analysed, when compared to e.g. standard microscopic evaluation.

Although the current conventional methods are not considered as the ultimate to predict the quality and fertilizing potential of a sperm sample, these methods can be used to establish the overall quality of a sample. It is important to recognise that not all sperm in a particular sample are capable of fertilizing. According to Holt & Van Look (2004), several studies suggest that most of the spermatozoa in a sample do not participate in the actual fertilization process. This implies that when samples are being considered for use in ART's, it is even more important to identify sperm samples that have a higher probability to ensure a successful fertilization outcome. Before any male is selected for use in a breeding program and whether his sperm samples can be used in ART's, it is crucial to establish the breeding soundness of the male (e.g. which can be influenced by nutrition and disease status) and the quality of the sperm samples produced by the male by using the methods mentioned above.

One of the drawbacks of the microscopic evaluation of sperm samples is the subjective nature of the quantification of the respective parameters (Auger, 1997; Gallego *et al*., 2018; Imbachi, 2018). Even though technicians are trained, there is an unavoidable degree of subjectivity involved. This prompted the development of CASA software to potentially provide a more objective evaluation of sperm sample quality. CASA can be used to simply aid in the determination of the quality of a sperm sample of a production animal and thus to obtain an overall indication of the male's fertility, but may also be used as part of a more detailed approach when a male is being assessed for suitability for use in an ART program. According to Rodriguez-Martinez (2003), current laboratory measures provide more of estimation, rather than a prediction of field fertility of a given sire. According to Amann (1989), it is important to have standardized lab tests that will as accurately as possible, predict the fertilizing potential of spermatozoa obtained from a male animal.

When the use of CASA in sperm sample evaluation is considered, it is important to note that the calibration of cut-off values needs to consider the ultimate purpose that the spermatozoa will be used for. The cut-off values for the determination of sperm motility during the evaluation of the breeding soundness of a male will differ considerably from the cut-off values used to quantify e.g., fast, medium- and slow-swimming spermatozoa in hyperactivation studies (Maree & Van der Horst, 2013). Even though CASA methods are more objective, the potential of the approach in determining the "perfect sperm" in terms of fertilization, still needs to be refined. Fertilization is a complex process and although it seems logic to qualify and quantify a set parameters to identify the "perfect" sperm sample, the heterogeneity (or inherent presence

of subpopulations) of a sample and how they interact, has been found critical for fertilization success (Rodríguez-Martínez, 2003).

The purpose of the meta-analysis is thus to consider all potential factors that can influence the potential of the sperm subpopulation approach to qualify and quantify sperm sample quality in animals. Firstly, the important components of a sperm cell that are used in the classification of subpopulations will be presented. This will be followed by processing factors that can influence the determination of cut-off values for subpopulation classes.

2.1 Qualification and quantification of sperm quality

Sperm sample quality consists of the determination of viability (% live and % normal sperm) and fertilizing potential (DNA- and acrosome integrity). The evaluation of a sample may be performed according to various procedures and includes micro- and macroscopic parameters. Macroscopic evaluation occurs most frequently under field conditions and entails the following:

- **Volume:** The volume of an ejaculate is influenced by various factors, such as the age of the male, the season of collection, the method of collection and the collection frequency. Additionally, the volume of semen produced will vary between species.
- **pH:** The pH measures the acidity or alkalinity of the sample and will differ slightly between different species. The sample's pH will influence the metabolic rate and thereby, the motility of the sperm.
- **Viscosity:** A sperm sample may be very viscous (concentrated) or more fluid (dilute) depending on the health of the male and the sample collection method e.g. samples collected via electro-ejaculation tend to be much less-viscous as the seminal plasma component forms part of the sample.
- **Sample colour:** Normal sperm sample colour after collection ranges from milky-white to creamy-white. Abnormal sample colour may be an indication of dead cells within the sample or contamination (blood and/or urine).

Microscopic evaluations, on the other hand, are of importance when the procedures require detailed information on the structural and functional integrity of the spermatozoa, such as is required for inseminations to be performed after the collection of a fresh semen sample or in the case where the semen will undergo long term storage (cryopreservation). These evaluations require a microscope and a trained technician and can be considered in terms of the sperm head, mid-piece and tail.

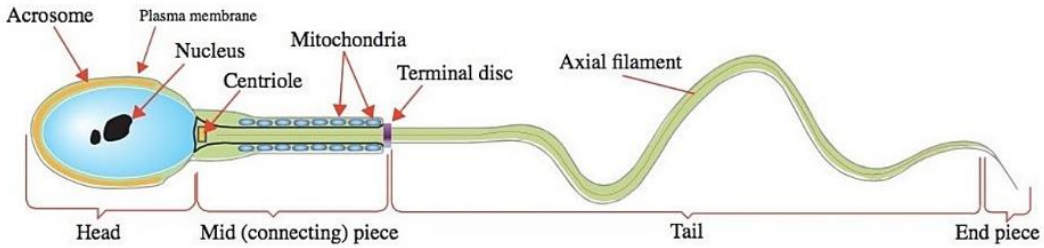

Figure 2.1 The structure of a sperm cell (Gizmodo.com.au, 2015).

2.1.1 Head

The mammalian sperm head contains genetic material in the form of chromosomes, found within the nucleus. DNA integrity is a crucial part of the reproductive potential toolkit as genetic integrity is essential to ensure healthy offspring. Sperm DNA damage has been closely associated with factors such as decreased embryo quality, lower fertilizing ability, implantation errors and malformations (Shamsi *et al.*, 2011).

The sperm head is surrounded by a plasma membrane and capped by an acrosome which contains specific enzymes, namely hyaluronidase, acrosin, zona lysine, esterases, and acid hydrolases, which enables the penetration of the *zona pellucida* that surrounds an ovum (Senger, 2003). Acrosome integrity is one of the most important sperm quality tests as it is strongly associated with fertility, due to the acrosome being involved in one of the most important reactions necessary for fertilization (Yániz *et al.*, 2015). An abnormal acrosome results in an inability for spermatozoa to penetrate the zona pellucida and fertilize an oocyte. Head malformations, in addition, contribute to a lower % normal sperm (Figure 2.2).

2.1.2 Mid-piece

The mid-piece houses the mitochondria that produce energy through a process called oxidative phosphorylation (Turner, 2003). Oxygen is converted into the energy currency of cells, (in the form of) ATP (adenosine triphosphate), that allow the sperm to travel/navigate through the female tract and reach the ovum. Abnormalities here too may occur and will affect the percentage normal sperm in a sample. Abnormalities include a bent or irregular mid-piece or the absence of mitochondria (Salvador & Fernandez, 2018).

2.1.3 Tail

The tail is an essential factor in the determination of the viability (or vitality) of a sperm sample due to this component of the sperm cell being crucial in the movement of a spermatozoon. Sperm motility is largely dependent on the availability of utilisable energy (ATP), supplied through the breakdown of glucose

molecules (glycolysis) in the tail, and was confirmed by Mukai & Okuno in 2004. Viability is measured as the percentage of sperm in a given sample which are alive, displaying progressive motility patterns and the percentage of sperm which display a normal morphology, therefore the viability is indicative of the sample´s quality and the fertilization potential (Rodríguez-Martínez, 2003). Live sperm are determined through either staining cells or through an osmotic swelling test, which provides information on the sperm's cell membrane integrity (WHO, 2010). Normal sperm are determined through staining. Samples with tail defects (Figure 2.2) leading to a viability of lower than 70% are often discarded.

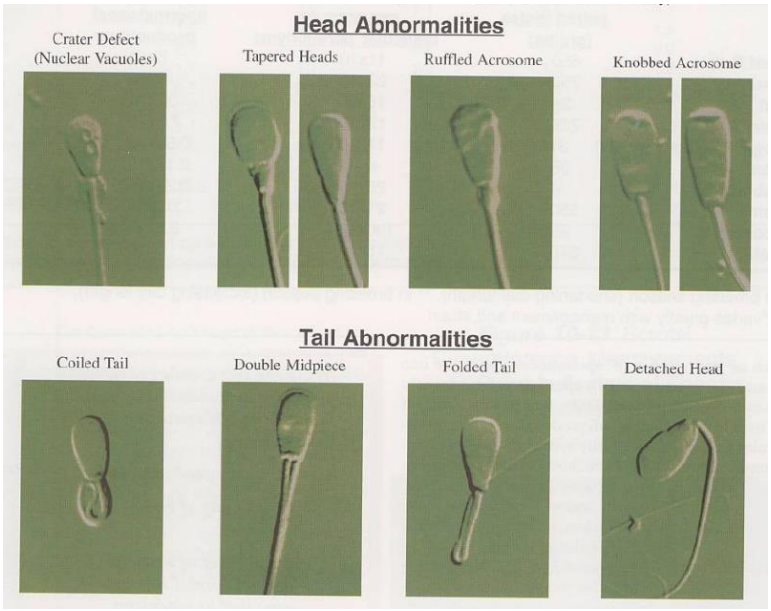

Figure 2.2 Head and tail abnormalities which may occur in bull sperm (Senger, 2003).

In terms of microscopic evaluation, the following two parameters also form a vital part of the determination of sample quality:

- **Sperm concentration:** A hemacytometer is generally used to determine the concentration of a sample. Samples containing a high concentration of sperm are found to be more viscous.
- **Sperm motility:** Sperm motility can be assessed in terms of progressive motility, individual motility and mass motility. Depending on the degree of motility, sperm are assigned a value of 0-5, with 0 assigned to samples displaying a majority of non-motile sperm and 5 assigned to samples with more than 80% of sperm displaying vigorous motion) (Hossain *et al*., 2012).

2.2 Processing and evaluation of spermatozoa

After sperm samples are collected and evaluated macroscopically, the samples can be stored in one of two forms. The ultimate use of the sperm sample will determine the type of storage method used. For short-term storage, sperm samples are maintained in a liquid state, whereas for long-term storage, sperm

samples are cryopreserved with and stored in liquid nitrogen. Storage inevitably influences the viability of sperm samples, regardless of the type of storage method selected.

Liquid storage may be carried out at various temperatures, depending on the species and extender in use. Most of the sperm quality parameters (%live, %normal and acrosome integrity) are influenced by this type of storage, however, Sadeghi *et al.* (2020) found that DNA fragmentation did not occur when this state of storage was applied. The extender, temperature and time of storage will further determine the degree to which the abovementioned parameters are influenced. Although the fertilizing potential of sperm stored in a liquid state is inevitably influenced, the maintenance of DNA integrity allows sperm to still be of use in ART's such as intra-cytoplasmic sperm injection (ICSI) that do not require spermatozoa to be progressively motile.

Despite cryopreservation currently being considered the most effective method of long-term storage for spermatozoa, it undoubtedly has a deleterious effect on sperm parameters such as motility, viability, and DNA integrity and acrosome integrity, respectively (Sharma *et al.*, 2015). Various studies have indicated sub-optimal conception rates when using frozen-thawed semen versus fresh semen (Cayan *et al.*, 2001; Lambo *et al*., 2012). The compromise in the fertilizing potential of spermatozoa can mainly be ascribed to the destabilization of the plasma lipid membrane caused by the freezing process, which in turn results in a loss of sperm function, subsequently decreasing the fertilizing potential of the spermatozoa for use in ART's.

2.3 Use of CASA in sperm subpopulation studies

A CASA system typically consists of hardware components (e.g. microscope with heated stage, camera, computer) and 3rd generation software which may be used to digitize and quantify sperm parameters in order to provide meaningful information on sperm cells and the subpopulations in a given sample (Amann & Katz, 2004). Each CASA system uses a customized slide that has a chamber(-s) with specific dimensions that will allow the spermatozoa to swim without any obstruction in the chamber.

Classification for sperm motility subpopulations include rapid, medium and slow. Additionally, sperm may be categorized as rapid progressive, medium progressive or non-progressive. CASA calculates the percentage sperm for each speed category based on the pre-determined cut-off values and although the same breed and medium is considered, there may be differing percentages of sperm found within each category. In terms of sperm morphometry subpopulation classification, sperm may be categorized per head shape. The subpopulation classification may indicate the sperm maturity as the head shape changes as the spermatozoon moves through the different sections of the epididymis (caput, corpus, cauda). Sperm are

rendered fully functional and capable to swim and fertilize successfully, once they have completed the full time period that needs to be spent in the sections of the epididymis.

The most commonly available CASA systems include:

- **Integrated Sperm Analysis System (ISAS)**

ISAS is an efficient system for use in various species, including both humans and animals for veterinary research. Parameters which can be measured are motility, morphometry, DNA fragmentation, vitality, concentration, and later versions being able to determine acrosome integrity. The chamber can be either 10µm or 20µm in depth, allowing more than 2000 sperm cells per field to be analysed, with a frame rate of up to 200 fps.

- **Sperm Class Analyzer (SCA®)**

The SCA® system measures kinematic parameters, sample concentration, sperm morphology, DNA fragmentation, vitality, and acrosome integrity, as well as detect distal droplets. The system can be used with positive phase, negative phase or fluorescence -microscopy and various editions available such as for human, veterinary and toxicology use. A chamber is used and several chamber types are available – 10µm (2 or 4 chambers) or 20µm (2, 4 or 8 chambers).

- **Internal Visual Optical System (IVOS)**

IVOS is currently the only sperm analyser with an internal optical system that uses an illumination strobe in order to eliminate motion blurring. The system, developed for animal semen analysis, measures morphometry, morphology, motility, doses, concentration, percentages motile and progressive sperm. A chamber of 20µm is used (4 or 8 chambers).

- **SpermVision**

SpermVision is a user-friendly software, ideal for small animal reproduction. A phase contrast system is implemented to measure parameters such as concentration, motility, morphology, and viability. Additionally, the software can calculate doses and amount of extender needed to process each ejaculate.

- **ImageJ**

ImageJ is a Java-based, open-access software developed by The National Institute of Health and The Laboratory for Optical and Computational Instrumentation. ImageJ allow for the annotation, editing, calibration, measurement, analysis and processing of digitized images. Since its release in 1997, it has contributed paramount to a variety of scientific projects.

- **AndroVision**

Optimal for mobile use, this CASA system can analyse the basic sperm parameters, such as motility, concentration, and morphology, and additionally can analyse sperm functionality (sperm viability, acrosome integrity, mitochondrial activity and DNA integrity) through fluorescence-based assessments. More than 1000 sperm can be analysed per field for a variety of species, including cattle, sheep, and horses. The system is marketed as delivering results which are both accurate and precise.

2.4 Approach to subpopulation classification

Subpopulations are clusters or groupings of sperm that are either morphometrically or kinematically distinct from one another, and co-exist within the same semen sample (Martinez-Pastor *et al*., 2011). The strategy of placing sperm from a single sample into different subpopulations was developed as a way to categorize classes of sperm found naturally within a heterogeneous sample (Yaniz *et al*., 2015).

The different CASA systems have been developed to analyse both sperm motility and morphometry subpopulations. However, the precision, accuracy and reliability of the results will depend on sample preparation, chamber depth (Ibanescu *et al.*, 2016) and all internal and external factors influencing the male animal (Hook & Fisher, 2020).

Sperm must be observed in a chamber with a depth that allows freedom of movement (natural movement) patterns to be displayed (Mortimer *et al*., 1998). A chamber that is too shallow, will prevent a spermatozoon from swimming normally and thus influence the measurements. Likewise, if the chamber dimensions are too large for the given concentration of sperm that has been loaded, the sperm will utilise energy reserves to swim in the chamber and upon evaluation, swim with a decreased velocity. The depth of the chamber will determine the concentration of sperm to be loaded.

Although all systems essentially measure the same parameters, differences in results have been found and there are significant differences for certain parameters between systems, for example the measurement of progressive motility (Proctor *et al.,* 2009; Boryshpolets *et al*., 2013). These differences are based on the algorithms developed, and the calibration of each system.

2.5 References

Amann, R.P. 1989. Can the fertility potential of a seminal sample be predicted accurately? J. Androl. 10(2),89–98.

Amann, R.P., & Katz, D.F. 2004. Reflections on CASA after 25 years. J. Androl. 25(3), 317–325.

Amiridis, G.S., & Cseh, S. 2012. Assisted reproductive technologies in the reproductive management of small ruminants. Anim. Reprod. Sci. 130, 152–161.

Auger, J. 1997. Subjective and objective evaluation of human sperm motility: Present and future. J. Androl.

433-442.

Benkhalifa, M. & Menezo, Y. Gamete and embryo quality role in fertilization failures and reproductive pathology: the contribution of Fluorescent *In Situ* Hybridization (FISH). 2019. [online] Available at: https://www.gfmer.ch/Books/Reproductive_health/Gamete_and_embryo_quality.html Accessed: 15 Dec. 2020

Boryshpolets, S., Kowalski, R.K., Dietrich, G.J., Dzyuba, B., & Ciereszko, A. 2013. Different computer-assisted sperm analysis (CASA) systems highly influence sperm motility parameters. Theriogenology 80, 758–765.

Cayan, S., Lee, D., Conaghan, J., Givens, C.A., Ryan, I.P., Schriock, E.D., & Turek, P.J. 2001. A comparison of ICSI outcomes with fresh and cryopreserved epididymal spermatozoa from the same couples. 16, 495–499.

Comizzoli, P., Paulson, E.E., & McGinnis, L.K. 2018. The mutual benefits of research in wild animal species and human-assisted reproduction. J. Assist. Reprod. Genet. 35, 551–560.

Eskandari, F., & Momeni, H.R. 2016. Silymarin protects plasma membrane and acrosome integrity in sperm treated with sodium arsenite. Int. J. Reprod. Biomed. 14(1), 47–52.

Food and Agriculture Organization (FAO) - The state of food security and nutrition in the world. 2019.[online] Available at http://www.fao.org/publications/sofi/2020/en/ Accessed 6 Dec 2020

Flesch, F.M., & Gadella, B.M. 2000. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochim. Biophys. Acta - Rev. 1469, 197–235.

Gallego, V., Herranz-Jusdado, J.G., Rozenfeld, C., Pérez, L., & Asturiano, J.F. 2018. Subjective and objective assessment of fish sperm motility: when the technique and technicians matter. Fish Physiol. Biochem. 44,1457–1467.

Gizmodo.com.au How sperm cells get stripped down and ready to race. [online] Available at https://www.gizmodo.com.au/2015/10/how-sperm-cells-get-stripped-down-and-ready-to-race/ Accessed Sept. 26 2020

Graczykowski, J.W., & Siegel, M.S. 1991. Influence of sperm processing on the fertilizing capacity and recovery of motile sperm from thawed human semen. Archives of Andrology. 26, 155-161.

Holt, W.V., & van Look, K.J. W. 2004. Concepts in sperm heterogeneity, sperm selection and sperm competition as biological foundations for laboratory test of semen quality. Reproduction 127, 527–535.

Hook, K.A., & Fisher, H.S. 2020. Methodological considerations for examining the relationship between sperm morphology and motility. Mol. Reprod. Dev. 87, 633–649.

Hossain, M., Khatun, M., Islam, M., & Miazi, O. 2012. Semen characteristics of breeding bulls at the Central Cattle Breeding and Dairy Farm of Bangladesh. Bangladesh J. Anim. Sci. 41, 1–5.

Ibanescu, I., Leiding, C., Ciornei, S.G., Rosca, P., Sfartz, I., & Drugociu, D. 2016. Differences in CASA output according to the chamber type when analyzing frozen-thawed bull sperm. Anim. Reprod. Sci. 166, 72-

79.

Imbachi, F.J., Zalazar, L., Pastore, J.I., Greco, M.B., Iniesta-Cuerda, M., Garde, J.J., Soler, A.J., Ballarin, V., & Cesari, A. 2018. Objective evaluation of ram and buck sperm motility by using novel sperm tracker software. Reproduction 156(1), 11-21.

Lambo, C.A., Grahn, R.A., Lyons, L.A., Bateman, H.L., Newsom, J., & Swanson, W.F. 2012. Comparative fertility of freshly collected vs frozen – thawed semen with laparoscopic oviductal artificial insemination in domestic cats. Reprod. Dom. Anim. 47, 284–288.

Larsson, B., & Rodríguez-Martínez, H. 2000. Can we use in vitro fertilization tests to predict semen fertility? Anim. Reprod. Sci. 60-61, 327–336.

Loutradi, K.E., Tarlatzis, B.C., Goulis, D.G., Zepiridis, L., Pagou, T., Chatziioannou, E., Grimbizis, G.F., Papadimas, I., & Bontis, I. 2006. The effects of sperm quality on embryo development after intracytoplasmic sperm injection. 23(2), 69-74.

Maree, L., & van der Horst, G. 2013. Quantification and identification of sperm subpopulations using computer-aided sperm analysis and species-specific cut-off values for swimming speed. Biotech. Histochem. 88, 181–193.

Martínez-Pastor, F., Tizado, E.J., Garde, J.J., Anel, L., & de Paz, P. 2011. Statistical Series: Opportunities and challenges of sperm motility subpopulation analysis. Theriogenology 75, 783–795.

Morrow, C.J., Penfold, L.M., & Wolfe, B.A. 2009. Artificial insemination in deer and non-domestic bovids. Theriogenology 71, 149–165.

Mortimer, S., Mortimer, D., & Fraser, L. 1998. Guidelines on the application of CASA technology in the analysis of spermatozoa. Hum. Reprod. 13, 142–145.

Mukai, C & Okuno, M. 2004. Glycolysis plays a major role for adenosine triphosphate supplementation in mouse sperm flagellar movement. Biol. Reprod. 71, 54-547.

Proctor, J.G., Boone, W.R., & Iii, H.L.H. 2009. Comparison of the manual , IVOS , and SCA® methods for semen analysis reporting. Fertil. Steril. 12, 10–12.

Purohit, G.N., Duggal, G.P., Dadarwal, D., Kumar, D., Yadav, R.C., & Vyas, S. 2000. Reproductive biotechnologies for improvement of buffalo : The current status. Asian-Aust. J. Anim. Sci. 16(7), 1071-1086.

Rodríguez-Martínez, H. 2003. Laboratory semen assessment and prediction of fertility: Still utopia? Reprod. Domest. Anim. 38(4), 312–318.

Salvador, Z. & Fernandez, S. 2018. What types of sperm morphological defects exist? [online] Available at: https://www.invitra.com/en/types-of-abnormal-sperm/#midpiece-defects Accessed 03/03/2021

Sadeghi, S., Del Gallego, R., Garcia-Colomer, B., Gomez, E., Yaniz, J., Gosalvez, J., Lopez-Fernandez, C., & Silvestre, M. 2020. Effect of sperm concentration and storage temperature on goat spermatozoa during liquid storage. 9, 1–13.

Sellem, E., Broekhuijse, M.L.W.J., Chevrier, L., Camugli, S., Schmitt, E., Schibler, L., & Koenen, E.P.C. 2015.

Use of combinations of *in vitro* quality assessments to predict fertility of bovine semen. Theriogenology 84(9), 1447-1454.

Senger., P.L. 2003. Pathways to Pregnancy and Parturition Revised 2nd Edition. Current Conceptions, Inc. USA.

Shamsi, M.B., Imam, S.N., & Dada, R. 2011. Sperm DNA integrity assays : diagnostic and prognostic challenges and implications in management of infertility. 28, 1073–1085.

Sharma, R., Kattoor, A.J., Ghulmiyyah, J., & Agarwal, A. 2015. Effect of sperm storage and selection techniques on sperm parameters. Syst. Biol. Reprod. Med. 61(1), 1-12.

Srivastava, N., Srivastava, S.K., Ghosh, S.K., Kumar, A., Perumal, P., & Jerome, A. 2013. Acrosome membrane integrity and cryocapacitation are related to cholesterol content of bull spermatozoa. 2(2), 126–131.

Turner, R.M. 2003. Tales from the tail: What do we really know about sperm motility? J. Androl. 24(6), 790–803.

United Nations – World Population Prospects 2017 [online] Available athttps://www.un.org/development/desa/publications/world-population-prospects-the-2017-revision.html#:~:text=The%20current%20world%20population%20of,Nations%20report%20being%20launched%20today Accessed 15 Dec. 2020.

United Nations - World Population Prospects 2019 [online] Available at https://www.who.int/publications/i/item/9789241547789 Downloaded 7 Dec. 2020 .

Warriach, H.M., Mcgill, D.M., Bush, R.D., Wynn, P.C., & Chohan, K.R. 2015. A review of recent developments in buffalo reproduction — A review. 28(3), 451–455.

WHO Laboratory Manual for the Examination and Processing of Human Semen 5th Edition. 2010. [online] https://www.who.int/publications/i/item/9789241547789 Downloaded 15 June 2020

Yániz, J.L., Soler, C., & Santolaria, P. 2015. Computer assisted sperm morphometry in mammals: A review. Anim. Reprod. Sci. 156, 1–12.

Chapter 3

Methodology

This section serves the purpose to provide insight into the use of sperm subpopulations to quantify fertility in terrestrial and aquatic animals. A meta-analysis approach was followed that allowed the quantitative analysis of the available research, with the aim to answer the research question. The information presented in this chapter provides information on the available literature consulted, the identification of the selection criteria, the extraction of the data and design of the database, and the respective statistical models that allowed for the analysis of the metadata.

No ethical approval was required for this study.

The research question posed for this study was whether a relationship exists between sperm subpopulations (motility and morphometric) and related fertility. In the context of this research question, the fertility of a male (sperm sample) was classified into two separate groups: the sample would be used in ART's or natural mating would take place (field fertility). All results and findings were based on the current available literature.

The following objectives were set:

- To determine the degree of standardization of experimental design used to study motility/morphometry subpopulations.
- To determine the degree of standardization regarding the cut-off values used to classify sperm motility subpopulations.
- To determine the degree of standardization regarding the cut-off values used to classify sperm morphometry subpopulations.
- To determine the influence of species on CASA-determined motility- and velocity-related parameters for fresh and frozen-thawed spermatozoa.
- To determine the influence of species on CASA-determined morphometry and morphometry-derived parameters for fresh and frozen-thawed spermatozoa.
- To determine the influence of type of CASA system used on motility- and velocity-related parameters for fresh and frozen-thawed spermatozoa.
- To determine the influence of type of CASA system used on morphometry and morphometry-derived parameters for fresh and frozen-thawed spermatozoa.
- To determine the influence of medium used on motility- and velocity-related parameters for fresh and frozen-thawed spermatozoa.

- To determine the influence of medium used on morphometry and morphometry-derived parameters for fresh and frozen-thawed spermatozoa.

3.1 Specification of the research topic and search criteria

Currently, there is limited information available regarding the cut-off values used for the classification of sperm subpopulations, and the relationship of the outcome of these classification approaches to the fertilizing ability and eventual outcome of spermatozoa allocated to such subpopulations. It is especially important to consider when sperm fertilizing ability and success is considered in the context of natural mating (also referred to as field fertility) or the use of the spermatozoa in ART's, and when the information generated is used to make management or selection decisions.

The objective of this meta-analysis study was therefore to collect as much related information as possible to gain insight in and draw conclusions from the available research regarding the research question. When the available literature was consulted, it became evident that there is a large degree of variation in the findings reported by the studies, as well as the classification and cut-off values used to classify sperm subpopulations.

Specific search criteria were thus established to assist in the identification of studies to include in the meta-analysis, with the criteria selected to allow for a focussed approach to provide an "answer" to the research question.

Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines were followed for both the sperm motility and morphometry datasets (Moher *et al*., 2009). An adapted version is depicted at Appendix C (Figure C.1).

3.2 Compilation of database for study

3.2.1 Establishment of search criteria and search interval

The set of scientific publications included in the study was identified by using a web-based literature approach, using specific search criteria.

Key words that were included, were the following:

- "sperm subpopulations and morphometry"
- "sperm subpopulations and morphology"
- "sperm subpopulations and motility"
- "sperm subpopulations and fertility"
- "sperm subpopulations and function"

3.2.2 Research databases consulted

Online databases accessed included:

- ScienceDirect : https://www.sciencedirect.com/
- PubMed: https://pubmed.ncbi.nlm.nih.gov/
- Google Scholar: https://scholar.google.com/
- Scopus: https://www.scopus.com/home.uri

3.2.3 Search interval and selection of publications

The web-based search was limited to the period of 1994 to February 2020.

Only peer-reviewed published publications were selected for inclusion in the study. Publications were only retained if they met the following two selection criteria, i.e. applying CASA and where subpopulations were determined using CASA-derived parameters. Exclusion criteria were not applicable for the initial data search i.e. all publications which met the two minimum inclusion criteria were included in the initial raw dataset. However, upon further investigation of the raw data, further publications were removed due to reasons as detailed in Table 3.2.

3.4 Categorization of studies

Sperm subpopulations are important to consider in terms of motility and morphometry. The publications that included the evaluation of both motility and morphometry as part of the same study were initially grouped together, and eventually recorded as individual entries in either the motility or the morphometry group. As there were more publications and data available regarding sperm subpopulation classification using motility, separate datasets for morphometry and motility were designed for statistical analysis.

Thereafter, the publications were categorized according to the species that was investigated or used in the study. In the cases where more than one species was included in the same study, individual entries were made per the correct category.

3.5 Transformation of publication information into datasets

The data obtained from the selected publications were combined into a database for exploration and analysis according to the classification approaches reported in the studies. The original database was thoroughly explored to ensure that unbiased analysis results would be achieved. Where bias was evident and essential combinations of variables were not sufficiently represented, these were removed from the dataset.

For the design of the motility- and morphometry datasets, results of fresh and frozen-thawed samples are presented separately. When reports included the use of chilled semen, data were included in the Fresh sample set. When a publication reported the standard deviation (SD) as a range, it was removed from the data set as the provided range was too wide and inflated the variation leading to possible bias.

A total of 30 publications were included for motility and 10 for morphometry.

3.5.1 Motility

Motility data was obtained for forty-four publications as shown in Table 3.1. Species or studies that were not sufficiently represented were removed (Table 3.2). Thirty of these forty-four publications provided sufficient data for statistical analysis. Publications represent data collected from 1999 to 2020.

Table 3.1 The complete list of all publications that matched the search criteria for sperm velocity-associated and capacitation status-associated parameters (determined using CASA) and that were used to compile the pre-analysis dataset.

	Author (-s)	**Year**	**Species**	**Journal**
1	Muino *et al.*	2008*a*	Bovine	Animal Reproduction Science
2	Ferraz *et al.*	2014	Bovine	Theriogenology
3	Muino *et al.*	2008*b*	Bovine	Animal Reproduction Science
4	Ibanescu *et al.*	2020	Bovine	Animal Reproduction Science
5	Barbas *et al.*	2018	Caprine	Cryobiology
6	Dorado *et al.*	2010	Caprine	Theriogenology
7	Vazquez *et al.*	2015	Caprine	Animal Reproduction Science
8	Dorado *et al.*	2013*a*	Equine	Theriogenology
9	Dorado *et al.*	2013*b*	Equine	Animal Reproduction Science
10	Giaretta *et al.*	2017	Equine	Animal Reproduction Science
11	Miro *et al.*	2009	Equine	Theriogenology
12	Miro *et al.*	2020	Equine	Journal of Equine Veterinary Science
13	Miro *et al.*	2005	Equine	Theriogenology
14	Ortega-Ferrusola *et al.*	2009	Equine	Reproduction Domestic Animals
15	Quintero-Moreno *et al.*	2003	Equine	Theriogenology
16	Beirao *et al.*	2009	Piscine	Theriogenology
17	Beirao *et al.*	2011	Piscine	Cryobiology
18	Caldeira *et al.*	2018	Piscine	Reproduction, Fertility and Development
19	Gallego *et al.*	2015	Piscine	Reproduction, Fertility and Development
20	Gallego *et al.*	2017	Piscine	Theriogenology
21	Kanuga *et al.*	2012	Piscine	Theriogenology
22	Cremades *et al.*	2005	Porcine	Journal of Andrology
23	Estrada *et al.*	2017	Porcine	Cryobiology
24	Flores *et al.*	2009	Porcine	Theriogenology
25	Ibanescu *et al.*	2018	Porcine	Journal of Reproduction and Development

26	Quintero-Moreno *et al.*	2004	Porcine	Theriogenology
27	Ramio *et al.*	2008	Porcine	Theriogenology
28	Rivera *et al.*	2006	Porcine	Reproduction Domestic Animals
29	Thurston *et al.*	2001	Porcine	Journal of Andrology
30	Garcia-Alvarez *et al.*	2013	Ovine	Reproduction, Fertility and Development
31	Bergstein-Galan *et al.*	2017	Ovine	Animal Reproduction Science
32	Bravo *et al.*	2011	Ovine	Animal Reproduction Science
33	Ledesma *et al.*	2017	Ovine	Animal Reproduction Science
34	O' Meara *et al.*	2008	Ovine	Theriogenology
35	Dorado *et al.*	2011*a*	Canine	Animal Reproduction Science
36	Dorado *et al.*	2011*b*	Canine	Animal Reproduction Science
37	Pena *et al.*	2012	Canine	Animal Reproduction Science
38	Nunez-Martinez *et al.*	2006*b*	Canine	Reproduction Domestic Animals
39	Martinez-Pastor *et al.*	2005	Deer	Theriogenology
40	Ramon *et al.*	2012	Deer	Theriogenology
41	Kemmer-Souza *et al.*	2018	Feline	Animal Reproduction Science
42	Maya-Soriano *et al.*	2015	Rabbit	Theriogenology
43	Flores *et al.*	2008	Equine, Porcine	Theriogenology
44	Abagair *et al.*	1999	Porcine, Gazelle	Biology of Reproduction

Table 3.2 A list of all publications that were excluded from the final pre-analysis sperm velocity-associated and capacitation status-associated parameters dataset, for reasons specified in the table.

	Author (-s)	**Year**	**Species**	**Reason for Exclusion**
1	Barbas *et al.*	2018	Caprine	Variation reported in the studies too large
2	Dorado *et al.*	2010	Caprine	Variation reported in the studies too large
3	Vazquez *et al.*	2015	Caprine	Variation reported in the studies too large
4	Giaretta *et al.*	2017	Equine	Only one velocity average output provided
5	Beirao *et al.*	2009	Piscine	Insufficient data for statistical analyses
6	Beirao *et al.*	2011	Piscine	Insufficient data for statistical analyses
7	Caldeira *et al.*	2018	Piscine	Insufficient data for statistical analyses
8	Gallego *et al.*	2015	Piscine	Insufficient data for statistical analyses
9	Gallego *et al.*	2017	Piscine	Insufficient data for statistical analyses
10	Kanuga *et al.*	2012	Piscine	Insufficient data for statistical analyses
11	Thurston *et al.*	2001	Porcine	Insufficient representation of CASA method used
12	Ibanescu *et al.*	2018	Porcine	Insufficient representation of CASA method used
13	O' Meara *et al.*	2008	Ovine	Insufficient representation of CASA method used
14	Pena *et al.*	2012	Canine	Insufficient representation of CASA method used
15	Nunez-Martinez *et al.*	2006	Canine	Subpopulation classification too wide
16	Martinez-Pastor *et al.*	2005	Deer	No standard deviations reported
17	Kemmer-Souza *et al.*	2018	Feline	Only one publication for this species

18	Abagair *et al.*	1999	Porcine, Gazelle	Insufficient representation of CASA system used
19	Maya-Soriano *et al.*	2015	Rabbit	Only one publication for this species

3.5.2 Morphometry

Table 3.3 presents the publications identified for morphometry subpopulation classification, based on matching of the publications with the determined search criteria, and according to author(-s), year of publication, species, and journal.

Table 3.3 The complete list of all published publications that matched the search criteria for sperm morphometry and subpopulation classification (determined by using CASA), and that were included in the compilation of the pre-analysis dataset.

	Author (-s)	**Year**	**Species**	**Journal**
1	Valverde *et al.*	2016	Bovine	Asian Journal of Andrology
2	Rubio-Guillen *et al.*	2007	Bovine	Reproduction Domestic Animals
3	Garcia-Herreros *et al.*	2014	Bovine	Systems Biology in Reproductive Medicine
4	Zaja *et al.*	2018	Caprine	Animal Reproduction Science
5	Maroto-Morales *et al.*	2015	Ovine	Theriogenology
6	Marti *et al.*	2011	Ovine	Theriogenology
7	Marti *et al.*	2012	Ovine	Theriogenology
8	Maroto-Morales *et al.*	2012	Ovine	Theriogenology
9	De Paz *et al.*	2011	Ovine	Theriogenology
10	Garcia-Herreros *et al.*	2016	Avian	Asian Journal of Andrology
11	Villaverde-Morcillo *et al.*	2017	Avian	Theriogenology
12	Beracochea *et al.*	2014	Deer	Animal Reproduction Science
13	Gravance *et al.*	1997	Equine	Journal of Reproduction and
14	Hidalgo *et al.*	2008	Equine	Animal Reproduction Science
15	Davis *et al.*	1994	Rodent	Reproductive Toxicology
16	Valle *et al.*	2012	Primate	Theriogenology
17	Valle *et al.*	2013	Primate	Animal Reproduction Science
18	Pena *et al.*	2005	Porcine	Journal of Andrology
19	Thurston *et al.*	1999	Porcine	Journal of Reproduction and Fertility
20	Vicente-Fiel *et al.*	2013	Bovine, Caprine, Porcine, Ovine	Animal Reproduction Science

Regarding the data analysis, studies that were not sufficiently represented were removed and are indicated in Table 3.4. Upon further investigation of the data, it was found that many of the species had limited numbers resulting in only two species, namely bovine and ovine, being used for the morphometric data.

Analysis was performed separately for each species due to all ovine samples being fresh and all bovine samples being frozen-thawed. Only subpopulations one to four were retained. Any further subpopulations were not included in the pre-analysis dataset.

Table 3.4 A list of all publications that were excluded from the final pre-analysis dataset for sperm morphometry and subpopulation classification, for reasons specified in the table.

	Author (-s)	**Year**	**Species**	**Reason for exclusion**
1	Zaja *et al.*	2018	Caprine	Insufficient representation of CASA system used
2	Garcia-Herreros *et al.*	2016	Avian	Insufficient representation of the species
3	Villaverde-Morcillo *et al.*	2017	Avian	Insufficient representation of the species
4	Beracochea *et al.*	2014	Deer	Only one publication for this species
5	Gravance *et al.*	1997	Equine	Insufficient representation of the species
6	Hidalgo *et al.*	2008	Equine	Subpopulations classified with CASA, however no values provided
7	Davis *et al.*	1994	Rodent	Only one publication for this species
8	Valle *et al.*	2012	Primate	Insufficient representation of the species
9	Valle *et al.*	2013	Primate	Insufficient representation of the species
10	Pena *et al.*	2005	Porcine	CASA parameters and subpopulations recorded, however not linked
11	Thurston *et al.*	1999	Porcine	Measured only tail lengths for subpopulation determination

Ultimately, ten publications provided sufficient data for statistical analysis and are presented in Chapter 5 (refer to Table 5.1). The publications represent data collected from 1994 to 2018.

Four publications reported sperm subpopulation results for both motility and morphometric parameters and are presented in Table 3.5. Only one publication from this group was excluded from the meta-analysis (Santolaria *et al*., 2015), due to the presentation of results being in a format which did not allow the extraction of sperm subpopulation information.

Table 3.5 The complete list of all published publications that matched the search criteria for both motility and morphometric subpopulations (determined by using CASA) and that were included in the initial compilation of the pre-analysis dataset.

	Author	**Year**	**Species**	**Journal**
1	Nunez-Martinez *et al.*	2006	Canine	Journal of Andrology
2	Ramon *et al.*	2013	Deer	Biology of Reproduction
3	Yaniz *et al.*	2015	Ovine	Animal Reproduction Science
4	Santolaria *et al.*	2015	Ovine	Animal Reproduction Science

3.6 Data analysis

In most of these studies for both morphology and motility, a multivariate analysis approach was followed. Most studies reported the standard deviation (SD) and not the standard error (SEM). Therefore, for uniformity of measuring variation between studies, where standard error was reported, these were converted to standard deviation:

$$SD = SEM \times \sqrt{n}$$; where n = number of ejaculates collected

To avoid confusion, SD from the publications was reported as SSD (study standard deviation) in this meta-analysis. The SD as seen anywhere throughout the thesis, was the SD calculated from the statistical analyses. Where the mean was measured in the publications, it was reported as only the variable name.

3.6.1 Establishment of statistical model

As part of the data analysis process, various statistical models were fitted. The model with the best fit was selected according to the adjusted-R^2, Akaike information criterion (AIC) values, and biological significance. Biological significance in this case relates to factors which influence the physiology of the sperm sample and therefore may influence the results.

Every possible parameter which could influence sperm motility or morphometry was recorded from the publications and is given in Tables E.1 (for the motility dataset) and E.2 (for the morphometry dataset). To establish the final model used in the analysis, variables were added in a stepwise manner until an as-complete a model was obtained. Three models were ultimately obtained, i.e. one model for the motility dataset, and two models for the morphometry dataset.

3.6.1.1 Motility

The final pre-analysis motility dataset comprised a total of 266 observations and represented 30 publications. Data obtained from the 30 publications were divided into separate fresh and frozen-thawed datasets as shown in Figure 3.1.

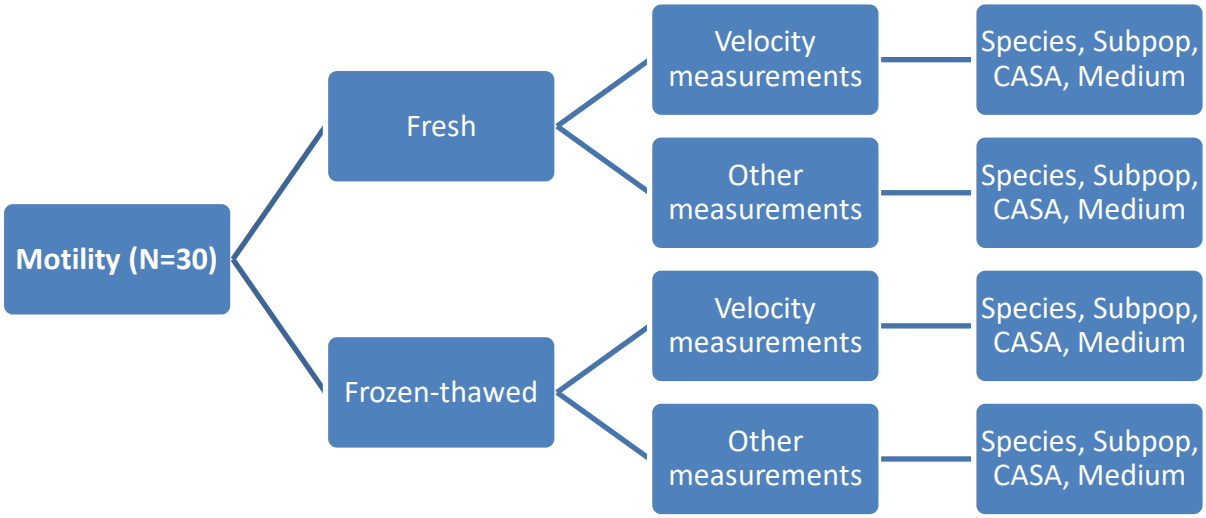

Figure 3.1 Flow chart illustrating the assignment of the motility data to separate datasets and the model selected and applied for each dataset.

The base model included only species and subpopulation as variables. The type of CASA system used was then added, followed by type of medium used, to consequently reach the final model for both the fresh and frozen-thawed datasets. Any further variables added to the model resulted in non-estimable values. The final model thus included species, subpopulation, and type of CASA system and type of medium used, respectively (Figures 3.2 and 3.3).

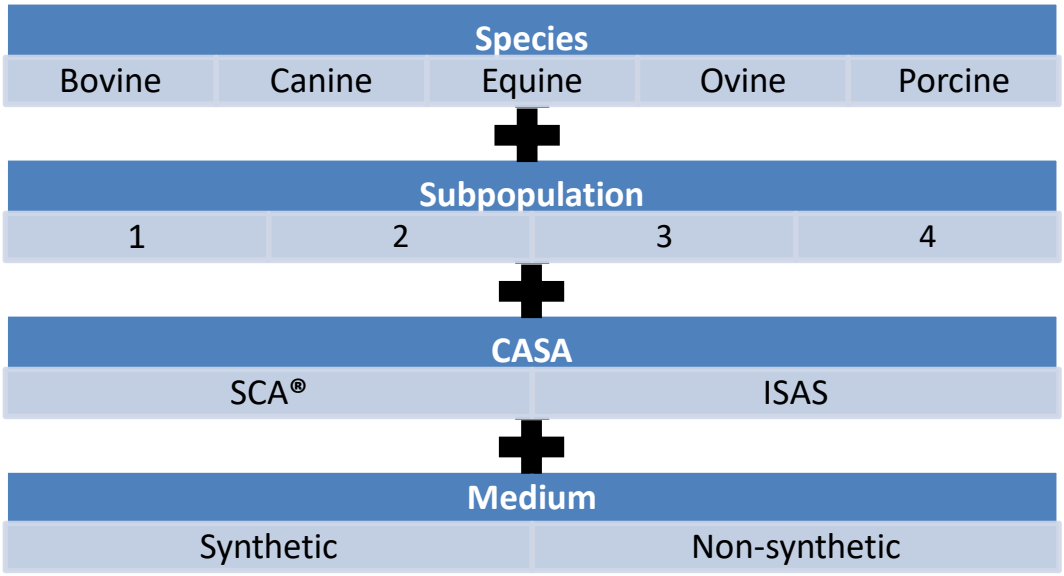

Figure 3.2 A graphic representation of the parameters and sub-parameters that constitute the model for the motility fresh dataset (**MODEL 1**). *Breed not included*

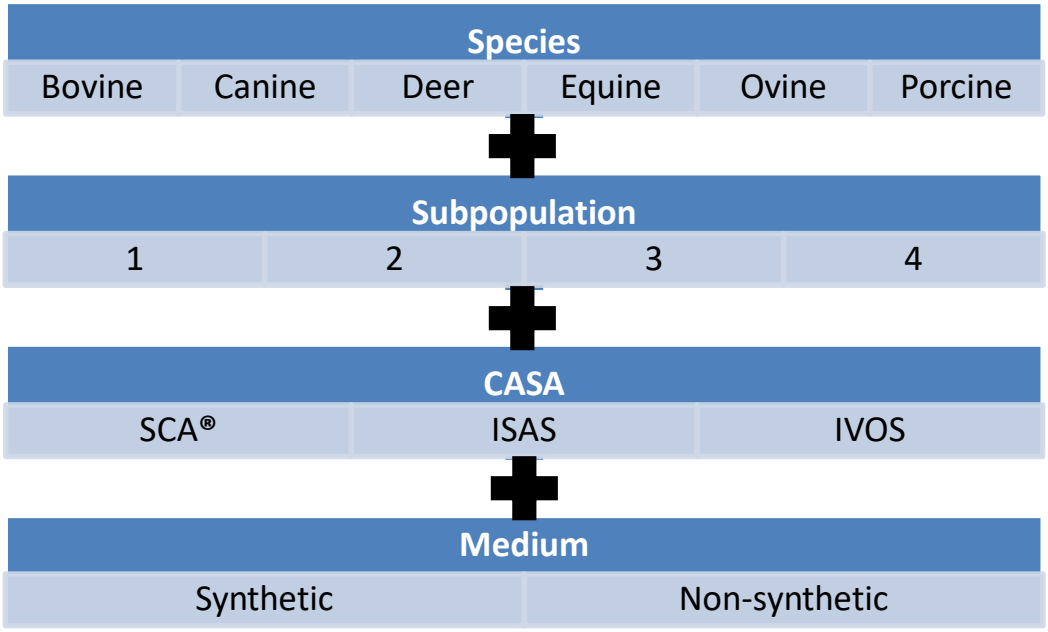

Figure 3.3 A graphic representation of the parameters and sub-parameters that constitute the model for the motility frozen-thawed dataset (**MODEL 2**). *Breed not included*

It should be noted that the sub-parameters for CASA and species differ between the fresh and frozen-thawed datasets. The deer species is not represented in Model 1 data (Figure 3.2). As the ImageJ and IVOS CASA method were poorly represented (one study for each) these observations were removed for the final analysis of the fresh data while the Hobson Sperm Tracker method was removed in the frozen-thawed dataset.

For further analysis of VAP and ALH means, the interaction between CASA and medium was also estimated. To determine an association between measurements, correlation coefficients were obtained for the motility data. The correlation coefficients were determined for the combination of each level of species and with each level of subpopulation, through the use of a correlation matrix calculated using XLSTAT. Only bovine, porcine, and equine samples were considered for correlations performed for the fresh data, while only bovine samples were considered for correlations performed for the frozen-thawed samples. These were the only species in the various categories with sufficient observations.
Dependant variables included average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), lateral head displacement (ALH), linearity coefficient (LIN), straightness of track (STR), wobble coefficient (WOB) and beat cross frequency (BCF) (Table A.1; Figure A.1).

Independent variables included species, subpopulation, CASA and medium.

3.6.1.2 Morphometry

There was considerably less data gleaned from the three publications for frozen-thawed (Bovine) data and seven publications for the fresh (Ovine) data.

Figure 3.4 represents the division of the data into separate datasets and the selected models applied for the individual measurements.

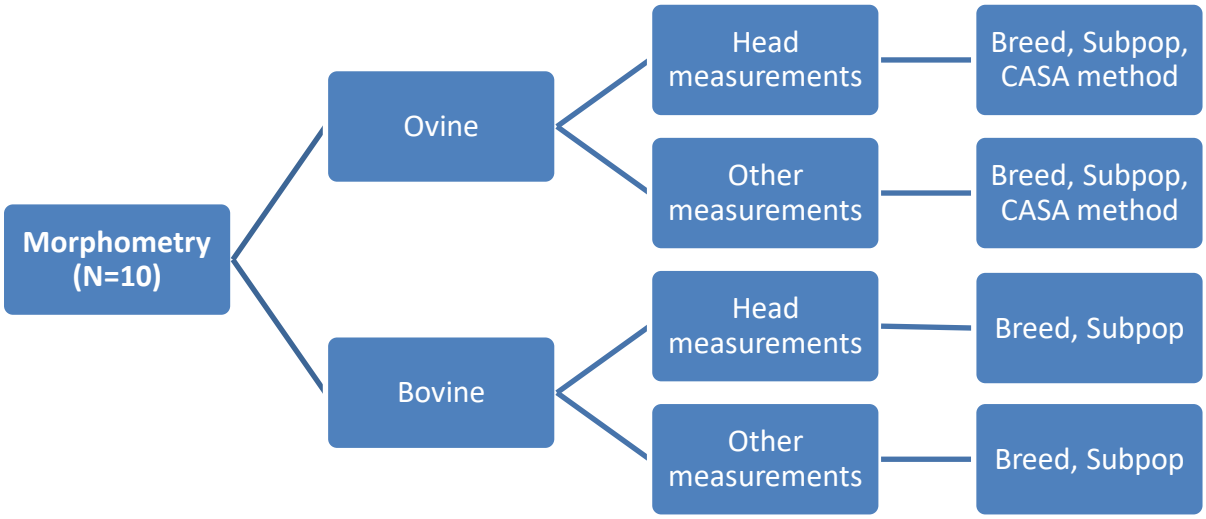

Figure 3.4 Flow chart illustrating the division of the morphometry data into separate datasets and the model selected and applied for each dataset.

The base model included only the variables species and subpopulation, and this was the only model that fitted the data for the bovine dataset and for which it was possible to obtain results. Thereafter, for the ovine dataset where more data were available, variables were included in a step-wise manner to reach the final model that included species and subpopulation (from the base model), as well as the type of CASA system used. The CASA variable could not be included in the model for the frozen-thawed bovine dataset as it was confounded with breed. Breed was better represented in the publications for the morphometry data and was thus included as a variable in the base model, instead of species as with the motility data, as can be seen in Figures 3.5 and 3.6.

Ellipticity and elongation were also reported by some of the authors, although less data for these variables were available and it was therefore excluded from the bovine dataset. The model is presented in Figure 3.5.

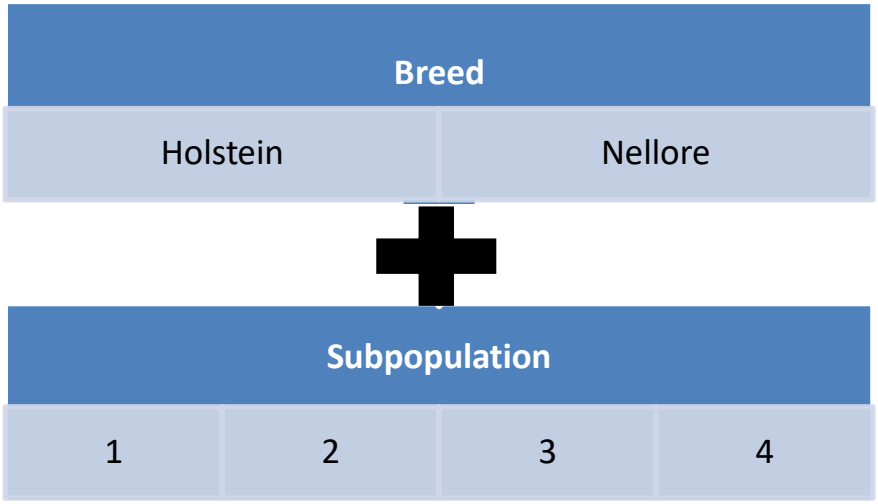

Figure 3.5 A graphic representation of the parameters and sub-parameters that constitute the model for the morphometry bovine dataset (**MODEL 3**). *Species, CASA, medium not included*

Due to better distribution for the ovine dataset, the variable CASA system type, could be included in the model. Other variables were found to be confounded with the type of CASA system used and were therefore excluded from the pre-analysis dataset. For the model, please refer to Figure 3.6.

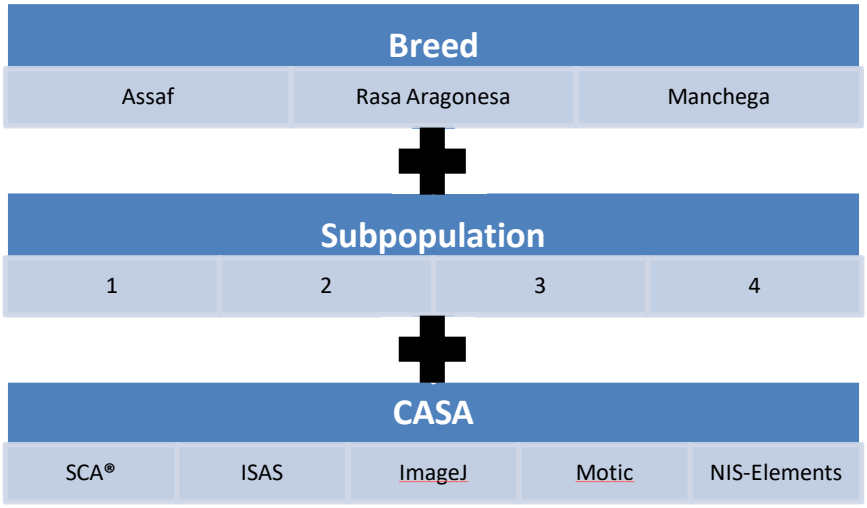

Figure 3.6 A graphic representation of the parameters and sub-parameters that constitute the model for the morphometry ovine dataset (**MODEL 4**). *Species and medium not included*

All the sperm head measurements, namely head length, head width, head area and head perimeter, were included as dependant variables in the bovine dataset (Table B.1; Figure B.1). The dependent variables ellipticity and elongation were additionally included in the ovine dataset as more data was available for these variables.

Initially breed and subpopulation were the only independent variables included in the model for the analysis of the bovine data. This was also the final model for the frozen-thawed data as the addition of any

other variables resulted in non-estimable values (see Figure 3.5). The type of CASA system used was included as an independent variable for the ovine dataset.

***Special note:** Variables that were recorded in the initial dataset, but not included in the final pre-analysis dataset due to non-estimable values included: the number of males in the study, the number of semen samples collected, the method of semen collection, the number of collections per week, the season of collection, the age of the males, equilibration and thawing rates of samples, sperm per sample analysed, concentration, microscope used, resolution, chamber depth, whether results were linked to fertility, and whether viability and acrosome integrity were evaluated.*

3.7 Statistical analysis

All analyses were performed using Microsoft XLSTAT (version 2020.4.1). Due to the data being unbalanced, for ANOVA analysis, the Type 3 sum of squares and Least square means were used to illustrate significance. Least square means were considered significant at $P \leq 0.05$.

Bonferonni *post hoc* tests were calculated for all variables.

Pearson correlation coefficients were estimated to determine the relationship /association between variables (VAP, VSL, VCL and ALH).

3.8 References

Caldeira, C., Garcia-Molina, A., Valverde, A., Bompart, D., Hassane, M., Martin, P., & Soler, C. 2018. Comparison of sperm motility subpopulation structure among wild anadromous and farmed male Atlantic salmon (*Salmo salar*) parr using a CASA system. Reprod. Fert. Dev. 30:897–906.

Abaigar, T., Holt, W.V, Harrison, R.A. P., & Barrio, G. 1999. Sperm subpopulations in boar (*Sus scrofa*) and gazelle (*Gazella dama mhorr*) semen as revealed by pattern analysis of Computer-Assisted Motility Assessments. 41, 32–41.

Barbas, J.P., Leahy, T., Horta, A.E., & García-Herreros, M. 2018. Sperm kinematics and subpopulational responses during the cryopreservation process in caprine ejaculates. Cryobiology 82, 137–147.

Beirao, J. 2009. Sperm quality evaluation in *Solea senegalensis* during the reproductive season at cellular level.Theriogenology 72, 1251–1261.

Beirão, J., Cabrita, E., Pérez-Cerezales, S., Martínez-Páramo, S., & Herráez, M.P. 2011. Effect of cryopreservation on fish sperm subpopulations. Cryobiology 62, 22–31.

Beracochea, F., Gil, J., Sestelo, A., Garde, J.J., Santiago-Moreno, J., Fumagalli, F., & Ungerfeld, R. 2014. Sperm characterization and identification of sperm sub-populations in ejaculates from pampas deer (*Ozotoceros bezoarticus*). Anim. Reprod. Sci. 149, 224–230.

Bergstein-Galan, T.G., Weiss, R.R., Kozicki, L.E., & Bicudo, S.D. 2017. Sperm subpopulations in ejaculated sperm and spermatozoa recovered from ovine epididymides up to 48 h after death. Anim. Reprod. Sci. 187, 20–27.

Bravo, J.A., Montanero, J., Calero, R., & Roy, T.J. 2011. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Ile de France rams. Anim. Reprod. Sci. 129, 22–29.

Cremades, T., Roca, J., Rodriguez-martinez, H., Abaigar, T., Vazquez, J.M., & Martinez, E. A. 2005. Kinematic changes during the cryopreservation of boar spermatozoa. J. Androl. 26, 610–618.

Davis, R.O., Gravance, C.G., Thal, D.M., & Miller, M.G. 1994. Automated analysis of toxicant-induced changes in rat sperm head morphometry. Reprod. Tox. 8(6):521–529.

De Paz, P., Mata-Campuzano, M., Tizado, E.J., Álvarez, M., Alvarez-Rodriguez, M., Herraez, P., & Anel, L. 2011. The relationship between ram sperm head morphometry and fertility depends on the procedures of acquisition and analysis used. 76, 1313–1325.

Dorado, J., Acha, D., Gálvez, M.J., Ortiz, I., Carrasco, J.J., Díaz, B., Gómez-arrones, V., Calero-Carretero, R., & Hidalgo, M. 2013a. Sperm motility patterns in Andalusian donkey (*Equus asinus*) semen: Effects of body weight , age , and semen quality. Theriogenology 79, 1100–1109.

Dorado, J., Acha, D., Ortiz, I., Gálvez, M.J., Carrasco, J.J., Díaz, B., Gómez-arrones, V., Calero-Carretero, R., & Hidalgo, M. 2013b. Relationship between conventional semen characteristics, sperm motility patterns and fertility of Andalusian donkeys (*Equus asinus*). Anim. Reprod. Sci. 143, 64–71.

Dorado, J., Alcaráz, L., Duarte, N., Portero, J.M., Acha, D., & Hidalgo, M. 2011a. Changes in the structures of motile sperm subpopulations in dog spermatozoa after both cryopreservation and centrifugation on PureSperm® gradient. Anim. Reprod. Sci. 125, 211–218.

Dorado, J., Gálvez, M.J., Murabito, M.R., Muñoz-Serrano, A., & Hidalgo, M. 2011b. Identification of sperm subpopulations in canine ejaculates: Effects of cold storage and egg yolk concentration. Anim. Reprod. Sci. 127, 106–113.

Dorado, J., Molina, I., Muñoz-Serrano, A., & Hidalgo, M. 2010. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Florida goats. Theriogenology 74, 795–804.

Estrada, E., Rivera del Álamo, M.M., Rodríguez-Gil, J.E., & Yeste, M. 2017. The addition of reduced glutathione to cryopreservation media induces changes in the structure of motile subpopulations of frozen-thawed boar sperm. Cryobiology 78, 56–64.

Ferraz, M.A.M.M., Morató, R., Yeste, M., Arcarons, N., Pena, A.I., Tamargo, C., Hidalgo, C. O., Muiño, R., & Mogas, T. 2014. Evaluation of sperm subpopulation structure in relation to in vitro sperm – oocyte interaction of frozen-thawed semen from Holstein bulls. Theriogenology 81, 1067–1072.

Flores, E., Fernández-Novell, J.M., Peña, A., & Rodríguez-Gil, J.E. 2009. The degree of resistance to freezing-thawing is related to specific changes in the structures of motile sperm subpopulations and mitochondrial activity in boar spermatozoa. Theriogenology 72, 784–797.

Flores, E., Taberner, E., Rivera, M.M., Peña, A., Rigau, T., Miró, J., & Rodríguez-Gil, J.E. 2008. Effects of freezing/thawing on motile sperm subpopulations of boar and donkey ejaculates. Theriogenology 70, 936–945.

Gallego, V., Vilchez, M.C., Penaranda, D.S., Perez, L., Herraez, M.P., Asturiano, J.F., & Martinez-Pastor, F. 2015. Subpopulation pattern of eel spermatozoa is affected by post-activation time, hormonal treatment and the thermal regimen. Reprod. Fert. Dev. 3:529-543.

Gallego, V., Cavalcante, S.S., Fujimoto, R.Y., Carneiro, P.C.F., Azevedo, H.C., & Maria, A.N. 2017. Fish sperm subpopulations: Changes after cryopreservation process and relationship with fertilization success in tambaqui (*Colossoma macropomum*). Theriogenology 87, 16–24.

Garcia-Alvarez, O., Maroto-Morales, A., Ramon, M., del Olmo, E., Jimenez-Rabadan, P., Fernandez-Santos, M.R., Anel-Lopez, L. Garde, J.J., & Soler, A.J. 2013. Dynamics of sperm subpopulations based on motility and plasma membrane status in thawed ram spermatozoa incubated under conditions that support *in vitro* capacitation and fertilisation. Reprod. Fertil. Dev. 5, 725-732.

García-Herreros, M. 2016. Sperm subpopulations in avian species: A comparative study between the rooster (*Gallus domesticus*) and Guinea fowl (*Numida meleagris*). Asian J. Androl. 18, 889–894.

García-Herreros, M., Leal, C.L.V, & Garcı, M. 2014. Comparative study of sperm washing and selection methods after cryopreservation and its influence on sperm subpopulational structure in a bovine model. Syst. Biol. Reprod. Med. 60(6), 338-347.

Giaretta, E., Munerato, M., Yeste, M., Galeati, G., Spinaci, M., Tamanini, C., Mari, G., & Bucci, D. 2017. Implementing an open-access CASA software for the assessment of stallion sperm motility: Relationship with other sperm quality parameters. Anim. Reprod. Sci. 176, 11–19.

Gravance, C.G., Champion, Z., Liu, I.K.M., & Casey, P.J. 1997. Sperm head morphometry analysis of ejaculate and dismount stallion semen samples. Anim. Reprod. Sci. 47, 149–155.

Hidalgo, M., Rodríguez, I., Dorado, J., & Soler, C. 2008. Morphometric classification of Spanish thoroughbred stallion sperm heads. Anim. Reprod. Sci. 103, 374–378.

Ibanescu, I., Leiding, C., & Bollwein, H. 2018. Cluster analysis reveals seasonal variation of sperm subpopulations in extended boar semen. J. Reprod. Dev. 64, 33-39.

Ibanescu, I., Siuda, M., & Bollwein, H. 2020. Motile sperm subpopulations in bull semen using different clustering approaches – Associations with flow cytometric sperm characteristics and fertility. Anim. Reprod. Sci. 215, 106-329.

Kanuga, M.K., Drew, R.E., Wilson-Leedy, J.G., & Ingermann, R.L. 2012. Subpopulation distribution of motile sperm relative to activation medium in steelhead (*Oncorhynchus mykiss*). Theriogenology 77, 916–925.

Ledesma, A., Zalazar, L., Fernández-Alegre, E., Hozbor, F., Cesari, A., & Martínez-Pastor, F. 2017. Seminal plasma proteins modify the distribution of sperm subpopulations in cryopreserved semen of rams with lesser fertility. Anim. Reprod. Sci. 184, 44–50.

Maroto-Morales, A., Ramón, M., García-álvarez, O., Montoro, V., & Garde, J.J. 2015. Sperm head phenotype and male fertility in ram semen. Theriogenology 84, 1536–1541.

Maroto-Morales, A., Ramón, M., García-Álvarez, O., Soler, A.J., Fernández-Santos, M.R., Roldan, E. R.S., Gomendio, M., Pérez-Guzmán, M.D., & Garde, J.J. 2012. Morphometrically-distinct sperm subpopulations defined by a multistep statistical procedure in Ram ejaculates: Intra- and interindividual variation. Theriogenology 77, 1529–1539.

Martí, J.I., Aparicio, I.M., & García-Herreros, M. 2011. Sperm morphometric subpopulations are differentially distributed in rams with different maturity age in cryopreserved ejaculates. Theriogenology 76, 97–109.

Martí, J.I., Aparicio, I.M., Leal, C.L.V., & García-Herreros, M. 2012. Seasonal dynamics of sperm morphometric subpopulations and its association with sperm quality parameters in ram ejaculates. Theriogenology 78, 528–541.

Martinez-Pastor, F., Diaz-Corujo, A.R., Anel, E., Herraez, P., Anel, L., & De Paz, P. 2005. Post mortem time and season alter subpopulation characteristics of Iberian red deer epididymal sperm. Theriogenology 64, 958–974.

Maya-Soriano, M.J., Taberner, E., Sabés-Alsina, M., Ramon, J., Rafel, O., Tusell, L., Piles, M., & López-Béjar, M. 2015. Daily exposure to summer temperatures affects the motile subpopulation structure of epididymal sperm cells but not male fertility in an in vivo rabbit model. Theriogenology 84, 384–389.

Meara, C.M.O., Hanrahan, J.P., Prathalingam, N.S., Owen, J.S., Donovan, A., Fair, S., Ward, F., Wade, M., Evans, A.C.O., & Lonergan, P. 2008. Relationship between *in vitro* sperm functional tests and *in vivo* fertility of rams following cervical artificial insemination of ewes with frozen-thawed semen. 69, 513–522.

Miro, Lobo, V., & Quintero-Moreno, A. 2005. Sperm motility patterns and metabolism in Catalonian donkey semen. 63, 1706–1716.

Miro, J., Morato, R., Vilagran, I., Taberner, E., Bonet, S., & Yeste, M. 2020. Preservation of epididymal stallion sperm in liquid and frozen states: Effects of seminal plasma on sperm function and fertility. J. Eq. Vet. Sci. 88, 102-109.

Miro, J., Taberner, E., Rivera, M., Pena, A., Medrano, A., Rigau, T., & Penalba, A. 2009. Effects of dilution and centrifugation on the survival of spermatozoa and the structure of motile sperm cell subpopulations in refrigerated Catalonian donkey semen. 72, 1017–1022.

Moher, D., Liberati, A., Tetzlaff, J.,Altman, D.G., & The PRISMA Group. 2009. Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. 6(7): 1-6.

Muiño, R., Rivera, M.M., & Rigau, T. 2008. Effect of different thawing rates on post-thaw sperm viability, kinematic parameters and motile sperm subpopulations structure of bull semen. 109, 50–64.

Muiño, R., Tamargo, C., Hidalgo, C.O., & Peña, A. I. 2008. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Holstein bulls: Effects of cryopreservation and between-bull variation. Anim. Reprod. Sci. 109, 27–39.

Muiño, R., Peña, A.I., Rodriguez, A., Tamargo, C., & Hidalgo, C.O. 2009. Effects of cryopreservation on the motile sperm subpopulations in semen from Asturiana de los Valles bulls. Theriogenology 72, 860–868.

Núñez-Martinez, I., Moran, J.M., & Peña, F.J. 2006. A three-step statistical procedure to identify sperm kinematic subpopulations in canine ejaculates: Changes after cryopreservation. Reprod. Dom. Anim. 41, 408-415.

Núñez Martínez, I., Morán, J.M., & Peña, F. J. 2006. Two-step cluster procedure after principal component analysis identifies sperm subpopulations in canine ejaculates and its relation to cryoresistance. J. Androl. 27, 596–603.

Ortega-Ferrusola, C. 2009. Identification of sperm subpopulations in stallion ejaculates: Changes after cryopreservation and comparison with traditional statistics. 423, 419–423.

Peña, A.I., Barrio, M., Becerra, J.J., Quintela, L.A., & Herradón, P. G. 2012. Motile sperm subpopulations in frozen-thawed dog semen: Changes after incubation in capacitating conditions and relationship with sperm survival after osmotic stress. Anim. Reprod. Sci. 133, 214–223.

Peña, F.J., Saravia, F., García-Herreros, M., Núñez-Martínez, I., Tapia, J.A., Johannisson, A., Wallgren, M., & Rodríguez-Martínez, H. 2005. Identification of sperm morphometric subpopulations in two different portions of the boar ejaculate and its relation to postthaw quality. J. Androl. 26, 716–723.

Quintero-Moreno, A., Miró, J., Teresa Rigau, A., & Rodríguez-Gil, J.E. 2003. Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. Theriogenology 59, 1973–1990.

Quintero-Moreno, A., Rigau, T., & Rodrı, J.E. 2004. Regression analyses and motile sperm subpopulation structure study as improving tools in boar semen quality analysis. 61, 673–690.

Ramió, L., Rivera, M.M., Ramírez, A., Concha, I.I., Peña, A., Rigau, T., & Rodríguez-Gil, J.E. 2008. Dynamics of motile-sperm subpopulation structure in boar ejaculates subjected to in vitro capacitation and further *in vitro* acrosome reaction. Theriogenology 69, 501–512.

Ramón, M., Martínez-Pastor, F., García-Álvarez, O., Maroto-Morales, A., Soler, A.J., Jiménez-Rabadán, P., Fernández-Santos, M.R., Bernabéu, R., & Garde, J.J. 2012. Taking advantage of the use of supervised learning methods for characterization of sperm population structure related with freezability in the Iberian red deer. Theriogenology 77, 1661–1672.

Ramón, M., Soler, A.J., Ortiz, J.A., García-Alvarez, O., Maroto-Morales, A., Roldan, E.R.S., & Garde, J.J. 2013. Sperm population structure and male fertility: An intraspecific study of sperm design and velocity in Red Deer. Biol. Reprod. 89, 1–7.

Rivera, M.M., Quintero-Moreno, A., Barrera, X., Rigau, T., & Rodriguez-Gil, J.E. 2006. Effects of constant, 9 and 16-h Light Cycles on sperm quality, semen storage ability and motile sperm subpopulations structure of boar semen. Reprod. Dom. Anim. 41, 386–393.

Rubio-Guillen, J., Gonzalez, D., Garde, J.J., Esteso, M.C., Fernandez-Santos, M.R., Rodriguez-Gil, J.E., Madrid-Bury, N., & Quintero-Moreno, A. 2007. Effects of cryopreservation on bull spermatozoa distribution in morphometrically distinct subpopulations. 42, 354–357.

Santolaria, P., Vicente-Fiel, S., Palacín, I., Fantova, E., Blasco, M.E., Silvestre, M.A., & Yániz, J.L. 2015. Predictive capacity of sperm quality parameters and sperm subpopulations on field fertility after artificial insemination in sheep. Anim. Reprod. Sci. 163, 82–88.

Souza, A.K., Trautwein, L.G.C., Paranzini, C.S., Perencin, F.M., Cardoso, G.S., & Martins, M.I.M. 2018. Influence of cooling temperature in sperm subpopulations of domestic cats. Anim. Reprod. Sci. 189, 1–10.

Thurston, L.M., Watson, P.F., & Holt, W.V. 1999. Sources of variation in the morphological characteristics of sperm subpopulations assessed objectively by a novel automated sperm morphology analysis system. J. Reprod. Fertil. 117, 271–280.

Thurston, L.M., Watson, P.F., Mileham, A.J., & Holt, W.V. 2001. Morphologically distinct sperm subpopulations defined by Fourier Shape descriptors in fresh ejaculates correlate. 22, 382–394.

Valle, R.R., Arakaki, P.R., Carvalho, F.M., Muniz, J.A.P. C., Leal, C.L.V., & García-Herreros, M. 2013. Identification of sperm head subpopulations with defined pleiomorphic characteristics in ejaculates of captive Goeldi's monkeys (*Callimico goeldii*). Anim. Reprod. Sci. 137, 93–102.

Valle, R.R., Nayudu, P.L., Leal, C.L.V., & García-Herreros, M. 2012. Sperm head morphometry in ejaculates of adult marmosets (*Callithrix jacchus*): A model for studying sperm subpopulations and among-donor variations. Theriogenology 78, 1152–1165.

Valverde, A., Arenán, H., Sancho, M., Contell, J., Yániz, J., Fernández, A., & Soler, C. 2016. Morphometry and subpopulation structure of Holstein bull spermatozoa: Variations in ejaculates and cryopreservation straws. Asian J. Androl. 18, 851–857.

Vázquez, A.J.F., Cedillo, M.J., Quezada, V.J., Rivas, A.C., Morales, E.C.L., Ayala, E.M.E., Hernández, M.J., González, R.A., & Aragón, M.A. 2015. Effects of repeated electroejaculations on kinematic sperm subpopulations and quality markers of Mexican creole goats. Anim. Reprod. Sci. 154, 29–38.

Vicente-Fiel, S., Palacín, I., Santolaria, P., & Yániz, J. L. 2013. A comparative study of sperm morphometric subpopulations in cattle, goat, sheep and pigs using a computer-assisted fluorescence method (CASMA-F). Anim. Reprod. Sci. 139, 182–189.

Villaverde-Morcillo, S., Soler, A.J., Esteso, M.C., Castaño, C., Miñano-Berna, A., Gonzalez, F., & Santiago-Moreno, J. 2017. Immature and mature sperm morphometry in fresh and frozen-thawed falcon ejaculates. Theriogenology 98, 94–100.

Yániz, J.L., Palacín, I., Vicente-Fiel, S., Sánchez-Nadal, J.A., & Santolaria, P. 2015. Sperm population structure in high and low field fertility rams. Anim. Reprod. Sci. 156, 128–134.

Zaja, I.Z., Vince, S., Milinkovic-Tur, S., Milas, P.N., Samardzija, M., Valpotic, H., Berta, V., Vilic, M., & Rakic, K. 2018. Exogenous melatonin in fluences distribution of French Alpine buck spermatozoa in morphometrically distinct subpopulations during the non-breeding season. 192, 154–163.

Chapter 4

RESEARCH CHAPTER: The relationship between sperm subpopulation classification parameters, recorded sperm motility, and sperm fertilizing potential

The results presented in this chapter will be discussed according to each model that was used to determine the potential of sperm subpopulation structure and associated computer-aided sperm analysis (CASA) parameters to determine or estimate the fertilizing ability of the spermatozoa in a sample. The velocity-associated measurements (VCL, VSL, VAP, ALH) were considered as and are presented as a unit, since these motility parameters directly pertain to the ability of a sperm to reach the site of fertilization as soon as possible after entry to the female reproductive tract. From the studies included in the analysis, these parameters also were presented in all the publications included in the analysis dataset.

The remainder of the motility-associated parameters (WOB, LIN, STR, BCF) were considered as being indicative of the capacitation status of spermatozoa, and where thus analysed and presented separately. The latter set of motility parameters were not equally represented in the publications considered for inclusion in the analysis dataset and are thus presented separately.

Table 4.1 presents all publications included in the motility dataset, regardless of whether the studies involved the use of fresh and/or frozen-thawed samples. The studies represented a variety of species and in all cases, the results reported in the respective studies were categorized into a minimum of three subpopulations.

Elucidation on the exclusion of the other publications in the dataset for this chapter, i.e. that were indicated in Chapter 3, will be presented as a chapter appendix.

Table 4.1 All publications that met the selection criteria and provided sufficient data to be included in motility dataset.

	Author (-s)	**Year**	**Species**	**Type of sample**
1	Ibanescu *et al.*	2020	Bovine	Frozen-thawed
2	Ferraz *et al.*	2014	Bovine	Fresh
3	Muino *et al.*	2008[a]	Bovine	Frozen-thawed
4	Muino *et al.*	2008[b]	Bovine	Frozen-thawed
5	Muino *et al.*	2009	Bovine	Fresh and Frozen-thawed
6	Nunez-Martinez *et al.*	2006	Canine	Fresh and Frozen-thawed

7	Dorado *et al.*	2011[a]	Canine	Fresh and Frozen-thawed
8	Dorado *et al.*	2011[b]	Canine	Fresh
9	Ramon *et al.*	2013	Deer	Frozen-thawed
10	Ramon *et al.*	2012	Deer	Frozen-thawed
11	Flores *et al.*	2008	Equine	Fresh and Frozen-thawed
12	Quintero-Moreno *et al.*	2003	Equine	Fresh
13	Ortega-Ferrusola *et al.*	2009	Equine	Fresh and Frozen-thawed
14	Miro *et al.*	2020	Equine	Frozen-thawed
15	Dorado *et al.*	2013[a]	Equine	Fresh
16	Dorado *et al.*	2013[b]	Equine	Fresh
17	Miro *et al.*	2009	Equine	Fresh
18	Miro *et al.*	2005	Equine	Fresh
19	Garcia-Alvarez *et al.*	2013	Ovine	Frozen-thawed
20	Bergstein-Galan *et al.*	2017	Ovine	Frozen-thawed
21	Bravo *et al.*	2011	Ovine	Fresh
22	Yaniz *et al.*	2015	Ovine	Fresh
23	Ledesma *et al.*	2017	Ovine	Frozen-thawed
24	Flores *et al.*	2008	Porcine	Fresh and Frozen-thawed
25	Cremades *et al.*	2005	Porcine	Frozen-thawed
26	Estrada *et al.*	2017	Porcine	Frozen-thawed
27	Quintero-Moreno *et al.*	2004	Porcine	Fresh
28	Flores *et al.*	2009	Porcine	Fresh and Frozen-thawed
29	Rivera *et al.*	2006	Porcine	Fresh
30	Ramio *et al.*	2008	Porcine	Fresh

[a,b] Different superscripts indicate different publications from the same first author published in the same year

4.1 RESULTS FOR VELOCITY-ASSOCIATED PARAMETERS FOR FRESH SAMPLES

The model with the best fit, according to adjusted-R^2 (R^2), Akaike Information Criterion (AIC) and biological significance, included the independent variables species, sperm subpopulation, CASA system used (SCA® or ISAS), and type of medium used (synthetic or non-synthetic).

Overall, an ANOVA analysis of the fresh sperm sample motility dataset indicated that R^2 values ranged from 30.5% (SSD=26.9%) for ALH, compared to 50.6% (SSD=51.8%) for VSL (Table 4.2).

Table 4.2 Coefficient of variation (R^2) calculated for the velocity-associated parameters for the fresh sample motility dataset.

Parameter	R^2 value	SSD value
VAP	0.418	0.541
VSL	0.506	0.518
VCL	0.467	0.571
ALH	0.305	0.269

4.1.1 Influence of species

Table 4.3 presents the respective velocity-associated parameters for fresh bovine, canine, equine, ovine, and porcine sperm samples, as determined by using Model 1 (please refer to Chapter 3 for model description). The number of ejaculates collected is indicated in brackets.

When the species representation is considered (i.e. bovine, ovine, equine, canine and porcine), boar samples constituted 51% of the dataset. The overall observed variation for the fresh motility dataset was considerable for the respective parameters, with the largest degree of variation observed in ovine samples. Ram sperm samples also exhibited the largest degree of variation for curvilinear velocity (VCL; Table 4.3). Equine samples exhibited the smallest degree of variation for the velocity-associated parameters, and the smallest degree of variation for the VSL parameter (Table 4.3).

Table 4.3 Computer-aided sperm velocity-related parameters (mean ± SD) reported for fresh sperm samples obtained from cattle, dogs, horses, sheep, and pigs, in the period 2003 to 2015, and presented per species.

Species	Parameter (unit of measurement)	Mean ± SD	Range	Coefficient of Variation
Bovine (n=75)	VAP ($\mu m.s^{-1}$)	75.04 ± 34.05	15.00 - 126.30	2.20
	VSL ($\mu m.s^{-1}$)	56.53 ± 31.99	8.90 - 110.80	1.77
	VCL ($\mu m.s^{-1}$)	107.18 ± 43.07	29.50 - 159.60	2.49
	ALH (μm)	3.85 ± 1.45	1.50 - 6.30	2.66
Canine (n=30)	VAP ($\mu m.s^{-1}$)	81.71 ± 36.55	31.14 - 130.02	2.23
	VSL ($\mu m.s^{-1}$)	61.50 ± 38.35	12.86 - 121.82	1.60
	VCL ($\mu m.s^{-1}$)	108.22 ± 44.66	27.80 - 165.49	2.42
	ALH (μm)	3.12 ± 1.07	1.94 - 5.13	2.91
Equine (n=~340)	VAP ($\mu m.s^{-1}$)	74.29 ± 42.54	16.50 - 183.40	1.75
	VSL ($\mu m.s^{-1}$)	61.18 ± 45.09	10.50 - 161.80	1.36
	VCL ($\mu m.s^{-1}$)	106.66 ± 54.38	30.70 - 203.10	1.96
	ALH (μm)	3.90 ± 2.25	1.36 - 10.98	1.73
Ovine	VAP ($\mu m.s^{-1}$)	91.66 ± 32.27	43.50 - 136.59	2.84

(n=311)	VSL (µm.s^{-1})	70.94 ± 34.52	36.45 - 129.33	2.06
	VCL (µm.s^{-1})	115.86 ± 35.07	58.06 - 147.23	3.30
	ALH (µm)	3.36 ± 1.32	2.13 - 5.25	2.55
Porcine	VAP (µm.s^{-1})	56.16 ± 25.16	15.20 - 121.10	2.23
(n=~265)	VSL (µm.s^{-1})	56.74 ± 36.81	9.80 - 154.70	1.54
	VCL (µm.s^{-1})	79.76 ± 36.17	25.50 - 140.90	2.21
	ALH (µm)	4.00 ± 1.76	1.40 - 10.34	2.28

~ to indicate where actual values were not reported in all publications and an estimate was calculated based on the provided values

Table 4.4 presents the least square means (LS means) and SSD values for the velocity-associated parameters, per species. Species significantly influenced most of the velocity-associated parameters (P≤0.004), with VAP (P=0.138) and ALH (P=0.318) being the exceptions (Table 4.4).

Table 4.4 The influence of species on the motility parameters (LS means ± SSD) associated with sperm velocity parameters, recorded for fresh sperm samples.

Species	**Parameter**			
	VAP	**VSL**	**VCL**	**ALH**
Bovine	73.60 ± 17.93	2.01^{d} ± 18.14	42.27^{c} ± 30.81	2.83 ± 0.98
Canine	71.05 ± 1.21	60.85^{bc} ± 5.72	110.69^{bc} ± 6.98	3.85 ± 0.45
Equine	70.10 ± 5.19	87.89^{b} ± 5.87	141.17^{ab} ± 6.87	4.06 ± 0.08
Ovine	97.16 ± 10.33	130.59^{a} ± 8.84	184.71^{a} ± 5.16	4.48 ± 0.87
Porcine	65.26 ± 3.08	57.83^{c} ± 0.86	75.64^{c} ± 0.51	3.22 ± 0.79

$^{a-d}$ Different superscripts in columns indicate significant differences (P≤0.05)

4.1.2 Influence of the number of sperm subpopulations

Table 4.5 presents the respective CASA velocity-associated parameters per sperm subpopulation for fresh sperm samples, as determined by using Model 1 (please refer to Chapter 3 for model description). The number of ejaculates collected is indicated in brackets.

When the number of sperm subpopulations that were reported is considered, a total of 22 publications reported on three sperm subpopulations, whilst only 18 publications included a fourth sperm subpopulation.

Table 4.5 Computer-aided sperm velocity-related parameters (mean ± SD) reported for fresh sperm samples obtained from cattle, dogs, horses, sheep, and pigs, in the period 2003 to 2015, and presented per sperm subpopulation.

Sperm subpopulation	Parameters	Mean ± SD	Range	Coefficient of Variation
SP1 (n=~1069)	VAP (µm.s^{-1})	45.54 ± 30.92	15.20 - 154.40	1.47
	VSL (µm.s^{-1})	28.26 ± 21.51	9.80 - 108.91	1.31
	VCL (µm.s^{-1})	62.96 ± 26.84	25.50 - 135.06	2.35
	ALH (µm)	2.86 ± 0.97	1.37 - 4.95	2.97
SP2 (n=~1069)	VAP (µm.s^{-1})	57.65 ± 26.18	16.50 - 136.59	2.20
	VSL (µm.s^{-1})	51.84 ± 24.05	10.50 - 129.33	2.15
	VCL (µm.s^{-1})	82.42 ± 32.92	27.80 - 153.40	2.50
	ALH (µm)	3.23 ± 1.10	1.79 - 6.30	2.92
SP3 (n=~1069)	VAP (µm.s^{-1})	71.38 ± 29.81	15.00 - 155.70	2.40
	VSL (µm.s^{-1})	62.40 ± 36.13	8.90 - 121.82	1.73
	VCL (µm.s^{-1})	108.97 ± 43.49	29.50 - 198.10	2.51
	ALH (µm)	4.81 ± 2.18	1.50 - 10.34	2.21
SP4 (n=~943)	VAP (µm.s^{-1})	95.16 ± 28.90	25.73 - 183.40	3.29
	VSL (µm.s^{-1})	94.84 ± 31.89	34.90 - 161.80	2.97
	VCL (µm.s^{-1})	120.64 ± 42.93	30.20 - 203.10	2.81
	ALH (µm)	4.46 ± 1.86	1.36 - 10.98	2.39

~ to indicate where actual values were not reported in all publications and an estimate was calculated based on the provided values

From the results presented in Table 4.5 and e.g. when the VAP parameter is considered, the smallest degree of variation was observed in sperm subpopulation 1 (SP1), when compared to sperm subpopulation 4 (SP4; 1.47 vs. 3.29). This general pattern was also evident for the other velocity-associated parameters (Table 4.5).

Table 4.6 presents the LS means and SSD values for the velocity-associated parameters, on a sperm subpopulation level. Sperm subpopulations differed significantly in terms of all the velocity-associated parameters ($P \leq 0.001$; Table 4.6).

Table 4.6 The influence of subpopulation on the motility parameters (LS means ± SSD) associated with sperm velocity parameters, recorded for fresh sperm samples.

Sperm subpopulation	Parameter			
	VAP	VSL	VCL	ALH
SP1	53.32^{c} ± 7.52	36.61^{c} ± 6.82	79.89^{b} ± 8.49	2.70^{b} ± 0.37
SP2	65.43^{bc} ± 7.27	60.19^{b} ± 6.76	99.34^{b} ± 9.09	3.06^{b} ± 0.41
SP3	79.16^{b} ± 7.70	70.75^{b} ± 7.23	125.89^{a} ± 9.92	4.64^{a} ± 0.41
SP4	103.82^{a} ± 7.70	103.78^{a} ± 9.36	138.48^{a} ± 11.94	4.36^{a} ± 0.62

$^{a.b.c}$ Different superscripts in columns indicate significant differences (P≤0.05)

4.1.3 Influence of CASA system used

When the type of CASA system used was considered, only the SCA® and ISAS systems were represented and evenly distributed (SCA® - 12 studies vs. ISAS - 9 studies).

Table 4.7 Computer-aided sperm velocity-related parameters (mean ± SD) reported for fresh sperm samples obtained from cattle, dogs, horses, sheep, and pigs, in the period 2003 to 2015. and presented per CASA system used.

CASA system	Parameter	Mean ± SD	Range	Coefficient of Variation
SCA®	VAP (µm.s^{-1})	73.72 ± 37.10	15.00 - 183.40	1.99
	VSL (µm.s^{-1})	66.14 ± 38.84	8.90 - 161.80	1.70
	VCL (µm.s^{-1})	113.55 ± 44.02	29.50 - 203.10	2.58
	ALH (µm)	3.82 ± 2.11	1.36 - 10.98	1.81
ISAS	VAP (µm.s^{-1})	59.55 ± 29.16	15.20 - 136.59	2.04
	VSL (µm.s^{-1})	55.79 ± 36.32	9.80 - 154.70	1.54
	VCL (µm.s^{-1})	84.38 ± 39.64	25.50 - 164.60	2.13
	ALH (µm)	3.81 ± 1.25	1.37 - 7.43	3.04

When the results presented in Table 4.7 are considered, it is evident that both systems (ISAS and SCA®) show a large degree of variation for each given parameter. A lack of standardization of CASA systems could contribute to this observation.

Table 4.8 presents the LS means and SSD values for the velocity-associated parameters reported by the SCA® and ISAS systems, respectively. The studies that used the SCA® system reported higher values than the ISAS system for the respective parameters.

Table 4.8 The influence of CASA on the motility parameters (LS means ± SSD) associated with sperm velocity parameters, recorded for fresh sperm samples.

CASA system	Parameter			
	VAP	VSL	VCL	ALH
SCA®	81.49 ± 7.09	95.63[a] ± 3.88	141.29[a] ± 3.61	3.83 ± 0.88
ISAS	69.38 ± 8.00	40.03[b] ± 11.20	80.50[b] ± 16.11	3.54 ± 0.03

[a.b] Different superscripts in columns indicate significant differences (P≤0.0001)

Except for the VAP and ALH parameters, there was a significant difference between the SCA® and ISAS values reported for VCL and VSL (Table 4.8; P≤0.0001).

Furthermore, the SCA® and ISAS systems did not differ in terms of the characterization of the respective sperm subpopulations (P≥0.05). This was further supported by a low R^2 value (VAP=0.42; ALH=0.31). Regarding the use of either the SCA® or ISAS system to characterise sperm subpopulations, it was observed that studies indicated a particular preference for the use a type of CASA system on species level. The SCA® system was the only CASA system used in the bovine studies, whilst the ISAS system was the only CASA system used in the ovine studies.

4.1.4 Influence of type of medium used

When the type of medium used was considered, more studies used synthetic mediums than non-synthetic mediums (12 studies vs. 10 studies). Table 4.9 presents the descriptive statistics for the respective velocity-associated motility parameters, as influenced by the type of medium used.

Table 4.9 Computer-aided sperm velocity-related parameters (mean ± SD) reported for fresh sperm samples obtained from cattle, dogs, horses, sheep, and pigs, in the period 2003 to 2015, and presented per medium used.

Type of medium	Parameter	Mean ± SD	Range	Coefficient of Variation
Synthetic	VAP (µm.s^{-1})	59.83 ± 26.94	15.00 - 126.30	2.22
	VSL (µm.s^{-1})	56.87 ± 35.84	8.90 - 154.70	1.59
	VCL (µm.s^{-1})	85.94 ± 39.85	25.50 - 159.60	2.16
	ALH (µm)	4.11 ± 1.92	1.40 - 10.98	2.14
Non-synthetic	VAP (µm.s^{-1})	80.65 ± 42.30	16.50 -183.40	1.91
	VSL (µm.s^{-1})	63.24 ± 40.05	10.50 -161.80	1.58
	VCL (µm.s^{-1})	108.27 ± 45.73	30.70 -203.10	2.37
	ALH (µm)	3.23 ± 1.31	1.36 -7.43	2.47

Table 4.10 presents the LS means and SSD values for the influence of medium on the velocity-associated parameters.

Table 4.10 The influence of medium on the motility parameters (LS means ± SSD) associated with sperm velocity-associated parameters, recorded for fresh sperm samples.

Type of medium	Parameter			
	VAP	VSL	VCL	ALH
Synthetic	70.82 ± 6.57	94.55[a] ± 8.19	145.42[a] ± 8.48	4.57[a] ± 0.36
Non-synthetic	80.04 ± 8.52	41.12[b] ± 6.89	76.38[b] ± 11.24	2.81[b] ± 0.55

[a.b] Different superscripts in columns indicate significant differences (P≤0.05)

Type of medium used significantly influenced the values reported for VSL, VCL, and ALH (P≤0.01; Table 4.10). Type of medium did not influence the VAP values reported in the respective studies (P≥0.05; Table 4.10).

4.2 Interaction between type of CASA system and medium used

The estimated interaction between type of CASA system and medium used is presented in Table 4.11 and was found to be significant (P≤0.0001). However, no clear pattern between the two variables was observed. For ALH, a significant difference between mediums used was observed, however no difference was found between the two CASA systems or the type of medium used. For VAP, there was a significant difference between the CASA systems (P=0.001).

Table 4.11 The interaction between CASA system and type of medium used on sperm velocity-related measurements, recorded for fresh sperm samples.

Interaction (CASA*Medium)	Parameter	
	VAP	ALH
SCA®*Non-synthetic	96.48[a] ± 7.29	2.75[b] ± 0.76
SCA®*Synthetic	76.92[ab] ± 6.12	4.65[a] ± 0.72
ISAS*Non-synthetic	53.54[b] ± 10.51	3.20[ab] ± 0.66
ISAS*Synthetic	77.34[ab] ± 6.08	4.17[ab] ± 0.33

[a.b] Different superscripts in columns indicate significant differences (P≤0.05)

4.3 Correlations for the motility dataset for fresh sperm samples

4.3.1 Subpopulation 1

Strong positive correlations were found between VAP and VCL for all the species included in the fresh dataset correlation test, namely for bovine (0.960), equine (0.998) and porcine (0.779) samples. Furthermore, all three species also showed strong positive correlations between VCL and ALH, with 0.967, 0.998 and 0.950 for bovine, equine and porcine respectively. The VSL and VCL parameters were also found to be positively correlated, however this was only the case with the values obtained from the bovine data

(0.944) and equine data (0.957). These two variables were negatively correlated in the case of porcine samples (-0.342).

4.3.2 Subpopulation 2

As with subpopulation one, strong positive correlations were found for all of the species for VAP and VCL for the fresh data (Bovine 0.750; equine 0.964; porcine 0.766). Porcine additionally showed a strong positive correlation between VAP and ALH (0.872) and equine between VAP and VSL (0.992).

4.3.3 Subpopulation 3

Bovine fresh data showed strong positive correlations between VSL and VCL (0.993) and between VSL and ALH (0.983). Porcine showed a strong negative correlation between VSL and VCL (-0.899). This was also the case between VAP and VCL (-0.711). A strong positive correlation was found for equine between VSL and ALH (-0.760). All species showed a strong positive correlation between VCL and ALH (Bovine 0.998; equine 0.995; porcine 0.936).

4.3.4 Subpopulation 4

Fewer correlations between variables were found for this subpopulation. Porcine showed only a strong negative correlation between VSL and VCL (-0.794). Equine again, as in subpopulation three, showed that VSL and ALH are negatively correlated (-0.869). However, a positive correlation existed between VAP and VSL (0.961), as well as between VAP and VCL (0.804). Bovine showed a positive correlation between VAP and VSL (0.995) and between VCL and ALH (0.863).

4.4 RESULTS FOR MOTILITY DERIVED PARAMETERS (FRESH SAMPLES)

Due to fewer publications measuring and presenting parameters associated with sperm capacitation status (versus parameters associated with sperm velocity), less data was available for this dataset and subsequently descriptive statistics are not provided for these measurements. The LS means and SSD values are provided for every independent variable included in Model 1.

4.4.1 Influence of species

Table 4.12 presents the LS means and SSD values for the motility parameters associated with the capacitation status of sperm, per species.

Table 4.12 The influence of species on the capacitation status-associated parameters (LS means ± SSD), recorded for fresh sperm samples.

Species	Parameter			
	LIN	STR	WOB	BCF
Bovine	58.69 ± 15.23	68.43^{ab} ± 15.58	74.68^{a} ± 11.68	3.20^{b} ± 3.41
Canine	47.38 ± 1.06	63.05^{ab} ± 2.24	56.38^{ab} ± 1.77	8.54^{a} ± 0.28
Equine	51.55 ± 4.33	70.61^{ab} ± 2.47	64.52^{ab} ± 2.26	10.65^{a} ± 1.09
Ovine	29.21 ± 6.17	49.12^{b} ± 6.29	41.92^{b} ± 4.79	12.19^{a} ± 2.28
Porcine	55.25 ± 4.44	75.95^{a} ± 3.13	77.24^{a} ± 2.19	8.60^{a} ± 1.19

$^{a.b}$ Different superscripts in columns indicate significant differences (P≤0.05)

Most of the parameters, except for LIN, were significantly influenced by species (P≤0.05; Table 4.12). The highest STR and WOB values were reported for boars, when compared to the other species (Table 4.12). The lowest STR and WOB values were reported for rams (49.12 and 41.92; Table 4.12).

4.4.2 Influence of the number of sperm subpopulations

Table 4.13 presents the LS means and SSD values for the motility parameters associated with the capacitation status of sperm, per sperm subpopulation. The sperm subpopulations differed significantly in terms of all the motility parameters associated with sperm capacitation status (P≤0.05; Table 4.13).

Table 4.13 The influence of subpopulation classification on the capacitation status-associated motility parameters (LS means ± SSD) of fresh sperm samples.

Sperm subpopulation	Parameter			
	LIN	STR	WOB	BCF
SP1	40.87^{b} ± 5.67	54.01^{c} ± 4.79	55.81^{b} ± 4.80	7.46^{b} ± 1.56
SP2	54.62^{a} ± 5.52	69.20^{ab} ± 5.52	64.35^{ab} ± 4.40	8.45^{ab} ± 1.28
SP3	40.95^{b} ± 6.56	62.26^{bc} ± 6.55	59.24^{b} ± 4.51	8.79^{ab} ± 1.75
SP4	57.22^{a} ± 7.24	76.25^{a} ± 6.91	72.39^{a} ± 4.43	9.85^{a} ± 1.57

$^{a.b.c}$ Different superscripts in columns indicate significant differences (P≤0.05)

4.4.3 Influence of type of CASA system used

Table 4.14 presents the LS means and SSD values for the motility parameters associated with the capacitation status of sperm, per CASA system used. There were significant differences between the ISAS and SCA® systems for both parameters of WOB (P=0.020) and BCF (P<0.0001).

Table 4.14 The influence of type of CASA system used on the capacitation status-associated motility parameters (LS means ± SSD) of fresh sperm samples.

CASA system	Parameter			
	LIN	STR	WOB	BCF
SCA®	47.26 ± 5.47	70.24 ± 4.81	68.81[a] ± 4.04	11.42[a] ± 1.64
ISAS	49.57 ± 7.023	60.62 ± 7.08	57.08[b] ± 5.03	5.85[b] ± 1.44

[a.b] Different superscripts in columns indicate significant differences (P≤0.05)

4.4.4 Influence of type of medium used

Table 4.15 presents the LS means and SSD values for the motility derived parameters,, per type of medium used. As seen above (Table 4.14), the only significant differences between mediums were found for parameters WOB and BCF (P<0.0001; Table 4.15). Straight-line velocity could not be estimated.

Table 4.15 The influence of type of medium used on the capacitation status-associated motility parameters (LS means ± SSD) of fresh sperm samples.

Type of medium	Parameter		
	LIN	WOB	BCF
Synthetic	42.85 ± 5.65	51.48[b] ± 5.17	9.88[a] ± 1.13
Non-synthetic	53.98 ± 6.84	74.42[a] ± 3.90	7.39[b] ± 1.95

[a.b] Different superscripts in columns indicate significant differences (P≤0.05)

4.5 RESULTS FOR VELOCITY-ASSOCIATED PARAMETERS FOR FROZEN-THAWED SAMPLES

Model 2 (please refer to Chapter 3 for a description of the model), was used in the analysis of velocity-associated motility parameters obtained for frozen-thawed samples. The model similarly included species, sperm subpopulation, type of CASA system used, and type of medium used, as fixed effects. In the publications considered for analysis with Model 2, bovine, deer, canine, equine, ovine, and porcine species were represented. Porcine studies constituted the bulk of the publications used for the analysis (41.7%).

Most of the studies reported on three sperm subpopulations (30 studies), with only 25 studies reporting on four sperm subpopulations.

Three CASA systems were represented in this dataset, namely the SCA®, ISAS and IVOS systems. The SCA® system was used in the most studies (9 studies), followed by the ISAS (7 studies), and IVOS (2 studies) systems.

Twelve of the studies used synthetic mediums, whilst six studies used non-synthetic mediums.

Overall, an ANOVA analysis of the frozen-thawed sperm sample motility dataset indicated that R^2 values ranged from 69.4% (SSD=92.1%) for ALH, compared to 37.9% (SSD=53.5%) for VSL (Table 4.16).

Table 4.16 Coefficient of variation (R^2) calculated for the velocity-associated parameters for the motility dataset for frozen-thawed samples.

Parameter	R^2 value	SSD value
VAP	0.502	0.706
VSL	0.379	0.535
VCL	0.627	0.727
ALH	0.694	0.921

4.5.1 Influence of species

Table 4.17 presents the respective CASA velocity-associated parameters for frozen-thawed bovine, canine, deer, equine, ovine, and porcine sperm samples, as determined by using Model 2 (please refer to Chapter 3 for model description). The number of ejaculates collected is indicated in brackets.

Table 4.17 Computer-aided sperm velocity-associated parameters (mean ± SD) for frozen-thawed sperm samples obtained from cattle, dogs, deer, horses, sheep, and pigs, in the period 2005 to 2020, presented per species

Species	Parameter	Mean ± SD	Range	Coefficient of Variation
Bovine (n=233)	VAP (µm.s^{-1})	74.35 ± 38.55	14.30 - 158.90	1.93
	VSL (µm.s^{-1})	55.56 ± 33.43	8.10 - 130.10	1.66
	VCL (µm.s^{-1})	121.10 ± 73.87	26.80 - 307.40	1.64
	ALH (µm)	5.02 ± 3.46	1.40 - 14.40	1.45
Canine (n=30)	VAP (µm.s^{-1})	53.78 ± 38.12	10.71 - 104.88	1.41
	VSL (µm.s^{-1})	55.78 ± 41.10	4.79 - 134.50	1.36
	VCL (µm.s^{-1})	94.27 ± 48.50	25.28 - 153.60	1.94
	ALH (µm)	2.56 ±1.28	1.48 - 4.56	2.01
Deer (n=37)	VAP (µm.s^{-1})	73.81 ± 38.14	16.41 - 128.09	1.94
	VSL (µm.s^{-1})	45.30 ± 32.99	9.13 - 112.10	1.37
	VCL (µm.s^{-1})	118.78 ± 50.05	34.73 - 179.55	2.37
	ALH (µm)	4.04 ± 1.74	1.83 - 5.92	2.33
Equine (n=~32)	VAP (µm.s^{-1})	46.53 ± 32.54	8.98 - 96.50	1.43
	VSL (µm.s^{-1})	31.73 ± 25.92	5.40 - 87.30	1.22
	VCL (µm.s^{-1})	76.77 ± 52.07	18.60 - 184.70	1.47
	ALH (µm)	3.45 ± 2.54	1.02 - 8.67	1.36
Ovine	VAP (µm.s^{-1})	85.61 ± 46.73	23.20 - 138.80	1.83

(n=~170)	VSL (µm.s^{-1})	80.13 ± 58.47	11.00 - 187.90	1.37
	VCL (µm.s^{-1})	139.87 ± 76.95	39.80 - 266.00	1.82
	ALH (µm)	3.81 ± 3.12	1.20 - 11.10	1.22
Porcine	VAP (µm.s^{-1})	53.64 ± 28.65	5.54 - 115.80	1.87
(n=~100)	VSL (µm.s^{-1})	80.68 ± 56.28	0.00 - 192.00	1.43
	VCL (µm.s^{-1})	45.24 ± 22.63	12.01 - 122.60	2.00
	ALH (µm)	3.17 ± 1.85	1.29 - 7.18	1.71

~ to indicate where actual values were not reported in all publications and an estimate was calculated based on the provided values

Similarly to what was observed for the fresh sample dataset, a large degree of variation was observed for the respective parameters, and for each species. The studies reporting in the respective parameters in deer demonstrated the largest degree of variation, whilst equine studies similarly when compared to the values for fresh samples, demonstrated the smallest degree of variation.

Table 4.18 presents the LS means and SSD values for the velocity-associated motility parameters for frozen-thawed samples.

Table 4.18 The influence of species on the velocity-associated motility parameters (LS means ± SSD) of frozen-thawed sperm samples.

	Parameter			
Species	**VAP**	**VSL**	**VCL**	**ALH**
Bovine	89.19 ± 20.19	71.18^{ab} ± 19.22	142.01^{a} ± 33.11	5.28 ± 1.72
Canine	84.08 ± 7.25	66.10^{ab} ± 7.98	139.42^{a} ± 13.99	4.47 ± 0.76
Deer	93.51 ± 8.74	64.83^{ab} ± 11.76	156.50^{a} ± 22.05	5.60 ± 0.83
Equine	50.31 ± 8.62	19.66^{b} ± 3.78	113.18^{ab} ± 13.96	6.25 ± 0.95
Ovine	79.39 ± 31.74	82.48^{a} ± 25.06	128.28^{ab} ± 39.56	4.33 ± 1.18
Porcine	54.74 ± 5.08	72.57^{a} ± 11.29	70.57^{b} ± 19.82	5.45 ± 1.05

$^{a.b.}$ Different superscripts in columns indicate significant differences (P≤0.05)

Species significantly influenced only the VCL and VSL parameters (P≤0.05; Table 4.18). Frozen-thawed deer sperm samples had the fastest curvilinear velocity compared to the rest of the species, whereas frozen-thawed ovine samples exhibited the highest straight-line velocity (P≤0.05; Table 4.18). Frozen-thawed equine spermatozoa had the slowest average path velocity, straight-line velocity and curvilinear velocity, and the largest amplitude for lateral sperm head displacement, when compared to the other species (P≤0.05; Table 4.18).

4.5.2 Influence of the number of sperm subpopulations

Table 4.19 presents the influence of sperm subpopulation classification on the velocity-associated motility parameters reported for frozen-thawed sperm samples.

Table 4.19 Computer-aided sperm velocity-associated parameters (mean ± SD) for frozen-thawed sperm samples obtained from cattle, dogs, deer, horses, sheep, and pigs, in the period 2005 to 2020, presented per subpopulation.

Sperm subpopulation	Measurement	Mean ± SD	Range	Coefficient of Variation
SP1	VAP (µm.s^{-1})	44.84 ± 24.26	8.98 - 110.00	1.85
(n=~592)	VSL (µm.s^{-1})	40.16 ± 23.65	4.79 - 100.10	1.70
	VCL (µm.s^{-1})	71.89 ± 37.69	18.60 - 152.20	1.91
	ALH (µm)	3.28 ± 2.24	1.02 - 9.10	1.46
SP2 (n=~592)	VAP (µm.s^{-1})	62.21 ± 28.76	15.86 - 133.30	2.16
	VSL (µm.s^{-1})	51.92 ± 32.99	5.59 - 124.30	1.57
	VCL (µm.s^{-1})	95.08 ± 51.67	32.70 - 266.00	1.84
	ALH (µm)	4.30 ± 2.09	1.50 - 11.10	2.06
SP3 (n=~592)	VAP (µm.s^{-1})	61.37 ± 46.55	5.54 - 158.90	1.32
	VSL (µm.s^{-1})	65.16 ± 55.81	0.00 - 187.90	1.17
	VCL (µm.s^{-1})	92.31 ± 82.24	12.01 - 294.10	1.12
	ALH (µm)	3.94 ± 3.34	1.29 - 12.30	1.18
SP4 (n=~355)	VAP (µm.s^{-1})	98.02 ± 25.80	23.20 - 140.10	3.80
	VSL (µm.s^{-1})	88.62 ± 45.31	11.00 - 192.00	1.96
	VCL (µm.s^{-1})	133.31 ± 75.91	23.90 - 307.40	1.76
	ALH (µm)	5.80 ± 4.04	1.20 - 14.40	1.44

* to indicate where actual values were not reported in all publications and an estimate was calculated based on the provided values

From the results presented in Table 4.19, and e.g. when the VSL parameter is considered, the smallest degree of variation was observed in sperm subpopulation 3 (SP3), when compared to SP4 (1.17 vs. 1.96).

Table 4.20 presents the LS means and SSD values for the velocity-associated parameters recorded for frozen-thawed spermatozoa, per subpopulation.

The sperm subpopulations differed significantly in terms of all the motility parameters associated with sperm capacitation status (P≤0.05; Table 4.20). It was further evident that SP4 classified sperm with the largest values for the dependent variables, compared to the rest of the subpopulations (e.g. SP4 VAP=106.21 vs. SP1 VAP=53.56). The opposite was true for SP1 that was classified with the smallest values. It was noted that this was however not the case for porcine samples, where SP3 represented sperm with

the smallest values for the velocity measurements, in comparison to the other subpopulations for this species.

Table 4.20 The influence of subpopulation on the velocity-associated motility parameters (LS means ± SSD) of frozen-thawed sperm samples.

Sperm subpopulation	Parameter			
	VAP	VSL	VCL	ALH
SP1	53.56^{b} ± 11.88	41.43^{b} ± 10.52	98.73^{b} ± 21.30	4.15^{b} ± 1.00
SP2	70.93^{b} ± 14.85	53.19^{b} ± 12.89	121.92^{b} ± 23.45	5.18^{b} ± 1.10
SP3	70.09^{b} ± 13.12	66.43^{ab} ± 12.01	119.15^{b} ± 22.93	4.81^{b} ± 1.01
SP4	106.21^{a} ± 14.39	90.17^{a} ± 17.31	160.16^{a} ± 27.31	6.78^{a} ± 1.21

$^{a.b}$ Different superscripts in columns indicate significant differences (P≤0.05)

4.5.3 Influence of type of CASA system used

Table 4.21 presents the influence of the CASA system used on the velocity-associated motility parameters reported for frozen-thawed sperm samples.

Table 4.21 Computer-aided sperm velocity-associated parameters (mean ± SD) for frozen-thawed sperm samples obtained from cattle, dogs, deer, horses, sheep, and pigs, in the period 2005 to 2020, presented per type of CASA system used.

CASA system	Parameter	Mean ± SD	Range	Coefficient of Variation
SCA®	VAP (µm.s^{-1})	60.04 ± 34.41	5.54 - 128.09	1.74
	VSL (µm.s^{-1})	48.30 ± 37.44	0.00 - 187.90	1.29
	VCL (µm.s^{-1})	87.19 ± 44.57	12.01 - 179.55	1.96
	ALH (µm)	3.32 ± 1.47	1.29 - 7.18	2.27
ISAS	VAP (µm.s^{-1})	58.90 ± 32.73	8.98 - 138.80	1.80
	VSL (µm.s^{-1})	73.24 ± 52.06	5.40 - 192.00	1.41
	VCL (µm.s^{-1})	71.37 ± 48.02	18.60 - 189.80	1.49
	ALH (µm)	2.86 ± 1.91	1.02 - 8.67	1.50
IVOS	VAP (µm.s^{-1})	101.69 ±40.945	28.50 - 158.90	2.48
	VSL (µm.s^{-1})	73.70 ± 33.76	16.30 - 130.10	2.18
	VCL (µm.s^{-1})	201.83 ± 83.01	70.20 -307.400	2.43
	ALH (µm)	9.33 ± 3.52	3.90 - 14.40	2.65

When the results presented in Table 4.21 are considered, it is evident that the IVOS system yielded values with a larger degree of variation, in comparison with the other CASA systems (e.g. ALH=2.65 vs. 2.27 for

SCA® and 1.50 for ISAS). The ISAS system showed the smallest degree of variation overall when the velocity-related measurements were considered.

Table 4.22 presents the LS means and SSD values for the velocity-associated parameters recorded for frozen-thawed spermatozoa, per CASA system used.

Table 4.22 The influence of type of CASA system used on the velocity-associated motility parameters (LS means ± SSD), recorded for frozen-thawed sperm samples.

Type of CASA system	Parameter			
	VAP	VSL	VCL	ALH
SCA®	$52.42^{b} \pm 17.39$	$42.76^{b} \pm 15.05$	$79.68^{b} \pm 36.08$	$3.50^{b} \pm 2.22$
ISAS	$73.93^{ab} \pm 14.14$	$76.35^{a} \pm 17.75$	$92.78^{b} \pm 24.01$	$2.44^{b} \pm 0.59$
IVOS	$99.26^{a} \pm 9.15$	$69.32^{ab} \pm 6.73$	$202.52^{a} \pm 11.14$	$9.75^{a} \pm 0.44$

$^{a.b}$ Different superscripts in columns indicate significant differences (P≤0.05)

There was a significant difference between the SCA®, ISAS and IVOS systems for all sperm velocity-related values reported (Table 4.22; P≤0.0001). The SCA® system consistently reported lower values for the velocity parameters compared to the other CASA systems, except for ALH, for which the ISAS system provided the lowest value of 2.44 (Table 4.22). The IVOS system was poorly represented in this dataset for the frozen-thawed samples, with only 2 studies utilising this CASA system.

***Note: there was not representation of each of the CASA systems for each of the species included in Model 2.*

4.5.4 Influence of type of medium used

Table 4.23 presents the influence of the type of medium used on the velocity-associated motility parameters reported for frozen-thawed sperm samples.

Table 4.23 Computer-aided sperm velocity-associated parameters (mean ± SD) for frozen-thawed sperm samples obtained from cattle, dogs, deer, horses, sheep, and pigs, in the period 2005 to 2020, presented per medium type used.

Type of medium	Parameter	Mean ± SD	Range	Coefficient of Variation
Synthetic	VAP ($\mu m.s^{-1}$)	63.53 ± 34.36	5.54 - 138.80	1.85
	VSL ($\mu m.s^{-1}$)	69.06 ± 50.23	0.00 - 192.00	1.37
	VCL ($\mu m.s^{-1}$)	80.84 ± 49.51	12.01 - 189.80	1.63
	ALH (µm)	3.26 ± 1.59	1.02 - 7.18	2.05

Non-synthetic	VAP (µm.s^{-1})	67.29 ± 40.46	10.71 - 158.90	1.66
	VSL (µm.s^{-1})	50.72 ± 34.40	4.79 - 130.10	1.47
	VCL (µm.s^{-1})	113.53 ± 77.82	25.28 - 307.40	1.46
	ALH (µm)	4.99 ± 3.63	1.17 - 14.40	1.37

The results in Table 4.23 reported the largest degree of variation for synthetic mediums, e.g. when considering ALH (2.05 vs. 1.37).

Table 4.24 presents the LS means and SSD values for the velocity-associated parameters recorded for frozen-thawed spermatozoa, per type of medium used.

Table 4.24 The influence of type of medium used on the velocity-associated motility parameters (LS means ± SSD), recorded for frozen-thawed sperm samples.

Type of medium	**Parameter**			
	VAP	**VSL**	**VCL**	**ALH**
Synthetic	82.72 ± 14.06	67.23 ± 12.73	137.60^{a} ± 23.14	5.41 ± 1.09
Non-synthetic	67.68 ± 13.06	58.38 ± 13.63	112.38^{b} ± 24.35	5.05 ± 1.08

Type of medium significantly influenced only the VCL parameter (P≤0.05; Table 4.24).

4.6 Interaction between type of CASA system and medium used

As was found for the fresh dataset, where an interaction was estimated between CASA and medium for velocity parameters for the frozen-thawed dataset, there were significant differences between velocity parameters for the various interactions (P≤0.05; Table 4.25). However, no clear pattern was found.

When the parameter VAP was considered, the combination of IVOS*non-synthetic differed significantly from SCA®*synthetic, and SCA®*non-synthetic (P≤0.05). For the parameter ALH, the IVOS*non-synthetic value differed significantly from the values reported by the other CASA systems (P≤0.05).

Table 4.25 The interaction between CASA system and type of medium used, and the effect on sperm velocity-associated parameters, recorded for frozen-thawed sperm samples.

Interaction CASA*Medium	**Parameter**			
	VAP	**VSL**	**VCL**	**ALH**
SCA®*Synthetic	59.93^{b} ± 9.99	47.13^{ab} ± 6.39	92.42^{b} ± 11.02	3.67^{b} ± 0.45
SCA®*Non-synthetic	45.29^{b} ± 6.29	36.82^{b} ± 5.71	64.27^{b} ± 9.02	3.11^{b} ± 0.38
ISAS*Non-synthetic	65.21^{ab} ± 20.20	76.99^{ab} ± 22.70	89.82^{b} ± 33.17	3.12^{b} ± 0.78

ISAS*Synthetic	81.50[ab] ± 14.51	80.21[a] ± 16.84	104.30[b] ± 22.50	2.58[b] ± 0.59
IVOS*Non-synthetic	91.96[a] ± 15.46	64,00[ab] ± 14.59	188.20[a] ± 34.93	9.46[a] ± 2.18

[a.b,c] Different superscripts in columns indicate significant differences (P≤0.05)

4.7 Correlations for frozen-thawed dataset

A correlation analysis was performed using species and subpopulation as subsamples.

4.7.1 Subpopulation 1

Strong positive correlations were found between VCL and ALH, for the frozen-thawed bovine data (0.968).

4.7.2 Subpopulation 2

When considering the frozen-thawed data, the bovine data showed a strong positive correlation between VAP and VSL (0.724), as was the case for VAP and ALH (0.797). Strong negative correlations were found between VSL and ALH for equine (-1.000) and porcine (-0.809). A moderate negative correlation was found between VCL and ALH (0.576) for porcine. A similar correlation, however much stronger, was found for equine (-1.000). Further, the frozen-thawed data for bovine showed strong positive correlations between VSL and VCL (0.791), as well as between VCL and ALH (0.890).

4.7.3 Subpopulation 3

As seen with subpopulation one and two, there was a strong positive correlation between VAP and VCL for bovine (0.999) and equine (0.973). When considering the frozen-thawed data for bovine, a strong positive correlation was found between VAP and VSL (1.000) and VAP and ALH (0.999). This was also the case between VSL and VCL (0.999) and VCL and ALH (1.000).

4.7.4 Subpopulation 4

From the frozen-thawed bovine data it was evident that, although VAP is strongly correlated with VCL (0.916) and ALH (0.875), it was weak negative correlated with VSL (-0.362).

4.8 RESULTS FOR MOTILITY DERIVED PARAMETERS (FROZEN-THAWED SAMPLES)

As with the parameters associated with sperm capacitation status for the fresh sperm samples, here too the limited reporting of results for these parameters in the publications did not allow descriptive statistics to be generated.

4.8.1 Influence of species

Table 4.26 presents the LS means and SSD values for the motility parameters associated with the capacitation status of sperm, per species.

Table 4.26 The influence of species on the motility parameters (LS means ± SSD) associated with sperm capacitation status.

Species	Parameter			
	LIN	STR	WOB	BCF
Bovine	51.96 ± 13.34	73.70[a] ± 14.73	64.27 ± 10.87	13.43[b] ± 5.04
Canine	51.93 ± 1.85	73.89[a] ± 2.97	62.98 ± 0.42	12.91[b] ± 2.55
Deer	38.85 ± 4.84	35.48[b] ± 3.81	70.97 ± 15.35	14.19[b] ± 3.19
Equine	30.44 ± 1.17	62.37[ab] ± 3.25	55.79 ± 2.37	16.76[b] ± 2.18
Ovine	49.46 ± 12.81	72.93[a] ± 15.56	65.47 ± 11.150	24.49[a] ± 9.92
Porcine	50.17 ± 14.62	56.75[ab] ± 14.05	70.97 ± 15.35	13.24[b] ± 5.45

[a.b] Different superscripts in columns indicate significant differences (P≤0.05)

There were significant differences between species for all of the reported parameters (P<0.0001; Table 4.25), with the exception of LIN (P=0.124) and WOB (P=0.342), for which species had no significant influence on the velocity parameters.

4.8.2 Influence of the number of sperm subpopulations

Table 4.27 presents the LS means and SSD values for the motility parameters associated with the capacitation status of sperm, per subpopulation.

Table 4.27 The influence of subpopulation classification on the motility parameters (LS means ± SSD) associated with sperm capacitation status.

Sperm subpopulation	Parameter			
	LIN	STR	WOB	BCF
SP1	46.44 ± 6.19	66.25 ± 5.98	63.99 ± 8.06	16.20 ± 5.08
SP2	42.32 ± 6.03	57.91 ± 9.12	62.60 ± 9.78	15.70 ± 4.53
SP3	42.20 ± 8.65	60.47 ± 9.74	62.64 ± 10.73	14.92 ± 4.52
SP4	50.91 ± 11.54	65.45 ± 11.40	71.07 ± 8.43	16.52 ± 4.75

There were no significant differences between subpopulations 1-4 for the given parameters (P>0.05; Table 4.27). There was a tendency for subpopulation four being used to classify the spermatozoa with the highest measurements for the velocity parameters related to sperm capacitation. However, this trend is not seen throughout the results (Table 4.27).

4.8.3 Influence of type of CASA system used

Table 4.28 presents the LS means and SSD values for the motility parameters associated with the capacitation status of sperm, per CASA system used.

Table 4.28 The influence of type of CASA system used on the motility parameters (LS means ± SSD) associated with sperm capacitation status.

Type of CASA system	Parameter			
	LIN	STR	WOB	BCF
SCA®	43.81[ab] ± 7.22	60.99 ± 7.66	68.81[a] ± 10.15	10.40[b] ± 2.35
ISAS	59.77[a] ± 12.61	64.19 ± 11.52	72.89[a] ± 12.00	9.90[b] ± 4.17
IVOS	32.83[b] ± 4.48	62.39 ± 8.01	53.52[b] ± 5.60	27.21[a] ± 7.65

[a.b] Different superscripts in columns indicate significant differences (P≤0.05)

There were significant differences between all the parameters, with the exception being STR (P<0.0001; Table 4.28).

4.8.4 Influence of type of medium used

Table 4.29 presents the LS means and SSD values for the motility parameters associated with the capacitation status of sperm, per type of medium used.

Table 4.29 The influence of type of medium used on the motility parameters (LS means ± SSD) associated with sperm capacitation status.

Type of medium	Parameter			
	LIN	STR	WOB	BCF
Synthetic	46.00 ± 7.44	63.39 ± 8.54	65.99 ± 9.15	15.51 ± 4.43
Non-synthetic	44.94 ± 8.76	61.65 ± 9.58	64.16 ± 9.35	16.16 ± 5.01

Despite there being no reported significant differences between the types of mediums used (P≥0.05; Table 4.29), sperm suspended in a synthetic medium generally provided larger values for the parameter values, and smaller values for the SSD's, e.g. LIN (synthetic=46.00 vs. non-synthetic=44.94; Table 4.29).

Appendix: Chapter 4

The following species were not included in the motility analyses as there were insufficient data to accurately compare subpopulations and/or CASA. A summary of what was available and the reasoning for their exclusion is summarised below.

Canine studies

The publications excluded from the final dataset were from the studies of (Peña *et al.*, 2012) and (Núñez-Martínez *et al.*, 2006). Nunez-Martinez *et al.* (2006) reported eleven subpopulations whereas Pena *et al*. (2012) reported only four subpopulations, after samples were collected via manual manipulation from males of different dog breeds. Although neither study linked subpopulations to fertility, both studies determined subpopulations from samples both pre- and post-thaw. Their findings concluded that although processing negatively influences sperm motility, the subpopulation of sperm which exhibited rapid and progressive motility were best able to survive processing and thus form the group of sperm with the highest fertilizing ability.

The variation from the study reporting eleven subpopulations was too large for even the first four subpopulations to be retained. The classification of subpopulations seems to be too wide, leading to discrepancies and an inaccuracy of the reported cut-values of the velocity measurements provided.

Caprine studies

Caprine was removed from the statistical analysis due to non-consensus regarding subpopulation classification from the publications (Dorado *et al*., 2010; Vázquez *et al*., 2015; Barbas *et al*., 2018). The model variation was greater when this species was included, yet it is unsure why the subpopulation classification for this species is so poor compared to the other species.

Deer studies

One publication by Martinez-Pastor *et al.* (2005) was excluded from the dataset. Samples were collected post-mortem from the epididymides of seventy-one male Iberian Red deer during different times of the year, providing 142 samples. Spermatozoa were loaded into a 10µm Makler chamber and SCA® software coupled to a negative-phase contrast microscope were used to analyse the spermatozoa. Four subpopulations were reported when samples were collected immediately post-mortem and three subpopulations were reported after 72hrs post-mortem. The research findings suggest that the quality of sperm samples decrease over time when samples are being harvested post-mortem and that sperm become less motile, or even immotile, the longer they remain within the epididymis post-mortem. It was

also reported that there was an increase in sperm quality during the transition and post-rut periods, to the extent that it can be concluded that season does influence sperm quality in deer.

Equine studies

One publication was excluded from the dataset for the equine species (Giaretta *et al.*, 2017). Velocity measurements were indicated for VCL, VAP, VSL, LIN, STR, WOB and ALH, and three subpopulations were reported based on CASA-generated VAP values, however, velocity measurements were not reported per subpopulation, making it impossible to link the provided values with the outcome of the sperm classification, therefore limiting the use of this study in the final analyses.

Nonetheless, interesting findings were reported. The objective of the study was to compare two CASA systems, namely a plugin CASA_bgm and the Hamilton-Thorn IVOS (Version 12). Twenty-five semen samples were collected from four stallions via AV and analysis was performed without cryopreservation. It was found that only BCF differed significantly between the two CASA systems, possibly due to differing system configurations. Additionally, a strong correlation was found between the total motile sperm percentage and mitochondrial membrane potential.

Feline studies

The publication by Kemmer-Souza *et al.* (2018) was excluded from the final dataset due to it being the only publication found in the search which used CASA to analyse feline semen samples, thus providing insufficient representation of the species and data to be included in the analyses.

Samples were collected via EE and 1560 sperm were analysed using IVOS in order to determine the existence of motile subpopulations. Three subpopulations were classified from samples before any cooling had taken place and three subpopulations from samples which had been chilled at -1°C for 24hrs and 48hrs respectively. The research group also identified two of the three subpopulations which were increasingly resistance to cold temperatures.

Ovine studies

A study by O' Meara *et al*. (2008) attempted to establish a link between sperm functionality and fertility, however the research group found no possible correlation between functional sperm tests and *in vivo* fertility in rams, suggesting that fertility is not easily quantifiable and a much more complex field than previously speculated.

The CASA method used for this study was the Hobson-Sperm Tracker, a CASA method which too few other studies utilized in order for there to be adequate representation of this analysis system. It was therefore excluded from the analyses.

Piscine studies

Fish were excluded from the analyses due to insufficient representation for this species. Although six publications were found in the initial search, Beirao *et al*., 2009; Beirão *et al*., 2011; Kanuga *et al.*, 2012; Gallego *et al*., 2015; Gallego *et al.*, 2017; Caldeira *et al*., 2018, when the data was extracted and explored, it became evident that the data provided an uneven representation of the species and the independent variables.

Aquatic species utilise a different means of fertilisation, namely external fertilisation, whereby sperm are expelled over already released ova. Subpopulation classification differs for terrestrial and aquatic species for this reason. All publications which investigated sperm from aquatic species reported only three subpopulations, with the exception of one study, which found four subpopulations. Two of the studies analysed the samples after the cryopreservation process – one study found that the subpopulations remained the same (Beirao *et al.,* 2011), however the other study reported one subpopulation less (Gallego *et al*., 2017). It has been found that sperm of internal fertilizers are more complex than those of external fertilizers and that the method of fertilization subsequently influences the sperm length and speed (Hook & Fisher, 2020).

Porcine studies

Two porcine publications were ultimately excluded from the analyses (Thurston *et al*., 2001; Ibanescu et al., 2018). The reason for the exclusion of these two studies is the same: both studies utilised CASA methods which did not have sufficient representation, namely the Hobson Morphology CASA system (Thurston *et al.*. 2001) and the SpermVision 3.7 CASA system (Ibanescu *et al.*. 2018), to be included in the final dataset. Despite Thurston *et al.* (2001) describing distinct morphometric sperm subpopulations, only velocity measurements were reported. Besides this discrepancy, the study showed that distinct sperm subpopulations do exist in fresh boar ejaculates and is influenced by cryopreservation. Ibanescu *et al*. (2018) investigated the significance of season on the occurrence and variation of boar subpopulations. It was concluded that seasonal variation does influence sperm subpopulations in boars, with summer and autumn negatively impacting the desired subpopulation of motile sperm, fast and linear sperm, the most.

Rabbit studies

As was the case for feline, only one publication was found for the rabbit species (Maya-Soriano *et al.*, 2015). Therefore it was excluded from the dataset due to insufficient representation of the species. The research group investigated the effects of heat on sperm subpopulation structure and fertility. It was reported that although increased temperatures increased the percentage of non-motile spermatozoa within a sample, it did not affect the fertility as was expressed by live births. Bucks were two months of age when they were sacrificed and epididymal sperm samples collected. ISAS and a dark field microscope at 100x magnification were used to analyse 2298 spermatozoa, leading to the research group reporting four subpopulations in total.

4.9 References

Caldeira, C., Garcia-Molina, A., Valverde, A., Bompart, D., Hassane, M., Martin, P., & Soler, C. 2018. Comparison of sperm motility subpopulation structure among wild anadromous and farmed male Atlantic salmon (*Salmo salar*) parr using a CASA system. Reprod. Fert. Dev. 30, 897–906.

Barbas, J. P., Leahy, T., Horta, A.E., & García-Herreros, M. 2018. Sperm kinematics and subpopulational responses during the cryopreservation process in caprine ejaculates. Cryobiology 82, 137–147.

Beirão, J., Soares, F., Herraez, M.P., Dinis, M.T., & Cabrita, E. 2009. Sperm quality evaluation in *Solea senegalensis* during the reproductive season at cellular level. Theriogenology 72, 1251–1261.

Beirão, J., Cabrita, E., Pérez-Cerezales, S., Martínez-Páramo, S., & Herráez, M. P. 2011. Effect of cryopreservation on fish sperm subpopulations. Cryobiology 62, 22–31.

Bergstein-Galan, T.G., Weiss, R.R., Kozicki, L.E., & Bicudo, S.D. 2017. Sperm subpopulations in ejaculated sperm and spermatozoa recovered from ovine epididymides up to 48 h after death. Anim. Reprod. Sci. 187, 20–27.

Bravo, J.A., Montanero, J., Calero, R., & Roy, T.J. 2011. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Ile de France rams. Anim. Reprod. Sci. 129, 22–29.

Dorado, J., Molina, I., Muñoz-Serrano, A., & Hidalgo, M. 2010. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Florida goats. Theriogenology 74, 795–804.

Cremades, T., Roca, J., Rodriguez-martinez, H., Abaigar, T., Vazquez, J.M., & Martinez, E. A. 2005. Kinematic changes during the cryopreservation of boar spermatozoa. J. Androl. 26, 610–618.

Dorado, J., Alcaráz, L., Duarte, N., Portero, J.M., Acha, D., & Hidalgo, M. 2011a. Changes in the structures of motile sperm subpopulations in dog spermatozoa after both cryopreservation and centrifugation on PureSperm® gradient. Anim. Reprod. Sci. 125, 211–218.

Dorado, J., Gálvez, M.J., Murabito, M.R., Muñoz-Serrano, A., & Hidalgo, M. 2011b. Identification of sperm subpopulations in canine ejaculates: Effects of cold storage and egg yolk concentration. Anim. Reprod. Sci. 127, 106–113.

Dorado, J., Acha, D., Gálvez, M.J., Ortiz, I., Carrasco, J.J., Díaz, B., Gómez-arrones, V., Calero-Carretero, R., & Hidalgo, M. 2013a. Sperm motility patterns in Andalusian donkey (*Equus asinus*) semen: Effects of body weight , age , and semen quality. Theriogenology 79, 1100–1109.

Dorado, J., Acha, D., Ortiz, I., Gálvez, M.J., Carrasco, J.J., Díaz, B., Gómez-arrones, V., Calero-Carretero, R., & Hidalgo, M. 2013b. Relationship between conventional semen characteristics , sperm motility patterns and fertility of Andalusian donkeys (*Equus asinus*). Anim. Reprod. Sci. 143, 64–71.

Estrada, E., Rivera del Álamo, M.M., Rodríguez-Gil, J.E., & Yeste, M. 2017. The addition of reduced glutathione to cryopreservation media induces changes in the structure of motile subpopulations of frozen-thawed boar sperm. Cryobiology 78, 56–64.

Ferraz, M.A.M.M., Morató, R., Yeste, M., Arcarons, N., Pena, A.I., Tamargo, C., Hidalgo, C. O., Muiño, R., & Mogas, T. 2014. Evaluation of sperm subpopulation structure in relation to *in vitro* sperm – oocyte interaction of frozen-thawed semen from Holstein bulls. Theriogenology 81, 1067–1072.

Flores, E., Taberner, E., Rivera, M.M., Peña, A., Rigau, T., Miró, J., & Rodríguez-Gil, J.E. 2008. Effects of freezing/thawing on motile sperm subpopulations of boar and donkey ejaculates. Theriogenology 70, 936–945.

Flores, E., Fernández-Novell, J.M., Peña, A., & Rodríguez-Gil, J.E. 2009. The degree of resistance to freezing-thawing is related to specific changes in the structures of motile sperm subpopulations and mitochondrial activity in boar spermatozoa. Theriogenology 72, 784–797.

Gallego, V., Vilchez, M.C., Penaranda, D.S., Perez, L., Herraez, M.P., Asturiano, J.F., & Martinez-Pastor, F. 2015. Subpopulation pattern of eel spermatozoa is affected by post-activation time , hormonal treatment and the thermal regimen. Reprod. Fert. Dev. 3, 529-543.

Gallego, V., Cavalcante, S.S., Fujimoto, R.Y., Carneiro, P.C.F., Azevedo, H.C., & Maria, A.N. 2017. Fish sperm subpopulations: Changes after cryopreservation process and relationship with fertilization success in tambaqui (*Colossoma macropomum*). Theriogenology 87, 16–24.

Garcia-Alvarez, O., Maroto-Morales, A., Ramon, M., del Olmo, E., Jimenez-Rabadan, P., Fernandez-Santos, M.R., Anel-Lopez, L. Garde, J.J., & Soler, A.J. 2013. Dynamics of sperm subpopulations based on motility and plasma membrane status in thawed ram spermatozoa incubated under conditions that support *in vitro* capacitation and fertilisation. Reprod. Fertil. Dev. 5, 725-732.

Giaretta, E., Munerato, M., Yeste, M., Galeati, G., Spinaci, M., Tamanini, C., Mari, G., & Bucci, D. 2017. Implementing an open-access CASA software for the assessment of stallion sperm motility : Relationship with other sperm quality parameters. Anim. Reprod. Sci. 176, 11–19.

Hook, K.A., & Fisher, H.S. 2020. Methodological considerations for examining the relationship between sperm morphology and motility. Mol. Reprod. Dev. 87, 633–649.

Ibanescu, I., Leiding, C., & Bollwein, H. 2018. Cluster analysis reveals seasonal variation of sperm subpopulations in extended boar semen. J. Reprod. Dev. 64(1), 33-39.

Ibanescu, I., Siuda, M., & Bollwein, H. 2020. Motile sperm subpopulations in bull semen using different

clustering approaches – Associations with flow cytometric sperm characteristics and fertility. Anim. Reprod. Sci. 215, 106-329.

Kanuga, M.K., Drew, R.E., Wilson-Leedy, J.G., & Ingermann, R.L. 2012. Subpopulation distribution of motile sperm relative to activation medium in steelhead (*Oncorhynchus mykiss*). Theriogenology 77, 916–925.

Kemmer-Souza, A.K., Trautwein, L.G.C., Paranzini, C.S., Perencin, F.M., Cardoso, G.S., & Martins, M.I.M. 2018. Influence of cooling temperature in sperm subpopulations of domestic cats. Anim. Reprod. Sci. 189, 1–10.

Ledesma, A., Zalazar, L., Fernández-Alegre, E., Hozbor, F., Cesari, A., & Martínez-Pastor, F. 2017. Seminal plasma proteins modify the distribution of sperm subpopulations in cryopreserved semen of rams with lesser fertility. Anim. Reprod. Sci. 184, 44–50.

Martinez-Pastor, F., Diaz-Corujo, A.R., Anel, E., Herraez, P., Anel, L., & De Paz, P. 2005. Post mortem time and season alter subpopulation characteristics of Iberian red deer epididymal sperm. Theriogenology 64, 958–974.

Maya-Soriano, M.J., Taberner, E., Sabés-Alsina, M., Ramon, J., Rafel, O., Tusell, L., Piles, M., & López-Béjar, M. 2015. Daily exposure to summer temperatures affects the motile subpopulation structure of epididymal sperm cells but not male fertility in an *in vivo* rabbit model. Theriogenology 84, 384–389.

Miro, Lobo, V., & Quintero-Moreno, A. 2005. Sperm motility patterns and metabolism in Catalonian donkey semen. 63, 1706–1716.

Miro, J., Morato, R., Vilagran, I., Taberner, E., Bonet, S., & Yeste, M. 2020. Preservation of epididymal stallion sperm in liquid and frozen states: Effects of seminal plasma on sperm function and fertility. J. Eq. Vet. Sci. 88, 102-109.

Miro, J., Taberner, E., Rivera, M., Pena, A., Medrano, A., Rigau, T., & Penalba, A. 2009. Effects of dilution and centrifugation on the survival of spermatozoa and the structure of motile sperm cell subpopulations in refrigerated Catalonian donkey semen. 72, 1017–1022.

Muiño, R., Rivera, M.M., & Rigau, T. 2008a. Effect of different thawing rates on post-thaw sperm viability , kinematic parameters and motile sperm subpopulations structure of bull semen. 109, 50–64.

Muiño, R., Tamargo, C., Hidalgo, C.O., & Peña, A. I. 2008b. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Holstein bulls: Effects of cryopreservation and between-bull variation. Anim. Reprod. Sci. 109, 27–39.

Muino, R., Pena, A.I., Rodriguez, A., Tamargo, C., & Hidalgo, C.O. 2009. Effects of cryopreservation on the motile sperm subpopulations in semen from Asturiana de los Valles bulls. Theriogenology 72, 860–868.

Núñez-Martinez, I., Moran, J.M., & Pena, F.J. 2006. A three-step statistical procedure to identify sperm kinematic subpopulations in canine ejaculates: Changes after cryopreservation. Reprod. Dom. Anim. 41, 408-415.

O´Meara, C.M.O., Hanrahan, J.P., Prathalingam, N.S., Owen, J.S., Donovan, A., Fair, S., Ward, F., Wade, M., Evans, A.C.O., & Lonergan, P. 2008. Relationship between *in vitro* sperm functional tests and *in vivo* fertility of rams following cervical artificial insemination of ewes with frozen-thawed semen. 69, 513–522.

Ortega-Ferrusola, C. 2009. Identification of sperm subpopulations in stallion ejaculates: Changes after cryopreservation and comparison with traditional statistics. 423, 419–423.

Núñez-Martínez, I., Moran, J.M., & Peña, F.J. 2006. A three-step statistical procedure to identify sperm kinematic subpopulations in canine ejaculates: Changes after cryopreservation. Reprod. Domest. Anim. 41, 408–415.

Peña, A.I., Barrio, M., Becerra, J.J., Quintela, L.A., & Herradón, P.G. 2012. Motile sperm subpopulations in frozen-thawed dog semen: Changes after incubation in capacitating conditions and relationship with sperm survival after osmotic stress. Anim. Reprod. Sci. 133, 214–223.

Quintero-Moreno, A., Miró, J., Teresa Rigau, A., & Rodríguez-Gil, J.E. 2003. Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. Theriogenology 59, 1973–1990.

Quintero-Moreno, A., Rigau, T., & Rodrı, J.E. 2004. Regression analyses and motile sperm subpopulation structure study as improving tools in boar semen quality analysis. 61, 673–690.

Ramón, M., Martínez-Pastor, F., García-Álvarez, O., Maroto-Morales, A., Soler, A.J., Jiménez-Rabadán, P., Fernández-Santos, M.R., Bernabéu, R., & Garde, J.J. 2012. Taking advantage of the use of supervised learning methods for characterization of sperm population structure related with freezability in the Iberian red deer. Theriogenology 77, 1661–1672.

Ramió, L., Rivera, M.M., Ramírez, A., Concha, I.I., Peña, A., Rigau, T., & Rodríguez-Gil, J.E. 2008. Dynamics of motile-sperm subpopulation structure in boar ejaculates subjected to in vitro capacitation and further *in vitro* acrosome reaction. Theriogenology 69, 501–512.

Ramón, M., Soler, A.J., Ortiz, J.A., García-Alvarez, O., Maroto-Morales, A., Roldan, E.R.S., & Garde, J.J. 2013. Sperm population structure and male fertility: An intraspecific study of sperm design and velocity in Red Deer. Biol. Reprod. 89, 1–7.

Thurston, L.M., Watson, P.F., Mileham, A.J., & Holt, W.V. 2001. Morphologically distinct sperm subpopulations defined by Fourier Shape Descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. J. Androl. 22(3), 382–394.

Rivera, M.M., Quintero-Moreno, A., Barrera, X., Rigau, T., & Rodriguez-Gil, J.E. 2006. Effects of constant, 9 and 16-h Light Cycles on sperm quality, semen storage ability and motile sperm subpopulations structure of boar semen. Reprod. Dom. Anim. 41, 386–393.

Vázquez, A.J.F., Cedillo, M.J., Quezada, V.J., Rivas, A.C., Morales, E.C.L., Ayala, E.M.., Hernández, M.J., González, R.A., & Aragón, M.A. 2015. Effects of repeated electroejaculations on kinematic sperm subpopulations and quality markers of Mexican Creole goats. Anim. Reprod. Sci. 154, 29–38.

Yániz, J.L., Palacín, I., Vicente-Fiel, S., Sánchez-Nadal, J.A., & Santolaria, P. 2015. Sperm population structure in high and low field fertility rams. Anim. Reprod. Sci. 156, 128–134.

Chapter 5

RESEARCH CHAPTER: The relationship between sperm subpopulation classification parameters, sperm head morphometry traits, and sperm fertilizing potential

The results presented in this chapter will be discussed according to each model that was used to investigate the relationship between sperm head morphometry traits and sperm subpopulation structure, and the potential of this relationship to estimate the fertilizing ability of the spermatozoa in a sample.

The results will be presented according to the models that were used to analyse the fresh and frozen-thawed datasets, respectively. The number of species reported on in this chapter differs from that reported on in Chapter 4, i.e. include only bovine and ovine studies. In addition, the ovine dataset is the only dataset that provided sufficient data to include sperm ellipticity and elongation in the analysis.

Elucidation on the exclusion of the other publications, i.e. publications indicated in Chapter 3, will be presented as a chapter appendix.

Table 5.1 presents the summary of the publications that met the selection criteria for the morphometry dataset, and that provided sufficient data to be included in the analysis.

Table 5.1 Publications that met the selection criteria for the sperm morphometry dataset.

	Author (-s)	**Year**	**Species**	**Type of Sample**
1	Valverde *et al.*	2016	Bovine	Frozen-thawed
2	Garcia-Herreros *et al.*	2014	Bovine	Frozen-thawed
3	Rubio-Guillen *et al.*	2007	Bovine	Frozen-thawed
4	Yaniz *et al.*	2015	Ovine	Fresh
5	Maroto-Morales *et al.*	2015	Ovine	Fresh
6	Maroto-Morales *et al.*	2012	Ovine	Fresh
7	De Paz *et al.*	2011	Ovine	Fresh
8	Marti *et al.*	2012	Ovine	Fresh
9	Santolaria *et al.*	2015	Ovine	Fresh
10	Vicente-Fiel *et al.*	2013	Ovine	Fresh

5.1 RESULTS FOR THE USE OF SPERM HEAD MORPHOMETRY PARAMETERS TO CLASSIFY SPERM SUBPOPULATIONS IN FROZEN-THAWED BOVINE SPERM SAMPLES

Initially, four publications were included in the bovine dataset. Three publications involved trials using frozen-thawed samples, with the fourth publication using only fresh samples. The latter publication was thus excluded from the dataset. The three publication consequently resulted in a small dataset of only 22 observations/measurements. Two cattle breeds were represented in these studies, i.e. the Holstein (*Bos taurus*) and Nellore (*Bos indicus*) breeds. All mediums used in the studies were non-synthetic, and all microscopy performed was with bright field microscope. All samples were collected by using an artificial vagina (AV). The base model for the frozen-thawed morphometric data included only the fixed effects breed and subpopulation. The type of CASA system used, could not be included in Model 3 due inclusion resulting in non-estimable values.

Overall, an ANOVA analysis of the morphometry dataset indicated that R^2 values ranged from 71.2% (SSD=5.8%) for head length, compared to 81.4% (SSD=70.5%) for head area (Table 5.2).

Table 5.2 Coefficient of variation (R^2) calculated for the sperm head morphometry-associated parameters for frozen-thawed bovine spermatozoa.

Parameter	R^2 value	SSD value
Head Length	0.712	0.058
Head Width	0.639	0.069
Head Area	0.814	0.705
Head Perimeter	0.735	0.057

5.1.1 Influence of breed

Table 5.3 presents the descriptive statistics for sperm head morphometric traits, i.e. length, width, area, and perimeter, recorded for frozen-thawed bovine spermatozoa in the three bovine publications used for the purpose of this study.

Table 5.3 Descriptive statistics for computer-aided sperm head morphometry parameters reported for frozen-thawed bovine sperm samples in the period 2007 to 2016.

Sperm morphometric parameter	No. of sperm analysed	Mean ± SD	Range	Coefficient of Variation
Head Length (µm)	28 431	8.07 ± 0.84	6.77 - 9.65	0.104
Head Width (µm)		4.20 ± 0.49	3.53 - 5.28	0.115
Head Area (µm^2)		29.42 ± 5.48	22.43 - 42.00	0.186
Head Perimeter (µm)		23.08 ± 4.61	18.41 - 35.30	0.200

Variation observed for the respective sperm head morphometric traits ranged from 10.4% to 20.0%.

Table 5.4 presents the LS means for the morphometric parameters, per breed. Breed significantly influenced the values recorded for sperm head length, -width, -area, and -perimeter (P≤0.05). Although the breeds are of similar size, in all cases the Holstein breed was represented by larger values for the respective parameters. However, it needs to be emphasised that this was a small dataset with only three studies and the Nellore data was obtained from one publication. The values recorded for the Nellore breed can thus not be considered as representative for the breed, but the values can be considered as approximate values. The SSD values are not reported due to insufficient data available from the publications.

Table 5.4 The influence of breed on the sperm head morphometry parameters (LS means), recorded for frozen-thawed bovine spermatozoa.

Breed	Sperm morphometric parameter			
	Head Length (µm)	Head Width (µm)	Head Area (µm^2)	Head Perimeter (µm)
Holstein	8.95^a	4.73^a	35.97^a	28.30^a
Nellore	7.62^b	3.94^b	26.12^b	20.44^b

^{a,b} Different superscripts in columns indicate significant differences (P≤0.05)

5.1.2 Influence of the number of sperm subpopulations

Table 5.5 presents the ranges for sperm head morphometric traits that were used to classify sperm subpopulations in frozen-thawed bovine sperm samples recorded in the three bovine publications used for the purpose of this study. The number of sperm analysed is indicated in brackets.

Table 5.5 Computer-aided sperm head morphometry parameters (LS means ± SSD) reported for frozen-thawed bovine sperm samples, in the period 2007 to 2016, and presented per sperm subpopulation.

Sperm subpopulation	Parameter	Mean ± SD	Range	Coefficient of Variation
SP1 (n=28 431)	Head Length (µm)	8.43 ± 0.81	7.51 - 9.65	10.45
	Head Width (µm)	4.25 ± 0.60	3.57 - 5.28	7.06
	Head Area (µm^2)	31.15 ± 5.82	25.33 - 42.02	5.36
	Head Perimeter (µm)	24.60 ± 5.52	20.26 - 35.30	4.46
SP2 (n=28 431)	Head Length (µm)	7.88 ± 0.57	7.26 - 8.62	13.73
	Head Width (µm)	4.27 ± 0.44	3.53 - 4.75	9.65
	Head Area (µm^2)	28.81 ± 3.86	23.67 - 34.40	7.46
	Head Perimeter (µm)	23.06 ± 4.74	19.61 - 32.12	4.87
SP3	Head Length (µm)	8.20 ± 0.82	7.33 - 9.24	10.02

(n=28 431)	Head Width (µm)	4.21 ± 0.57	3.67 - 4.94	7.36
	Head Area (µm^2)	30.08 ± 6.49	24.48 - 38.31	4.63
	Head Perimeter (µm)	23.11 ± 4.61	19.68 - 31.18	5.02
SP4	Head Length (µm)	7.61 ± 1.22	6.77 - 9.40	6.24
(n=25 758)	Head Width (µm)	4.03 ± 0.34	3.79 - 4.52	11.83
	Head Area (µm^2)	26.77 ± 6.44	22.43 - 36.23	4.16
	Head Perimeter (µm)	20.81 ± 3.74	18.41 - 26.34	5.57

A large degree of variation was observed for the parameter of head length for each subpopulation (SP1 head length=10.45, SP2=13.73, SP3=10.02, SP4=6.24). This was also the parameter with the largest degree of variation in comparison with the other parameters, except for SP4 where a larger degree of variation was observed for head width versus head length (11.83 vs. 6.24; Table 5.5).

Table 5.6 present the average head length, -width, -area, and perimeter values, as influenced by sperm subpopulation classification. The subpopulations did not differ in terms of the respective parameters (P≥0.05; Table 5.6).

Table 5.6 The cut-off values for sperm head morphometry parameters (LS means ± SSD) used to classify the sperm subpopulation structure of frozen-thawed bovine sperm samples.

Sperm subpopulation	**Sperm morphometric parameter**			
	Head Length (µm)	**Head Width (µm)**	**Head Area (µm^2)**	**Head Perimeter (µm)**
SP1	8.655 ± 0,425	4.384 ± 0,240	32.793 ± 2,270	25.909 ± 1,745
SP2	8.106 ± 0,410	4.397 ± 0,245	30.455 ± 2,225	24.366 ± 1,815
SP3	8.426 ± 0,395	4.342 ± 0,240	31.721 ± 1,885	24.424 ± 1,390
SP4	7.947 ± 0,460	4.226 ± 0,200	29.232 ± 2,440	22.771 ± 1,460

5.2 RESULTS FOR THE USE OF SPERM HEAD MORPHOMETRY PARAMETERS TO CLASSIFY SPERM SUBPOPULATIONS IN FRESH OVINE SPERM SAMPLES

The sperm morphometry analysis dataset for fresh sperm samples included samples obtained from rams only. A total of eight ovine studies were identified, of which one study used frozen-thawed semen samples. This latter study was therefore not included in the sperm morphometry dataset.

Three sheep breeds were represented in the studies that were included in the sperm head morphometry dataset, i.e. the Assaf, Rasa Aragonesa, and Manchega breeds. All animals were adults at the time of collection, and all samples were collected using the AV method. The base model for the ovine sperm

morphometry dataset included the fixed effects breed, number of sperm subpopulations, and CASA system used.

Overall, an ANOVA analysis of the ovine sperm head morphometry dataset indicated that R^2 values ranged from 37.3% (SSD=76.3%) for head width, compared to 80.0% (SSD=86.5%) for head perimeter (Table 5.7).

Table 5.7 Coefficient of variation (R^2) calculated for the sperm head morphometry parameters for fresh ovine sperm samples.

Parameter	R^2 value	SSD value
Head Length	0.773	0.684
Head Width	0.373	0.763
Head Area	0.756	0.784
Head Perimeter	0.800	0.865

Table 5.8 presents the descriptive statistics for sperm head morphometric traits, i.e. length, width, area, and perimeter, recorded for fresh ovine spermatozoa.

Table 5.8 Descriptive statistics for computer-aided sperm head morphometry parameters reported for fresh ovine sperm samples, in the period 2011 to 2015.

Sperm morphometric parameter	No. of sperm analysed	Mean ± SD	Range	Coefficient of Variation
Head Length (µm)	~19 364	7.99 ± 0.93	6.04 - 9.64	0.12
Head Width (µm)		4.57 ± 0.36	3.30 - 5.08	0.08
Head Area mean (µm^2)		31.15 ± 4.11	23.71 - 39.93	0.13
Head Perimeter (µm)		23.14 ± 3.67	18.02 - 32.98	0.16

~ to indicate where actual values were not reported in all publications and an estimate was calculated based on the provided values

5.2.1 Influence of breed

A total of seven studies were included in the sperm head morphometry dataset, with four studies reporting only three subpopulations, whilst three studies reported a fourth subpopulation. Table 5.9 present the influence of breed on the respective sperm head morphometry parameters.

Breed significantly influenced the sperm head morphometry parameters, with Assaf rams producing spermatozoa with the largest sperm heads, when compared to the other two breeds ($P \leq 0.05$; Table 5.9).

Table 5.9 The influence of breed on the sperm head morphometry parameters (LS means ± SSD), recorded for fresh ovine sperm samples.

Breed	Sperm morphometric parameter			
	Head Length (µm)	Head Width (µm)	Head Area (µm^2)	Head Perimeter (µm)
Assaf	8.50 ± 0.49	4.73 ± 0.23	36.31^a ± 2.24	26.33^a ± 1.12
Manchega	7.80 ± 0.24	4.48 ± 0.25	28.72^b ± 1.75	22.45^b ± 0.78
Rasa Aragonesa	7.80 ± 0.24	4.48 ± 0.25	28.72^b ± 1.75	22.45^b ± 0.78

^{a,b} Different superscripts in columns indicate significant differences (P≤0.05)

5.2.2 Influence of the number of sperm subpopulations

Table 5.10 presents the descriptive statistics for sperm head morphometry traits that were used to classify sperm subpopulations in fresh ovine sperm samples, recorded in the six ovine publications used for the purpose of this study.

Table 5.10 Descriptive statistics for computer-aided sperm head morphometry parameters (mean ± SD) reported for fresh ovine sperm samples, in the period 2011 to 2015, and presented per sperm subpopulation.

Sperm subpopulation	Parameter	Mean ± SD	Range	Coefficient of Variation
SP1 (n=~19 364)	Head Length (µm)	8.41 ± 0.70	7.60 - 9.57	11.96
	Head Width (µm)	4.55 ± 0.42	3.70 - 4.99	10.87
	Head Area (µm^2)	32.87 ± 4.04	27.92 - 39.93	8.15
	Head Perimeter (µm)	23.62 ± 3.22	20.62 - 30.02	7.34
SP2 (n=~19 364)	Head Length (µm)	7.97 ± 0.69	6.99 - 8.95	11.62
	Head Width (µm)	4.55 ± 0.46	3.30 - 5.08	9.85
	Head Area (µm^2)	30.94 ± 3.82	23.71 - 35.29	8.10
	Head Perimeter (µm)	22.59 ± 2.75	18.82 - 27.18	8.22
SP3 (n=~19 364)	Head Length (µm)	7.70 ± 0.86	6.04 - 8.77	8.94
	Head Width (µm)	4.59 ± 0.24	4.16 - 5.08	18.68
	Head Area (µm^2)	30.28 ± 3.43	26.51 - 37.88	8.84
	Head Perimeter (µm)	22.34 ± 2.18	19.63 - 25.59	10.26
SP4 (n=~6450)	Head Length (µm)	7.82 ± 1.89	6.15 - 9.64	4.13
	Head Width (µm)	4.60 ± 0.13	4.47 - 4.71	34.54
	Head Area (µm^2)	29.42 ± 6.55	23.74 - 35.23	4.49
	Head Perimeter (µm)	25.51 ± 8.63	18.02 - 32.98	2.96

~ to indicate where actual values were not reported in all publications and an estimate was calculated based on the provided values

Table 5.11 present the average head length, -width, -area, and perimeter values, as influenced by sperm subpopulation classification.

Table 5.11 The cut-off values for sperm head morphometry parameters (LS means ± SSD) used to classify sperm subpopulations in fresh ovine sperm samples.

Sperm subpopulation	Sperm morphometric parameter			
	Head Length (µm)	Head Width (µm)	Head Area (µm^2)	Head Perimeter (µm)
SP1	8.59^{a} ± 0.39	4.56 ± 0.24	33.42^{a} ± 2.33	24.07 ± 1.16
SP2	8.06^{ab} ± 0.25	4.57 ± 0.18	31.49^{ab} ± 1.48	23.04 ± 0.78
SP3	7.80^{b} ± 0.31	4.61 ± 0.21	30.84^{ab} ± 1.70	22.79 ± 0.84
SP4	7.69^{b} ± 0.36	4.54 ± 0.34	29.24^{b} ± 2.14	25.06 ± 0.80

^{a,b} Different superscript letters indicate significant differences (P≤0.05)

The subpopulations differed significantly for head length and –area (P≤0.05; Table 5.11). The variation obtained in the studies were consistent with the exception of the SSD for head width and head area where there were significant differences between subpopulations two and four and one and two respectively. The head measurements in subpopulation one were generally higher than rest of the subpopulations, however, this was only a trend. Furthermore, it was shown that the largest percentages of sperm were consistently reported in subpopulation two.

5.2.3 Influence of type of CASA system used

Table 5.12 presents the influence of the CASA system used on the head length, -width, -area and perimeter values, as reported for fresh ovine sperm samples. The large differences in variation between CASA systems is partly explained by the amount of data available for each system. Where less data was available, e.g. for the CASA system NIS Elements, a subsequently smaller variation was observed.

Table 5.12 Descriptive statistics for computer-aided sperm head morphometry parameters (mean ± SD) reported for fresh ovine sperm samples, in the period 2011 to 2015, and presented per CASA system used.

CASA system	Sperm morphometric parameter	Mean ± SD	Range	Coefficient of Variation
SCA®	Head Length (µm)	9.08 ± 0.51	8.34 - 9.64	17.75
	Head Width (µm)	4.79 ± 0.13	4.66 - 4.99	35.59
	Head Area (µm^2)	35.21 ± 1.98	32.68 - 37.96	17.78
	Head Perimeter (µm)	28.31 ± 2.99	25.58 - 32.98	9.47
ISAS	Head Length (µm)	8.39 ± 0.34	7.95 - 8.77	24.58
	Head Width (µm)	4.76 ± 0.23	4.38 - 5.08	21.15
	Head Area (µm^2)	33.87 ± 4.71	27.38 - 39.93	7.19
	Head Perimeter (µm)	24.18 ± 3.07	21.45 - 30.02	7.89
Motic	Head Length (µm)	7.27 ± 0.59	6.15 - 7.91	12.38
	Head Width (µm)	4.47 ± 0.14	4.16 - 4.65	31.40

	Head Area (µm^2)	27.82 ± 2.32	23.74 - 31.32	12.00
	Head Perimeter (µm)	19.95 ± 1.07	18.02 - 21.43	18.71
ImageJ	Head Length (µm)	8.21 ± 0.14	8.04 - 8.42	57.09
	Head Width (µm)	4.62 ± 0.10	4.42 - 4.67	46.26
	Head Area (µm^2)	30.01 ± 1.15	27.92 - 31.28	26.18
	Head Perimeter (µm)	22.34 ± 0.44	21.69 - 22.67	51.11
NIS-Elements	Head Length (µm)	7.31 ± 1.02	6.04 - 8.80	7.16
	Head Width (µm)	4.19 ± 0.68	3.30 - 5.08	6.13
	Head Area (µm^2)	30.26 ± 4.43	23.71 - 35.29	6.82
	Head Perimeter (µm)	21.85 ± 1.89	18.82 - 24.13	11.58

Table 5.13 presents the influence of the type of CASA system used to measure sperm head morphometry parameters. The type of CASA system used significantly influenced the values reported in the respective studies, with the SCA® system consistently producing higher values for the respective sperm head morphometry parameters (P≤0.05; Table 5.13).

Table 5.13 The influence of CASA system used on the sperm head morphometry parameters (LS means ± SSD), recorded for fresh ovine sperm samples.

Type of CASA system	**Sperm morphometric parameter**			
	Head Length (µm)	**Head Width (µm)**	**Head Area (µm^2)**	**Head Perimeter (µm)**
SCA®	9.32^a ± 0.38	4.87^a ± 0.34	37.74^a ± 3.07	29.60^a ± 1.99
ISAS	8.32^b ± 0.27	4.71^a ± 0.18	31.94^b ± 1.51	23.97^b ± 0.61
Motic	7.48^c ± 0.58	4.55^a ± 0.33	30.17^{bc} ± 2.75	21.37^{bc} ± 1.17
ImageJ	8.33^b ± 0.13	4.70^a ± 0.05	31.87^b ± 0.45	24.07^b ± 0.17
NIS-Elements	6.72^c ± 0.27	4.01^b ± 0.32	24.53^c ± 1.78	19.70^c ± 0.54

^{a,b,c} Different superscripts in columns indicate significant differences (P≤0.05)

5.3 RESULTS: THE USE OF SPERM HEAD MORPHOMETRY PARAMETERS TO CALCULATE SPERM HEAD ELLIPTICITY AND –ELONGATION

The data presented in this section was obtained from the same dataset as was used in section 5.2. Therefore, the breeds and CASA systems, as well as the number of sperm analysed, represented in this section remains the same.

Table 5.14 presents the descriptive statistics for sperm head morphometric traits, i.e. ellipticity and elongation, recorded for fresh ovine spermatozoa.

Table 5.14 Descriptive statistics for computer-aided sperm head ellipticity and elongation parameters reported for fresh ovine sperm samples, in the period 2011 to 2015.

Sperm morphometric parameter	No. of sperm analysed	Mean ± SD	Range	Coefficient of Variation
Ellipticity	~19 364	1,77 ± 0,25	1,37 - 2,42	7,08
Elongation		0,27 ± 0,07	0,15 - 0,41	4,11

~ to indicate where actual values were not reported in all publications and an estimate was calculated based on the provided values

From the results presented in Table 5.14, it was observed that the degree of variation for ellipticity was almost double the degree of variation for elongation (7.08 vs. 4.11).

5.3.1 Influence of breed

Table 5.15 presents the LS means for the morphometric parameters, per breed. There was no significant influence by breed on the values recorded for sperm head ellipticity and elongation (P≥0.05).

Table 5.15 The influence of breed on the sperm head ellipticity and -elongation parameters (LS means ± SSD), calculated for fresh ovine spermatozoa.

Breed	Sperm morphometric parameter	
	Ellipticity	Elongation
Assaf	1.92 ± 0.13	0.30 ± 0.04
Manchega	1.71 ± 0.10	0.26 ± 0.03
Rasa Aragonesa	1.71 ± 0.10	0.26 ± 0.03

5.3.2 Influence of the number of sperm subpopulations

Table 5.16 presents the LS means for the morphometric parameters, per subpopulation. There was no significant influence by subpopulation on the values recorded for sperm head ellipticity and elongation (P≥0.05; Table 5.16). However, it was evident that subpopulation one showed the largest values for both measurements of ellipticity and elongation.

Table 5.16 The sperm head ellipticity and -elongation values (LS means ± SSD) calculated per sperm subpopulation for fresh ovine sperm samples.

Sperm subpopulation	Sperm morphometric parameter	
	Ellipticity	Elongation
SP1	1.96 ± 0.13	0.32 ± 0.03
SP2	1.79 ± 0.09	0.28 ± 0.02
SP3	1.70 ± 0.10	0.26 ± 0.03
SP4	1.65 ± 0.13	0.23 ± 0.04

5.3.3 Influence of type of CASA system used

Table 5.17 presents the LS means for the morphometric parameters, per CASA system used. There were no significant differences observed between CASA systems for the two variables (P≥0.05, Table 5.17). It was further found that the CASA system SCA® tended to provide larger values versus the other CASA systems represented in this dataset.

Table 5.17 The influence of type of CASA system used on the sperm head ellipticity and -elongation values (LS means ± SSD) calculated for fresh ovine spermatozoa.

Type of CASA system	Sperm morphometric parameter	
	Ellipticity	Elongation
SCA®	1.96 ± 0.15	0.32 ± 0.04
ISAS	1.80 ± 0.08	0.28 ± 0.02
Motic	1.69 ± 0.16	0.24 ± 0.04
ImageJ	1.80 ± 0.03	0.28 ± 0.01
NIS- Elements	1.63 ± 0.14	0.23 ± 0.05

Appendix: Chapter 5

The following species were not included in the sperm head morphometry dataset, for the studies did not provide sufficient data to meet the selection criteria, and to allow for statistical analysis of the data. A summary of the publications that were initially selected, and why they were eventually excluded, is summarised below.

Avian studies

Two studies were excluded for the avian species, which included three different avian breeds (i.e. chickens, falcons, and guinea fowl; (García-Herreros, 2016; Villaverde-Morcillo *et al*., 2017). All birds were mature and considered fertile and healthy. Both studies were excluded from the dataset due to insufficient data being available from only two publications, however, a basic exploration of the available data showed that the degree of variation between these studies is high, possibly due to there being such a large variety of breeds (i.e. domesticated as well as wild species). However, when a correlation analysis was performed, a negative correlation existed between head length and head area. This was supported through the fact that avian species' sperm have especially long/elongated heads and therefore also cannot be compared with other species in this analysis.

The same CASA method was used for both studies and collected all samples manually by abdominal massage. Voluntary false copulation was also used in the case of falcons. Both studies used bright field microscopy. Despite different medium compositions being used, all mediums were synthetic. Neither study examined links with fertility. One of the studies analysed fresh samples (García-Herreros, 2016), while the other study analysed samples which were both fresh and frozen-thawed (Villaverde-Morcillo *et al.*, 2017).

Porcine studies

Two porcine studies were excluded (Thurston *et al*., 1999; Peña *et al*., 2005), for different reasons. The Pena *et al.* (2005) publication measured sperm parameters using CASA and reported sperm subpopulations, however, failed to link the subpopulations with the measured parameters. Thurston *et al.* (1999) classified spermatozoa into subpopulations based on tail lengths, the only publication to have classified spermatozoa according to this sperm component. The two studies reported different numbers of subpopulations, possibly due to tail lengths and head lengths being used respectively to determine the subpopulations.

The breeds included in these studies were from various genetic lines, including Pietrain, Large White and Landrace-Meishan. Different CASA systems (ImageJ and Hobson Morphology) were used for each study; however, all samples were collected manually. Both studies used brightfield microscopy to analyse the spermatozoa.

Caprine studies

One study was excluded for the caprine species (Zaja *et al.*, 2018). As is the case for most excluded species, insufficient representation of the species led to the exclusion of caprine from the dataset. Additionally, the study analysed samples using CASA system SFROM, the only study which was found using this system. All males were adults and considered sexually mature at the time of collection.

The study reported only three subpopulations after sperm from adult French Alpine bucks was collected via AV and analysed without cryopreservation of the samples taking place. The effects of melatonin treatment were found to significantly influence the sperm subpopulations.

Equine studies

Two publications for equine sperm morphometry analysis was removed from the dataset as they were the only two studies found (Gravance *et al*., 1997; Hidalgo *et al*., 2008), resulting in insufficient data available for analyses. Various breeds were included in the studies and five subpopulations were found using for the study by Gravance *et al.* (1996), which investigated the difference between sperm subpopulations from fertile and sub-fertile stallions and found that the mean values for head length, width, area, and perimeter, differed significantly between the two groups. The study by Hidalgo *et al*. (2008) classified six subpopulations using SCA®.

Deer studies

One study was found and therefore excluded for deer morphometric sperm subpopulation classification (Beracochea *et al.*, 2014). The breed represented was Pampas deer (*Ozotoceros bezoarticus*). The study collected samples by electro-ejaculation during the breeding season. Fresh samples were analysed using SCA® and three distinct morphometric subpopulations were found, and these were consistent across the different sample collections. Positive relationships were reported for all head measurements.

Primate studies

Two studies were excluded for primate, the only two studies available for this species, therefore warranting their exclusion from the final dataset. Both studies were from the same author, however with different years of publication (Valle *et al*., 2012; Valle *et al.*, 2013). The breeds included were marmosets (*Callithrix jacchus*) and Goeldi's monkeys (*Callimico goeldii*). All samples were collected from reproductively mature, healthy males. One study collected samples by penile vibrostimulation apparatus, while the other was by electro-ejaculation. Both used a CASA software provided by Motic.

Rodent studies

The rodent species was removed from dataset as there was only one study found for this species (Davis *et al.*, 1994) resulting in insufficient data available for analyses. The study was performed with the breed Sprague Dawley, using epididymal samples and the Hamilton-Thorn CASA system.

5.4 References

Beracochea, F., Gil, J., Sestelo, A., Garde, J. J., Santiago-Moreno, J., Fumagalli, F., & Ungerfeld, R. 2014. Sperm characterization and identification of sperm sub-populations in ejaculates from pampas deer (*Ozotoceros bezoarticus*). Anim. Reprod. Sci. 149, 224–230.

Davis, R.O., Gravance, C.G., Thal, D.M., & Miller, M.G. 1994. Automated analysis of toxicant-induced changes in rat sperm head morphometry. Reprod. Tox. 8(6), 521–529.

De Paz, P., Mata-Campuzano, M., Tizado, E.J., Álvarez, M., Alvarez-Rodriguez, M., Herraez, P., & Anel, L. 2011. The relationship between ram sperm head morphometry and fertility depends on the procedures of acquisition and analysis used. 76, 1313–1325.

García-Herreros, M. 2016. Sperm subpopulations in avian species: A comparative study between the rooster (*Gallus domesticus*) and Guinea fowl (*Numida meleagris*). Asian J. Androl. 18, 889–894.

García-Herreros, M., Leal, C.L.V, & Garcı, M. 2014. Comparative study of sperm washing and selection methods after cryopreservation and its influence on sperm subpopulational structure in a bovine model. Syst. Biol. Reprod. Med. 60(6), 338-347.

Gravance, C.G., Champion, Z., Liu, I.K.M., & Casey, P.J. 1997. Sperm head morphometry analysis of ejaculate and dismount stallion semen samples. Anim. Reprod. Sci. 47, 149–155.

Hidalgo, M., Rodríguez, I., Dorado, J., & Soler, C. 2008. Morphometric classification of Spanish thoroughbred stallion sperm heads. Anim. Reprod. Sci. 103, 374–378.

Maroto-Morales, A., Ramón, M., García-Álvarez, O., Soler, A.J., Fernández-Santos, M.R., Roldan, E. R.S., Gomendio, M., Pérez-Guzmán, M.D., & Garde, J.J. 2012. Morphometrically-distinct sperm subpopulations defined by a multistep statistical procedure in Ram ejaculates: Intra- and interindividual variation. Theriogenology 77, 1529–1539.

Maroto-Morales, A., Ramón, M., García-álvarez, O., Montoro, V., & Garde, J.J. 2015. Sperm head phenotype and male fertility in ram semen. Theriogenology 84, 1536–1541.

Martí, J.I., Aparicio, I.M., Leal, C.L.V., & García-Herreros, M. 2012. Seasonal dynamics of sperm morphometric subpopulations and its association with sperm quality parameters in ram ejaculates. Theriogenology 78, 528–541.

Peña, F.J., Saravia, F., García-Herreros, M., Núñez-Martínez, I., Tapia, J.A., Johannisson, A., Wallgren, M., & Rodríguez-Martínez, H. 2005. Identification of sperm morphometric subpopulations in two different portions of the boar ejaculate and its relation to postthaw quality. J. Androl. 26, 716–723.

Rubio-Guillen, J., Gonzalez, D., Garde, J.J., Esteso, M.C., Fernandez-Santos, M.R., Rodriguez-Gil, J.E., Madrid-Bury, N., & Quintero-Moreno, A. 2007. Effects of cryopreservation on bull spermatozoa distribution in morphometrically distinct subpopulations. 42, 354–357.

Santolaria, P., Vicente-Fiel, S., Palacín, I., Fantova, E., Blasco, M.E., Silvestre, M.A., & Yániz, J.L. 2015. Predictive capacity of sperm quality parameters and sperm subpopulations on field fertility after artificial insemination in sheep. Anim. Reprod. Sci. 163, 82–88.

Thurston, L.M., Watson, P.F., & Holt, W.V. 1999. Sources of variation in the morphological characteristics of sperm subpopulations assessed objectively by a novel automated sperm morphology analysis system. J. Reprod. Fertil. 117, 271–280.

Valle, R.R., Arakaki, P.R., Carvalho, F.M., Muniz, J.A.P.C., Leal, C.L.V., & García-Herreros, M. 2013. Identification of sperm head subpopulations with defined pleiomorphic characteristics in ejaculates of captive Goeldi's monkeys (*Callimico goeldii*). Anim. Reprod. Sci. 137, 93–102.

Valle, R.R., Nayudu, P.L., Leal, C.L.V., & García-Herreros, M. 2012. Sperm head morphometry in ejaculates of adult marmosets (*Callithrix jacchus*): A model for studying sperm subpopulations and among-donor variations. Theriogenology 78, 1152–1165.

Valverde, A., Arenán, H., Sancho, M., Contell, J., Yániz, J., Fernández, A., & Soler, C. 2016. Morphometry and subpopulation structure of Holstein bull spermatozoa: Variations in ejaculates and cryopreservation straws. Asian J. Androl. 18, 851–857.

Vicente-Fiel, S., Palacín, I., Santolaria, P., & Yániz, J. L. 2013. A comparative study of sperm morphometric subpopulations in cattle, goat, sheep and pigs using a computer-assisted fluorescence method (CASMA-F). Anim. Reprod. Sci. 139, 182–189.

Villaverde-Morcillo, S., Soler, A.J., Esteso, M.C., Castaño, C., Miñano-Berna, A., Gonzalez, F., & Santiago-Moreno, J. 2017. Immature and mature sperm morphometry in fresh and frozen-thawed falcon ejaculates. Theriogenology 98, 94–100.

Yániz, J.L., Palacín, I., Vicente-Fiel, S., Sánchez-Nadal, J.A., & Santolaria, P. 2015. Sperm population structure in high and low field fertility rams. Anim. Reprod. Sci. 156, 128–134.

Zaja, I.Z., Vince, S., Milinkovic-Tur, S., Milas, P.N., Samardzija, M., Valpotic, H., Berta, V., Vilic, M., & Rakic, K. 2018. Exogenous melatonin in fluences distribution of French Alpine buck spermatozoa in morphometrically distinct subpopulations during the non-breeding season. 192, 154–163.

Chapter 6

Discussion, General Conclusions & Recommendations

The objective of this meta-analysis was to investigate the degree of standardization of the classification of sperm subpopulations using CASA-determined sperm motility and morphometry parameters in available literature on animal species. The potential use of sperm subpopulations in the prediction of sperm fertilizing ability of males, whether in the wild (natural environment or extensive production conditions) or in intensive production systems/breeding programs, where gene flow is more regulated, was also investigated.

Regardless of the type of production system involved, the sustainable use of land-, water- and animal resources is becoming an ever-increasing critical matter when food security is concerned. To meet the demand for food by 2050, it is imperative that animals are identified and selected that demonstrate an ability to cope with their changing environment, especially where the increase in ambient temperature is concerned. This will assist livestock producers and wildlife conservationists to select for animals that are resilient to changes that can affect their production and reproduction ability, thus maintaining homeostasis under challenging conditions. This in turn will contribute to food safety (livestock) and ecosystem stability (wildlife).

6.1 DISCUSSION: The relationship between sperm subpopulation classification parameters, recorded sperm motility, and sperm fertilizing potential

6.1.1 MOTILITY PARAMETERS ASSOCIATED WITH SPERM VELOCITY

Velocity-associated parameters of fresh samples: A total of 30 peer-reviewed publications, published between 2005 and 2020, were included in the motility dataset for fresh sperm samples. The statistical model for the velocity-associated parameters included the fixed effects species, sperm subpopulation, CASA system used (SCA® or ISAS), and type of medium used (synthetic or non-synthetic). The R^2 values for the velocity-associated parameters ranged from 30.5% for ALH, compared to 50.6% for VSL.

Velocity-derived parameters of fresh samples: Considerably fewer studies reported on these parameters, and consequently descriptive statistics could not be provided for this dataset. The statistical model included the fixed effects species, sperm subpopulation, CASA system used (SCA® or ISAS), and type of medium used (synthetic or non-synthetic).

Velocity-associated parameters of frozen-thawed samples: The statistical model included species, sperm subpopulation, type of CASA system used (SCA®, ISAS and IVOS systems), and type of medium used (synthetic and non-synthetic), as fixed effects. The dataset contained bovine, deer, canine, equine, ovine, and porcine studies, with porcine studies comprising 41.7% of the dataset. The R^2 ranged from 69.4% for ALH, compared to 37.9% for VSL.

Thirty studies reported on three sperm subpopulations, with 25 studies reporting on a fourth sperm subpopulation. Synthetic mediums were used in 12 studies, whilst six studies used non-synthetic mediums.

Velocity-derived parameters of frozen-thawed samples: Similar to what was found for the fresh samples, fewer studies reported on these parameters, and consequently descriptive statistics could not be provided for this dataset. The statistical model included the fixed effects species, sperm subpopulation, CASA system used (SCA® or ISAS), and type of medium used (synthetic or non-synthetic).

6.1.1.1 Influence of species on the velocity-associated parameters

Fresh sperm samples: The motility dataset for the velocity-associated parameters (VAP, VSL, VCL, and ALH) demonstrated a considerable difference between the number of ejaculate samples used per study, i.e. 30 samples for the canine studies, compared to 340 samples for the equine studies. The R^2 values demonstrated a moderate to large degree of variation, e.g. from 30.5% for ALH, compared to 50.6% for VSL.

When species representation is considered (i.e. bovine, ovine, equine, canine and porcine), boar samples constituted 51% of the dataset. The variation observed for the respective parameters was moderate to large, with the largest degree of variation observed in ovine samples. Ram sperm samples also exhibited the largest degree of variation for VCL. Equine samples exhibited the smallest degree of variation for the velocity-associated parameters, and the smallest degree of variation for the VSL parameter.

Frozen-thawed sperm samples: The statistical model included species, sperm subpopulation, type of CASA system used, and type of medium used, as fixed effects. Bovine, deer, canine, equine, ovine, and porcine species were represented in the frozen-thaw sample dataset, with porcine studies that comprised 41.7% of the publications used for the analysis. There was a considerable difference in the number of ejaculates collected i.e. 30 samples for the canine studies vs. 233 samples for the bovine studies. The R^2 values reported for the frozen-thawed samples ranged from 37.9% for VSL to 69.4% for ALH. This is a larger degree of variation in comparison to the fresh samples.

Discussion

As expected, species had a significant effect on the various velocity-associated motility parameters. The species reported on in this study, all differ in terms on the length of spermatogenesis, and the time the spermatozoa needs to spend in the epididymis to undergo maturation and thus acquire the ability to swim and fertilize (Dvořáková *et al.*, 2005). The journey of spermatozoa upon entry into the epididymis from the testis, is characterized by specific changes that need to occur in the *caput-*, *corpus-* and *cauda epididymis*, respectively. Disruption of these processes and shortening of the duration of the epididymal transit period, can all contribute to changes in the swimming and fertilizing ability of spermatozoa (Gervasi & Visconti, 2018).

According to Talluri *et al.* (2017), species also differ in terms of the composition of seminal plasma, which is the transport fluid of the spermatozoa during ejaculation and up to the uterus in the female reproductive tract after mating (Hawk, 1982). The composition of seminal plasma can also differ between males of a particular species, with the variation in inorganic and organic components of the seminal plasma that can influence spermatozoa on a molecular level, thus ultimately resulting in different results when it comes to sperm analysis and comparison within a species, and between species (Druart *et al.*, 2013).

In addition to the composition of seminal plasma, factors such as sperm head shape can also influence the swimming pattern and ultimately time it will take for a spermatozoon to reach the oocyte. This can be considered as an evolutionary consequence to ensure species survival. As example here one can refer to the sperm head design of two abalone species, *Haliotis midae* and *H. spadiceae.* Upon comparison of these two species, differences in sperm head dimensions were evident (Visser-Roux, 2011). This difference in head shape is considered an evolutionary consequence to prevent hybridization between species, and on a species-level sperm head shape may potentially also influence the fertilizing potential of spermatozoa of males of a particular species. Sperm head shape has been found to influence the speed at which sperm are able to swim (Boshoff, 2014).

Species also differ in terms of when a male will enter puberty (i.e. initiation of spermatogenic processes in the testes, and production of the gonadotropins required for gamete production) and eventually become sexually mature. The age at which a male animal enters puberty can be manipulated by nutrition, Barth *et al.* (2008) reported on the positive effects that a superior plane of nutrition provided to bull calves, resulted in larger testes at yearling age and earlier onset of spermatogenesis. The nutritional status of an animal also affects the growth and development of the reproductive organs positively (Widiyono *et al.*, 2017). Reproductive factors such as spermatogenesis, hormones, and seminal plasma composition can all be influenced by the type and amount of feed provided. Under-nutrition has been found to negatively influence semen quality (Martin et al., 2010) and subsequently affecting fertility. Almost all the studies

used sexually mature males, with only one study by Marti *et al*. (2011) that included young rams (<12 months) as part of the experimental design to investigate the influence of age on sperm motility. They found that frozen-thawed ram sperm samples were characterized by a specific subpopulation structure, when compared to fresh samples, and was related to ram age and ultimately sample quality, which in turn was important to consider in terms of the fertilizing ability of the spermatozoa.

The genetic selection of production species for specific production purposes, can indirectly affect the quality of spermatozoa, by affecting the expression of genes that code for sperm cytoskeleton. The sperm cytoskeleton is a highly specialized part of mammalian spermatozoa, and distribution of cytoskeleton components such as actin, spectrin, and vimentin is thought to be correlated to surface specializations, which in turn can influence sperm motility and function (Dvořáková *et al.*, 2005). Sandenbergh (2015) reported on how selection for prolificacy in sheep can influence, on a micro-genetic level, the expression of genes that code for the formation of the sperm cytoskeleton, which in turn can result in a change in the ability of spermatozoa to tolerate and cope with the changes induced by cryopreservation. This finding corroborated the findings of Boshoff (2014) who used the same resource flock, where a change in sperm morphometric traits were observed post-thaw.

Season and temperature greatly influence the sperm production potential and quality of spermatozoa produced by species that occur in temperate zones, where reproduction is driven by an important *zeitgeber*, i.e. the availability of food for offspring once they are born. Most animals are seasonal to a certain extent where their breeding is concerned, relying on biorhythms and coordinated production and secretion of the gonadotropins and steroid hormones such as testosterone and oestrogen, to initiate and maintain reproduction-related processes (Rivera *et al.*, 2006). A study by Ibanescu *et al.* (2018) found that boar sperm quality markedly decreased during warmer months, resulting in reduced fertility, with this phenomenon often defined as ´´seasonal infertility´´. This potential modification of sperm depending on the season, may lead to a marked variation in the distribution of sperm in the different subpopulations classes, and the interpretation of what these results mean in terms of male fertility.

Viruses and bacteria have an influence on fertility as disease ultimately affects sperm shape, motility, and impairs proper sperm function. The reproductive potential of a male may be affected by a disease directly or from the secondary effects of immune-suppressive drug treatment (Tiseo *et al.*, 2016). Crucial physiological processes necessary for sperm production and function may be negatively influenced by a fever response and a consequent in body core temperature, with the latter that is considered as a line of defence when the body has been invaded by a foreign and potentially harmful organism (Chastant & Saint-Dizier, 2019).

From the statistical analysis for species, the smallest degree of variation for the frozen-thawed dataset (as seen for the fresh dataset) was presented for the equine species. The protocols for the cryopreservation of stallion sperm are well-established due to ART's and specifically the use of AI that is the standard breeding method in horses, after being initiated as a breeding method for this species at the end of the 19th century (Aurich, 2012). Semen is shipped internationally for the purpose of breeding sport horses, such as Hanoverians (Klug, 1992), which necessitated the refinement of the freeze-thaw process over many years, ultimately potentially contributing to the observed lower degree of variation observed in this study for equids.

The composition of seminal plasma in terms of seminal plasma proteins can influence whether there are differences between and within species for how they cope with freezing injury, also known as sperm resilience (Rickard *et al.*, 2013; 2016). Sperm resilience has a large genetic component, due to various sperm mechanisms, including heat shock proteins and anti-oxidants, being coded for by the DNA found within the sperm head. These molecules assist the sperm cell to tolerate different types of intrusions brought about by processing (heat shock, cold shock, osmolarity, pH etc.) (Highland, 2016).

6.1.1.2 Influence of the number of sperm subpopulations on the velocity-associated parameters

Fresh sperm samples: A lack of uniformity and standardization for subpopulation classification was evident in all the studies, with this supported by the way in which the data were reported. Most studies reported on three subpopulations, whilst equine (Ortega-Ferrusola *et al*., 2009), ovine (Bravo *et al.*, 2011) and canine (Núñez-Martínez *et al.*, 2006) studies reported on four and more subpopulations. Various authors reported the SD as wide ranges, which inflated the variation observed for the dataset.

The chosen cut-off values reported in the studies was often not provided by authors and motivations were not provided for the classification of sperm into the respective subpopulations. It seems that most of the studies included in the meta-analysis, based their cut-off values on previous research and what had been done for previous studies that were similar in design and nature, and not necessarily motivated by scientific review. The variation observed for SP1 was smaller than what was observed for SP4 in this study, potentially indicating that the cut-off values for SP1 are better defined, when compared to the other subpopulations. Few studies linked subpopulations to ultimate fertility.

Frozen-thawed sperm samples: The R^2 values ranged from 69.4% for ALH, compared to 37.9% for VSL. This degree of variation was larger than what was observed for the fresh samples. Similar to what was observed for the fresh sample dataset, most studies reported the use of three subpopulations, as opposed to four subpopulations. The fact that all four subpopulations differed significantly from each other indicates that the classification into four groups is justified for this dataset. Additionally, a good indication of uniformity is

provided through the fact that subpopulation four classified all the sperm with the fastest velocities and subpopulation one classified spermatozoa which were the least progressive. A greater degree of consensus regarding the classification of sperm into clusters and the respective cut-off values per cluster seems to be evident for this dataset.

Discussion

The reason for collection and ultimate use of a semen sample, e.g. for use in ART's, will determine to a large degree how subpopulations fit into the context of the assessment of the fertilizing potential of spermatozoa, and ultimately male fertility. When natural mating is the preferred breeding method, sperm enter the female reproductive tract directly from the penis, without any sample collection taking place. In instances like this, subpopulation determination is vital in the establishment of the prolificacy/fecundity of males that can deliver trusted results in terms of offspring, when in the field and under extensive production conditions. When processing and cryopreservation is involved for the preparation of samples to be used for ART's, the resulting decrease in sperm motility and overall quality necessitates the development of approaches to identify males that produce spermatozoa that are resilient to changes induced by processing and cryopreservation (Flores *et al*., 2009), to ensure the successful application of ART's.

The classification of subpopulations and thus the selection of cut-off values potentially needs to be revised to allow for the use of only three sperm subpopulations in the quantification of sperm fertilizing potential, as influenced by motility. This modification to the current approach is supported by the fact that in in this study, most studies reported on three subpopulations, and fewer reported a fourth subpopulation. Sperm subpopulations one and two may be merged as one population, as their cut-off values lie near to each other. The functional relevance of have four or more subpopulations also needs to be considered, especially when livestock managers and conservationists need to make sound selection decisions in terms of the selection of more prolific animals in their systems.

The distribution of sperm between the different subpopulations has been reported to change as a sample is collected, processed, and cryopreserved. Studies that measured sperm velocity variables for fresh and post-thaw samples, reported a significant increase in the number of sperm classified in "lower quality subpopulations" after thawing (Flores *et al.*, 2008). This is partly due to cryopreservation-induced changes that negatively influence sperm kinematics (Flores *et al.*, 2009). Caution is also advised in the comparison of fresh with frozen-thawed samples in terms of the velocity-associated parameters, for the freeze-thawing process can significantly influence the classification of subpopulations due to a progressive decrease in sperm motility during storage as well as caused by cryopreservation (Quintero-Moreno *et al.*, 2003).

6.1.1.3 Influence of type of CASA system used on the velocity-associated parameters

Fresh sperm samples: There was an even distribution of the two represented CASA systems (12 publications for SCA® vs. 9 publications for ISAS). The ISAS system, however, generated values which exhibited larger variation, e.g. an R^2 of 3.04 was reported for ALH, compared to the SCA®-generated value of 1.81. The SCA® system generated higher values than the ISAS system for the respective velocity-associated parameters. The type of CASA system significantly influenced the VCL and VSL, and tended to influence the value of the VAP parameter. The two system did not differ in terms of the characterization of the respective sperm subpopulations, which implies that either system can be used to classify sperm subpopulations. A certain preference, however, was observed in the use of the respective systems, i.e. the SCA® system was the only CASA system used in the bovine studies, whilst the ISAS system was the only CASA system used in the ovine studies.

Frozen-thawed sperm samples: Three CASA systems were used to analyse frozen-thawed samples, i.e. SCA®, ISAS, and the IVOS system. The dataset included nine SCA® studies, seven ISAS studies, and two IVOS studies. The values reported by the IVOS system demonstrated the largest degree of variation e.g. ALH=2.65 vs. 2.27 for SCA® and 1.50 for ISAS. Contrary to what was observed for fresh samples, the smallest variation in the parameters reported was for values generated by the ISAS system.

Discussion

A difference in CASA system design (i.e. components included or software design and development, system settings and calibration methods used per system) can potentially contribute to the differences observed for the two systems. The preference for a particular type of CASA system potentially is influenced by the availability/accessibility of the system in a country or to a research group.

One aspect that may influence the values generated by a CASA system, apart from the calibration settings, is the chamber depth of the slide used to load the sample for analysis. Spermatozoa must be observed in a chamber that allows freedom of movement and the natural movement patterns of the spermatozoa to be exhibited (Mortimer *et al.*, 1998). Too small chamber dimensions will prevent spermatozoa from swimming normally, and result in inaccurate motility measurements. Likewise, if the chamber dimensions are too large for the concentration of sperm that has been loaded, the spermatozoa will use internal energy reserves, which in turn will affect their ability to participate in fertilization (Gloria *et al*., 2013). Commonly used chamber depths include 10µm and 20µm. It is important to know the sperm concentration being loaded as this will determine whether the sperm can exhibit normal motility or not.

A CASA system consists of software and various parts of hardware, such as a microscope with a heated stage (essential to minimize the effects of thermal shock on the sperm cells), a video camera, and a

computer. Commonly used microscopes include bright field and phase contrast microscopes. Phase contrast microscopy requires special phase contrast objectives and a special phase contrast condenser (positive and negative). This method is useful for observing unstained specimens that lack colour, and it is particularly important in biology, as it allows many cellular structures that are invisible with a bright-field, to be visualised (Murphy *et al.,* online article). It is preferable to bright field microscopy when high magnifications (400X, 1000X) are required, for example in the analysis of cilia and flagella. However, equipment costs associated with phase contrast microscopes may be higher when compared to bright field and dark field microscopes, respectively. Different oculars may be used with the chosen type of microscope. The most common magnifications at which spermatozoa were analysed included 10X, 40X, and 100X. The magnification at which spermatozoa is analysed, may influence how much detail is visible, and thus the morphometric measurements that are recorded. The experience of the technician is also important to take into consideration in this regard.

6.1.1.4 Influence of type of medium used on velocity-associated parameters

Fresh sperm samples: There was a clear preference for synthetic medium to be used in the studies, with 11 publications using this type of medium compared to only 9 publications which made use of non-synthetic mediums. When considering the degree of variation, it was found that there was a larger degree of variation reported in the studies that used synthetic mediums, e.g. when considering ALH (2.05 vs. 1.37). The largest velocity parameters for VSL, VCL and ALH were also reported for studies using synthetic mediums, with the VAP parameter not influenced by the type of medium used.

The increased use of synthetic mediums reported in the publications is possibly due to these mediums having a longer shelf-life, being more accessible and a decreased risk for the contamination of a sample compared to non-synthetic mediums. The composition of the medium in which the sperm sample is suspended and the molecules found within the medium can influence the sperm cells by interacting with the sperm surface proteins (Anbari *et al.*, 2016) hereby also affecting the fertility outcome.

Frozen-thawed sperm samples: The degree of variation in the number of studies which suspended samples in the different mediums was increasingly evident for the frozen-thawed dataset. 12 publications used a synthetic medium, while only 6 publications were reported to use a non-synthetic medium. When all of the velocity-related parameters are considered, only curvilinear velocity was found to be significantly influenced by the type of medium used.

Discussion

Two important factors when considering medium are the temperature at which the medium is maintained at during processing of the sample, and the osmolarity of the medium. Both these factors can lead to

temperature- and chemical shock if not within acceptable ranges. Certain spermatozoa are more resilient to external stressors, such as osmotic stress, than others (Flores *et al.*, 2009), but not much is currently known about this specific field. An isotonic medium prevents hypo- or hyperosmotic shock. Additionally, the rate of dilution may influence the sperm. Higher rates of dilution have been found to influence sperm fertility prediction, based on a Sperm Quality Index (SQI) (Parker & McDaniel, 2006). In a study conducted by Parker & McDaniel (2006), it appeared that excess ATP was being produced by sperm diluted from 2- to 50- fold. This is mainly due to dilution causing sperm activation, which can lead to sperm exhaustion at high dilution rates.

Most sperm media contain a variety of additional energy sources and nutrients. Synthetic mediums do not contain any animal products. Non-synthetic mediums contain animal products, such as milk, egg yolk and/or bovine serum albumin. A good semen extender is characteristically one which is isotonic, a good buffer, minimizes cold damage/shock, provides the appropriate nutrients, prevents microbial growth, maintains sperm viability and is relatively low in cost (Senger, 2003). There is no culture medium that can be considered a generic medium, i.e. is suitable to use optimal for all species, due to sperm from diverse species having adapted to different environments, as well as differences in seminal plasma composition stemming from genetic differences between species. As seen for the fresh dataset, medium was found to also influence sperm velocity measurements for the frozen-thawed data.

The cryopreservation process is usually performed to properly and safely store donor samples for cases where ART's are the preferred method of breeding. An added benefit of storing sperm in this manner, is for cases where the laboratory is far away from the collection site and sperm viability needs to be maintained over an extended time period. Before the sperm sample is plunged into liquid nitrogen, there is a time period of equilibration whereby sperm are in contact with the cryoprotectant, before undergoing freezing. In spite of the fact that the cryoprotectant functions to prevent intracellular ice crystal formation, plasma membrane damage causes many spermatozoa to die during this stage of the freezing process. However, certain spermatozoa are more resistant to the freeze-thaw process than others (Rodríguez-Martínez, 2003). Thawing rates differed slightly between species included in the dataset, however in general the thawing rate was 37°C for 30-40 seconds.

The level of experience of the technician has been found to have an influence on the results obtained. Bias may happen because of the technician´s own judgement on sperm quality (Freund *et al.*, 1964; Amann & Waberski, 2014).

6.1.2 MOTILITY DERIVED PARAMETERS

When the available publications were considered in terms of the parameters that are associated with sperm capacitation status, i.e. LIN, STR, WOB, and BCF, insufficient publications were available for the fresh and frozen-thawed sample datasets to allow for the determination of the descriptive statistics. Consequently, only the LS means, and SSD values were provided for the fixed effects. The statistical model used included species, sperm subpopulation, type of CASA system used, and type of medium used, as fixed effects.

6.1.2.1 Influence of species on the motility derived parameters

Fresh sperm samples: Most of the parameters, except for LIN, were significantly influenced by species. The highest STR and WOB values were reported for boars, and the lowest STR and WOB values were reported for rams.

Frozen-thawed sperm samples: Species significantly influenced STR and BCF, with no influence reported for LIN and WOB recorded for the frozen-thawed samples.

6.1.2.2 Influence of the number of sperm subpopulations on the motility derived parameters

Fresh sperm samples: Sperm subpopulations differed in terms of the sperm parameters associated with capacitation status.

Frozen-thawed sperm samples: No differences were observed between the respective sperm parameters. A tendency, however, was observed for SP4 being characterized by the highest values for this dataset. This was not observed for any of the other fixed effects.

6.1.2.3 Influence of type of CASA system used on the motility derived parameters

Fresh sperm samples: The type of CASA system used significantly affected the BCF and WOB values reported.

Frozen-thawed sperm samples: The STR values reported was the only parameter that was not influenced by the type of CASA systems used.

6.1.2.4 Influence of type of medium used on the motility derived parameters

Fresh sperm samples: The type of medium used significantly influenced the WOB and BCF parameters. It is noteworthy that STR could not be estimated.

Frozen-thawed sperm samples: Despite no reported significant influence of medium, it was observed that sperm suspended in a synthetic medium generally resulted in higher values reported for the LIN parameter.

Discussion

The parameters associated with sperm capacitation (sperm head ellipticity and elongation) are especially of importance with regards to *in vitro* studies, where the values obtained for these parameters provide an indication of how close a sperm is from undergoing the capacitation process. The capacitation process should ideally only take in proximity to the site of fertilization, where certain changes of the acrosome will allow the sperm to penetrate the zona pellucida of the oocyte and reach the cytoplasm (Badawy *et al.,* 2006). No clear pattern for any of the fixed effects were observed, placing the status of results for the capacitation-related parameters in a similar position as the velocity-related motility parameters.

Albeit in the context of natural mating, where certain males´ spermatozoa undergo capacitation at a faster rate (Ostermeier *et al.,* 2017), or in the context of ART's (where premature capacitation needs to be prevented up and to the point of *in vitro* fertilization), correct study design is of utmost importance for studies to truly reap the benefits of measuring the above-mentioned parameters. Standardisation of such protocols related to the measurement of parameters of sperm capacitation will subsequently allow the direct comparison and establishment of linkages between laboratories and selected production animals, which will furthermore lead to the development of breeding indices.

6.2 DISCUSSION: The relationship between sperm subpopulation classification parameters, sperm head morphometry traits, and sperm fertilizing potential

The morphometry dataset was limited to bovine studies that used frozen-thawed samples (with the exception of one study that used fresh samples), and ovine studies that used fresh samples. A possible explanation for this could be that cattle have the longest history with ART's and therefore the protocols for frozen-thawed samples are refined and are widely adopted in the bovine industry as standard operational protocols to use.

The three bovine publications represented a small dataset of only 22 observations/measurements. The Holstein (*Bos taurus*) and Nellore (*Bos indicus*) breeds were represented in these studies (i.e. two studies and one study, respectively), and non-synthetic mediums were used in all the studies. All samples were collected using the AV method, as were analysed using bright field microscopy. The statistical model for the frozen-thawed dataset included only the fixed effects breed and subpopulation. The R^2 values varied considerably, ranging from 71.2% for sperm head length to 81.4% for -head area.

The morphometry dataset for fresh sperm samples comprised of seven studies where samples were obtained from adult rams using the AV method. From the initially selected publications, one ovine study used frozen-thawed samples, and were not included in this dataset. The Assaf- (one study), Rasa Aragonesa- (four studies), and Manchega- (two studies) sheep breeds were represented in the morphometry dataset. The statistical model included breed, number of sperm subpopulations, and CASA system used, as fixed effects. The R^2 values varied moderately to considerably, from 37.3% for sperm head width to 80.0% for sperm head perimeter.

6.2.1 Influence of breed on the sperm head morphometry parameters

Frozen thawed bovine sperm samples: Breed significantly influenced the morphometry parameters, with the Holstein breed characterized by larger and broader sperm heads than Nellore bulls, which is considered a Zebu breed. Beletti *et al.* (2005) reported smaller head sizes for Zebu breeds, that is also characterized by a more elliptical shape, than sperm of Holstein, although findings were based on fresh samples. This finding, combined with the results from this dataset, motivates the importance of considering the breed when quantifying sperm morphometric characteristics.

Fresh ovine sperm samples: Assaf rams produced spermatozoa with the largest sperm heads, when compared to the other two breeds. It needs to be noted that only one study using Assaf rams was available for inclusion in the dataset. Nonetheless, even not representative for the Assaf breed, the significant size difference in sperm head morphometry parameters warrants that breed needs to be considered when analysing and interpreting sperm head morphometry parameters, and their relationship with sperm fertilizing ability.

Discussion

Although the number of studies for the bovine morphometry dataset was limited, the observed moderate to considerable degree of variation in the results reported was surprising, since the expectation would be that a small degree of variation will be reported based on the fact that the development and application of ART's is more advanced in cattle than in any other species. Due to the SSD not being reported in all of the studies, it was difficult to determine whether the subpopulation classification is similar or different between the studies.

Certain animal breeds are selected for certain production traits, which may be for economic or production efficiency reasons, and are thus considered as "developed" breeds in different parts of the world, where different external factors may impact their reproductive characteristics and parameters related to gametogenesis (Wilson, 1962; Teodoro *et al.*, 2013). Another possible explanation for the significant

differences observed between the breeds, is that certain breeds are managed more intensively and therefore selected more intensively, while others are left to natural mating. These external factors influence the gene pool of a breed, which in turn influence the expression of genes involved in spermatogenesis and fertility.

6.2.2 Influence of the number of sperm subpopulations on the sperm head morphometry parameters

Frozen-thawed bovine sperm samples: The sperm head length parameter varied considerably between subpopulations (35.2%), with SP2 being characterized by the highest values in this regard. Sperm head length varied also more than -head width, -area, and -perimeter.

Fresh ovine sperm samples: The classified ovine sperm subpopulations differed significantly in terms of sperm head length and –area, with no differences reported for -width and –perimeter respectively. Even though not significant, it is worth noting that the sperm head measurements for SP1 tended to be the largest for almost all the parameters. It is also worth noting that SP2 was characterized by a higher number of sperm than SP1, SP3 or SP4.

Discussion

Results for the bovine dataset indicate that the classification of sperm subpopulations in cattle is poorly defined. Although insignificant, a pattern was observed where the largest sperm were classified in SP1, and the smallest spermatozoa in SP4. However, the poor definition/selection of cut-off values to classify sperm subpopulations is supported by the observations in this study where SP2 and SP3 is concerned.

The limited number of bovine studies included in the dataset, however, prohibit drawing any conclusion regarding the influence of sperm subpopulation on the respective morphometric traits. No significant influence of sperm subpopulation was reported in this dataset, but this needs to be verified by conducting more studies similar in design.

The similar lack of consistency in the classification of sperm subpopulations was observed for the ovine dataset as well. The ovine studies were characterized by too wide ranges for the respective subpopulations, due to poorly defined cut-off values. Additionally, no clear difference between SP3 and SP4 potentially indicates that only three sperm subpopulations need to be considered for morphometry analysis.

6.2.3 Influence of type of CASA system used on sperm head morphometry parameters

Since analysis could not be carried out for the frozen-thawed bovine sperm sample (i.e. results confounded by breed and subpopulation), only the ovine dataset was analysed. In addition to the respective sperm head morphometry parameters, the two calculated parameters ellipticity and elongation will also be reported.

A wide variety of CASA systems were represented for this dataset, namely the SCA® (2 publications), ISAS (1 publication), Motic (1 publication), ImageJ (2 publications), and NIS-Elements (1 publication) systems. The type of CASA system used significantly influenced the values reported in the respective studies, with the SCA® system consistently producing higher values for the respective sperm head morphometry parameters. The large variation between CASA systems can in part be explained by the data available for each system. Where less data was available, e.g. for the CASA system NIS-Elements, a subsequently smaller variation was observed.

Ellipticity and elongation were not influenced by breed, subpopulation of type of CASA system used. It needs to be noted that SP1 was characterized by the highest values for both ellipticity and elongation. The SCA® system tended to generate higher values for ellipticity and elongation.

Although not included in the model, there was an even distribution of the microscopy type used, bright field versus fluorescence (4 publications for bright field vs. 3 publications for fluorescence). The lesser representation of fluorescence microscopy could be since not all researchers have access to a fluorescence microscope and the use involves fluorescent probes which may be radioactive. Bright field is considered more affordable, accessible and allows the technician to visualise more structures.

Discussion

There is currently a large variety of CASA systems commercially available for analysis of animal sperm (Amann & Waberski, 2014). The software is used to track the spermatozoa in order to quantify the set parameters (Mortimer, 2000) in the case of sperm motility, whereas staining and fixation are general practice when sperm morphometry is being evaluated. Although CASA systems deliver similar results in the quantification of sperm (Hook & Fisher, 2020), the large variation observed between and within the CASA systems is mainly due to different components, based on optics and software, and different calibrations depending on the breed being studied (Bompart *et al.*, 2019) being used. Thus it remains vital that the correct setup and cut-off values are used depending on the circumstances.

The shape of the sperm head changes as it moves through the epididymis during maturation (Gervasi & Visconti, 2017). Thus the ellipticity and elongation, which are directly determined by the sperm head shape, are vital parameters which should be included and measured as part of a standard CASA analysis. Both of these parameters can be linked to the swimming speed of the sperm and the ability of sperm to penetrate the zona pellucida after reaching the ovum (Sailer & Evenson, 1996; Hirai *et al*., 2001).

6.2.4 Processing factors influencing sperm motility and morphometry

The preparation and processing of a sample prevents premature capacitation of sperm, a reaction that ultimately should only take place (a) immediately prior to fertilization and (b) after the sperm reservoir has been established in the oviduct (Holt & Van Look, 2004). There are multiple factors which affect the accuracy and precision of results when using CASA for subpopulation analyses and are therefore very important to consider:

Frame rate

Each CASA system possesses a configurations menu where important parameters, such as the frame rate, can be selected. The frame rates used differed between the studies, with certain studies measuring sperm parameters at 25fps and others at 50fps, with the IVOS system at either 30fps or 60fps. More recent CASA systems allow for analyses using frame rates of up to 200fps. Measurements such as VCL and VAP are especially influenced by the frame rate and different results will be obtained, for example, between 25fps and 50fps.

Medium

After semen collection, the sample is re-suspended in an artificial medium in order to maintain the integrity and viability of the cells and additionally to allow time for transport to the laboratory for evaluation. Most media contain a variety of additional energy sources and nutrients, despite the constituents of the medium. Synthetic mediums do not contain any animal products, adversely non-synthetic mediums contain animal products, such as milk, egg yolk and/or bovine serum albumin. A variety of mediums are utilized throughout the studies, such as phosphate buffered saline (PBS),

Two important factors when considering the medium are the temperature at which the medium is worked with and the osmolarity of the medium. Both of these can lead to temperature- and chemical shock of the spermatozoa if not within acceptable ranges and drastically influence the kinematics of sperm

Staining

Staining is performed for morphometric evaluations, enabling the cell's membranes and organelles of interest to become easier to visualise. A variety of stains are used, however commonly used stains and dyes include methylene blue, DiffQuick and Harriss' hematoxylin. Staining affects the morphology of cells by causing the sperm head to either shrink or swell (Maree *et al.*, 2010). Banaszewska et al. (2015) found significant differences between head measurements depending on the staining method which was used. Thus it is crucial to use a stain which is isotonic and isosmotic to preserve the normal size and form of the sperm. Additionally, staining protocols have been developed in a species-specific manner as sperm across various species react differently to different stains, subsequently certain stains are more suitable to be used for certain species' sperm.

6.3 GENERAL CONCLUSIONS AND RECOMMENDATIONS

This study presented both conflicting and inconsistent findings regarding the potential of CASA- generated parameters to classify sperm subpopulations. There were not sufficient breeds represented per species to allow the potential identification of base-line values for a species or a particular type of sample or production system.

From the "snapshot" provided on the potential factors that can affect sperm motility and morphometry, it is imperative that the potential of CASA systems to quantify the influence of all these factors, as well as link it to the fertilizing ability need to determined and validated. An in-depth study of the publications that met the selection criteria, did not provide any indication of a relationship between species, sperm subpopulation classification for that species, and the fertilising ability of the spermatozoa assigned to a sperm subpopulation. A positive relationship between sperm subpopulations and fertilizing ability have been noted in studies, where fertilizing ability was determined using either pregnancy rates, birth rates, percentage embryos obtained, or zona pellucida binding assays.

The two most common reasons for conducting sperm evaluation studies are firstly for research purposes to investigate and better understand sperm form and function, and secondly to determine the potential of ART's and CASA to assist livestock producers and conservationists to manage their animal resources, as sustainable and cost-efficient as possible. Currently, studies are being carried out without asking the question of what exactly the goal of sperm subpopulation classification is, and how it fits into the bigger picture of assessment of sperm fertilising ability, male fertility, cost-efficient and optimal animal production, and ultimately food security.

It is important to consider the ultimate use of a given sperm sample and the environment in which the animal from which the sample was collected. This will simplify the standardization of CASA-generated values between different production systems as well as research and commercial laboratories. One of the most important factors associated with CASA analyses is the cut-off values used to classify motility and morphometry parameters, as well as subpopulations. Cut-off values that are used to determine the respective parameters can to a large extent influence the interpretation of results, which in turn can influence the management decisions that are based on these findings. For example, the fate of spermatozoa, i.e. whether they are used for ART's or research purposes (to improve our knowledge of what happens to spermatozoa in situ and in vivo) will potentially differ. However, from the findings of this study, it is evident that future studies need to carefully and responsibly consider the selection of cut-off values for CASA-generated motility and morphometry parameters. Standardization of CASA protocols will allow for the comparison of findings between producers and laboratories, which in turn will contribute positively to ensuring the optimal management of animal and wildlife resources in terms of production and reproduction. Standardization of protocols may potentially assist in the calculation of breeding indexes for species, which in turn will contribute to the optimal management of animal genetic resources.

Future CASA studies should place more emphasis on solidifying potential associations and links between subpopulations and fertility. The relatively more objective approach of CASA will contribute to the standardization of protocols for species. The large degree of variation reported in this study warrants studies that will assist in the determination of baseline and relevant cut-off (i.e. related to fate of sperm sample) values that will contribute to optimal management of animal production resources, ensuring food security. From the results of this study, it is not possible to determine whether one CASA systems is currently more reliable than another.

It is recommended that because fertilization is a multi-factorial process, future studies need to include both motility and morphometry parameters in the study design. The findings reported for morphometry indicated a lack of conformity for these parameters, potentially a result of the fewer studies that reported on morphometric parameters. Evaluating both motility and morphometry can additionally prevent the incorporation of intra-male variation into results, allowing a more refined model for investigating the possible link between sperm motility and morphometry, and sperm function.

It is also advised that authors need to include more details on the preparation, processing, and imaging of samples, and that the choice of cut-off values to determine subpopulation categories should be universally applicable, scientifically sound, and justified. In addition, it is recommended that the environmental

variables are provided to allow a more efficient comparison between independent studies, and in this way, can lead to the development of optimized protocols for species.

The findings from the study highlighted the need for a device that is robust, simple, and relatively easy to use in field/extensive conditions to aid in the generation of CASA-associated results. Ideally such a device should additionally be accessible using smartphone-based technologies. Two such systems have already been developed, i.e. iSperm by Aidmics Biotechnology and YO Sperm Test, Medical Electronic Systems.

6.4 References

Amann, R.P., & Waberski, D. 2014. Computer-assisted sperm analysis (CASA): Capabilities and potential developments. Theriogenology 81, 5-17.

Anbari, F., Halvaei, I., Nabi, A., Ghazali, S., Khalili, M.A., & Johansson, L. 2016. The quality of sperm preparation medium affects the motility, viability, and DNA integrity of human spermatozoa. J. Hum. Reprod. Sci. 9, 254–258.

Aurich, J.E. 2012. Artificial insemination in horses – more than a century of practice and research. J. Eq. Vet. Sc. 32(8), 458-463.

Badawy, S.Z.A., Shue, F., & Chohan, K.R. 2006. Sperm capacitation: Effect of assisted reproductive technology. Middle East Fertil. Soc. J. 11(2), 77-81.

Banaszewska, D., Czubaszek, M., Walczak-Jędrzejowska, R., & Andraszek, K. 2015. Morphometric dimensions of the stallion sperm head depending on the staining method used. Bull. Vet. Inst. Pulawy 59, 263–270.

Barth, A.D., Brito, L., & Kastelic, J.P. 2008. The effect of nutrition on sexual development of bulls. Theriogenology 70(3), 485-494.

Beletti, M.E., da Fontoura Costa, L., & Viana M.P. 2005. A comparison of morphometric characteristics of sperm from fertile *Bos taurus* and *Bos indicus* bulls in Brazil. Anim. Reprod. Sci. 85, 105-106.

Bompart, D., Vázquez, R.F., Gómez, R., & Valverde, A. 2019. Combined effects of type and depth of counting chamber , and rate of image frame capture , on bull sperm motility and kinematics. Anim. Reprod. Sci. 209, 106-169.

Boshoff, N. H. 2014. The influence of genotype on sperm motility and sperm head morphometry of Merino (*Ovis aries*) sheep. MSc (Agric) Thesis, Stellenbosch University.

Bravo, J.A., Montanero, J., Calero, R., & Roy, T.J. 2011. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Ile de France rams. Anim. Reprod. Sci. 129, 22–29.

Chastant, S., & Saint-dizier, M. 2019. Inflammation: Friend or foe of bovine reproduction? Proceedings of the 35th Annual Meeting of the European Embryo Transfer Association (AETE). Murcia, Spain, September 12th and 14th, 2019.

Druart, X., Rickard, J.P., Mactier, S., Kohnke, P.L., Kershaw-Young, C.M., Barthgate, R., Gibb, Z., Crossett, B., Tsikis, G., Labas, V., Harichaux, G., Grupen, C.G., & de Graaf S.P. 2013. Proteomic characterization and cross-species comparison of mammalian seminal plasma. J Proteom. 91, 13-22.

Dvořáková, K., Moore, H.D.M., Šebková, N., & Paleček, J. 2005. Cytoskeleton localization in the sperm head prior to fertilization. Reproduction 130, 61–69.

Flores, E., Fernández-Novell, J.M., Peña, A., & Rodríguez-Gil, J.E. 2009. The degree of resistance to freezing-thawing is related to specific changes in the structures of motile sperm subpopulations and mitochondrial activity in boar spermatozoa. Theriogenology 72, 784–797.

Flores, E., Taberner, E., Rivera, M.M., Peña, A., Rigau, T., Miró, J., & Rodríguez-Gil, J.E. 2008. Effects of freezing/thawing on motile sperm subpopulations of boar and donkey ejaculates. Theriogenology 70, 936–945.

Freund, M., Carol, B., & York, N. 1964. Factors affecting haemocytometer counts of sperm concentration in human semen. J. Reprod. Fertil. 8, 149–155.

Gervasi, M.G., & Visconti, P.E. 2018. Molecular changes and signalling events occurring in sperm during epididymal maturation. HHS Public Access 5, 204–218.

Gloria, A., Carluccio, A., Contri, A., Wegher, L., Valorz, C., & Robbe, D. 2013. The effect of the chamber on kinetic results in cryopreserved bull spermatozoa. Andrology 1, 879–885.

Hawk, H.W. Sperm survival and transport in the female reproductive tract. J. Dairy Sci. 66, 2645–2660.

Highland, H. 2016. Environmental stressors: Is the sperm cell vulnerable or resilient? Andrology-Open Access. 5(1), 1-5.

Hirai, M., Boersma, A., Hoeflich, A., Wolf, E., Ller, R.A., & Braun, J. 2001. Objectively measured sperm motility and sperm head morphometry in boars (Sus scrofa): Relation to fertility and seminal plasma growth factors. J. Androl. 22(1), 104–110.

Holt, W.V., & Van Look, K.J.W. 2004. Concepts in sperm heterogeneity, sperm selection and sperm competition as biological foundations for laboratory test of semen quality. Reproduction 127, 527–535.

Hook, K.A., & Fisher, H.S. 2020. Methodological considerations for examining the relationship between sperm morphology and motility. Mol. Reprod. Dev. 87, 633–649.

Klug, E. 1992. Routine AI application in the Hannoverian Sport Horse Breeding Association. Anim. Reprod. Sc. 28, 39-44.

Maree, L., Du Plessis, S.S., Menkveld, R., & Van Der Horst, G. 2010. Morphometric dimensions of the human sperm head depend on the staining method used. Hum. Reprod. 25(6), 1369–1382.

Marti, J.I., Aparicio, I.M., & García-Herreros, M. 2011. Sperm morphometric subpopulations are differentially distributed in rams with different maturity age in cryopreserved ejaculates. Theriogenology 76, 97–109.

Martin, G.B., Blache, D., Miller D.W., & Vercoe, P.E. 2010. Interactions between nutrition and reproduction in the management of the mature male ruminant. Animal 4(7), 1214-1226.

Mortimer, S.T. 2000. CASA — Practical Aspects. J. Androl. 21, 515–524.

Mortimer, S., Mortimer, D., & Fraser, L. 1998. Guidelines on the application of CASA technology in the analysis of spermatozoa. Hum. Reprod. 13, 142–145.

Murphy, D.B., Oldfield, R., Schwartz, S., & Davidson, M.W. 2020. Introduction to phase contrast microscopy [online] Available at: https://www.microscopyu.com/techniques/phase-contrast/introduction-to-phase-contrast-microscopy Accessed 5 Nov. 2020].

Núñez-Martínez, I., Moran, J. M., & Peña, F. J. 2006. A three-step statistical procedure to identify sperm kinematic subpopulations in canine ejaculates: Changes after cryopreservation. Reprod. Domest. Anim. 41, 408–415.

Ortega-Ferrusola, C., Garcia, B.M., Rama, V.S., Bolanos-Gallardo, J.M., Gonzalez-Fernandez, L., Tapia, J.A., Rodrifuez-Martinez, H., & Pena, F.J. 2009. Identification of sperm subpopulations in stallion ejaculates: changes after cryopreservation and comparison with traditional statistics. Reprod. Domest. Anim. 44, 419–423.

Ostermeier, G.C., Cardona, C., Moody, M.A., Simpson, A.J., Mendoza, R., Seaman, E., & Travis, A.J. 2018. Timing of sperm capacitation varies reproducibly among men. Mol. Reprod. Dev. 85(5), 387-396.

Parker, H.M., & McDaniel, C.D. 2006. The immediate impact of semen diluent and rate of dilution on the sperm quality index, ATP utilization, gas exchange, and ionic balance of broiler breeder sperm. Poult. Sci. 85, 106–116.

Quintero-Moreno, A., Miró, J., Teresa Rigau, A., & Rodríguez-Gil, J.E. 2003. Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. Theriogenology 59, 1973–1990.

Rickard, J.P., Schmidt, R.E., Maddison, J.W., Bathgate, R. Lynch, G.W., Druart, X., & de Graaf, X.P 2013. The effect of seminal plasma from rams with varied freezing resilience on the cryosurvival of ram spermatozoa. Reprod. Dom. Anim. 17th Annual Conference of the European Society for Domestic Animal Reproduction, Sep 2013, Bologna, Italy.

Rickard, J.P., Schmidt, R.E., Maddison, J.W., Bathgate, R., Lynch, G.W., Druart, X., & de Graaf, X.P. 2016 Variation in seminal plasma alters the ability of ram spermatozoa to survive cryopreservation. Reprod. Fertil. Dev. 28(4), 516-523.

Rodríguez-Martínez, H. 2003. Laboratory semen assessment and prediction of fertility: Still utopia? Reprod. Domest. Anim. 38, 312–318.

Sailer, B.L., Jost, L.K., & Evenson, D.P. 1996. Bull sperm head morphometry related to abnormal chromatin structure and fertility. Cytometry 24, 167-173.

Sandenbergh, L. 2015. Identification of SNPs associated with robustness and greater reproductive success in the South African Merino sheep using SNP chip technology. PhD (Agric) Thesis, Stellenbosch University.

Senger., P.L. 2003. Pathways to Pregnancy Revised 2nd Edition. Current Conceptions, Inc. USA.

Sudano, M.J., Crespilho, A.M.I., Fernandes, C.B., Junior, A.M., Papa, F.O., Rodrigues, J., MacHado, R., & Landim-Alvarenga, F.D.C. 2011. Use of Bayesian Inference to correlate in vitro embryo production and in vivo fertility in Zebu bulls. Vet. Med. Int. 2011, 10–12.

Talluri, T.R., Mal, G., & Ravi, S.K. 2017. Biochemical components of seminal plasma and their correlation to the fresh seminal characteristics in Marwari stallions and Poitou jacks. Vet. World 10, 214–220.

Teodoro, S.M., Manuel, A., Pereira, F., Dantas, J., & Filho, G. 2013. Influence of availability of shade on testicular characteristics of Santa Ines rams. 35(4), 441–446.

Tiseo, B.C., Cocuzza, M., Srougi, M., & Silva, C. A. 2016. Male fertility potential alteration in rheumatic diseases: a systematic review . 42, 11–21.

Vicente-Fiel, S., Palacín, I., Santolaria, P., & Yániz, J.L. 2013. A comparative study of sperm morphometric subpopulations in cattle, goat, sheep and pigs using a computer-assisted fluorescence method (CASMA-F). Anim. Reprod. Sci. 139, 182–189.

Visser-Roux, A. 2011. Reproduction of the South African abalone, Haliotis midae. PhD (Agric) Thesis, Stellenbosch University.

Widiyono, I., Sarmin, Putro, P.P., & Astuti, P. 2017. Effects of nutritional status on semen characteristics of Kacang goats. Pakistan J. Nutr. 16, 678–683.

Wilson, C. 1962. Some factors influencing semen production in rams. PhD (Agric) Thesis, Louisiana State University.

Appendix A

Table A.1 Measured parameters when assessing sperm motility using CASA. (Compiled from Ramio *et al*., 2008; Martinez-Pastor *et al*. 2011)

Parameter	Unit	Description
Curvilinear velocity (VCL)	µm.s $^{-1}$	The mean path velocity of the sperm head along its actual trajectory
Linear velocity (VSL)	µm.s $^{-1}$	The mean path velocity of the sperm head along a straight line from its first detected position to its last detected position
Mean velocity (VAP)	µm.s $^{-1}$	The mean velocity of the sperm head along its average trajectory
Linearity coefficient (LIN)	%	The linearity of the curvilinear trajectory (VSL/VCL)x100
Wobble coefficient (WOB)	%	(VAP/VCL)x100
Straightness of track (STR)	%	(VSL/VAP)
Mean amplitude of lateral head displacement (ALH)	µm	The amplitude of variations of the actual sperm head trajectory along its average trajectory
Frequency of head displacement (BCF)	Hz	The frequency with which the actual sperm trajectory crosses its average path trajectory

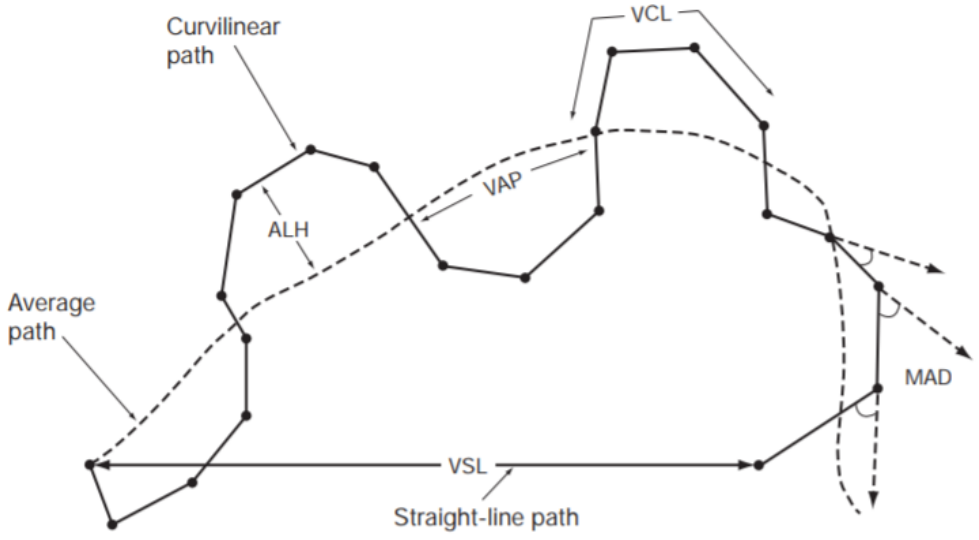

Figure A.1 Graphic depiction of sperm motility terminology for parameters measured by CASA systems. (WHO Sperm Manual, 2010)

References

Martinez-Pastor, F., Tizado, E.J., Garde, J.J., Anel, L., & De Paz, P. 2011. Statistical series: Opportunities and challenges of sperm motility subpopulation analysis. Theriogenology 75, 783-795.

Ramio, L., Rivera, M.M., Ramirez, A., Concha, I.I., Pena, A., Rigau, T., & Rodriguez-Gil J.E. 2008. Dynamics of motile-sperm subpopulation structure in boar ejaculates subjected to in vitro capacitation and further in vitro acrosome reaction. Theriogenology 69, 501-512.

Appendix B

Table B.1 Measured parameters when assessing sperm morphometry using CASA.

Parameter	Unit	Description
Head length	µm	L
Head width	µm	W
Head area	μm^2	A
Head perimeter	µm	P
Ellipticity	N/A	L/W
Elongation	N/A	(L-W)/(L+W)
Regularity	N/A	πLW/4A
Rugosity	N/A	$4\pi A/P^2$

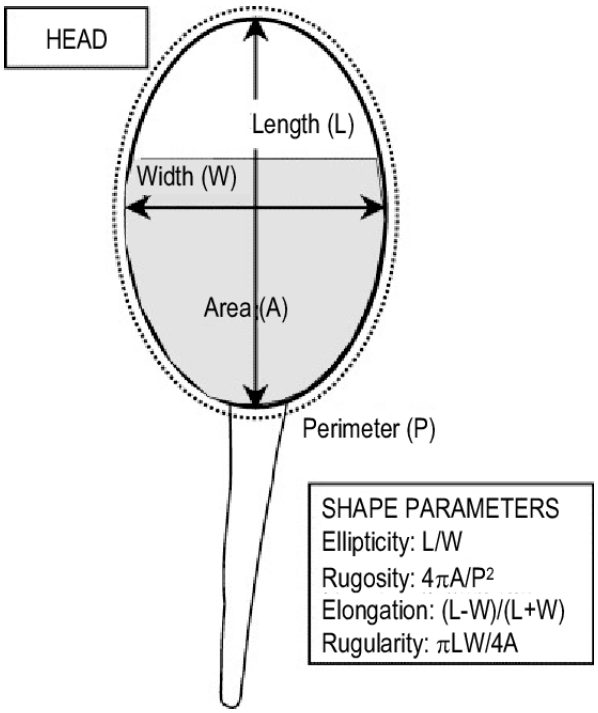

Figure B.1 Graphic depiction of how sperm head parameters are measured using CASA. (Valverde *et al.*, 2020)

Reference

Valverde, A., Barquero, V., & Soler, C. 2020. The application of computer-assisted semen analysis (CASA) technology to optimize semen evaluation: A review. J. Anim. Feed Sc. 29, 189-198.

Appendix C

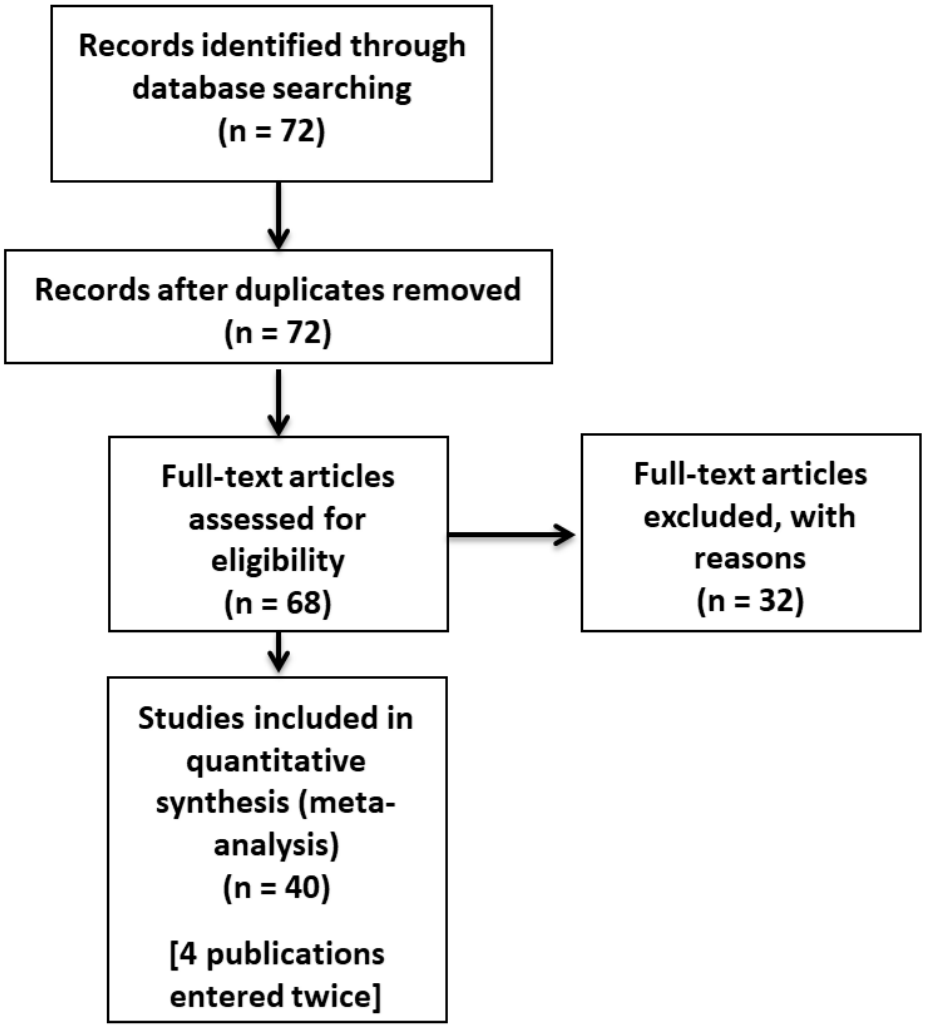

Figure C.1 PRISMA diagram illustrating the flow of studies from start to finish, for both the motility and morphometry datasets (adapted from the original by Moher *et al*., 2009).

Reference

Moher, D., Liberati, A., Tetzlaff, J.,Altman, D.G., & The PRISMA Group. 2009. Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. 6(7), 1-6.

Appendix D

Table D.1 A list of all parameters extracted and recorded from the motility publications and that were included in the establishment of the initial motility raw dataset.

Parameter	
Year(-s) of trial	Viability stain
Age of males	Equilibration temperature (°C)
Number of males	Equilibration time (min)
Frequency of collection	Sample state analysed
Season of collection	Chamber depth (µl)
Country of collection	Chamber type
Collection method	Cryopreservation (manual/automatic)
Number of ejaculates collected	Straw volume (ml)
Breed(-s) included	Thawing temperature if FT (°C)
CASA system	Thawing time if FT (sec)
Linked to fertility (Yes/No)	Frame rate (Hz)
Method to link fertility	Number of fields analyzed
Number of subpopulations reported (Frozen-thawed)	Cluster analysis used
Number of subpopulations reported (Fresh)	Aliquot volume (µl)
Microscope	Sperm per sample
Magnification	Total sample size
Extender used	Total motility (%)
Flow cytometry (Yes/No)	Concentration – initial (spz/ml)
Acrosome stain	Concentration – final (spz/ml)

Table D.2 A list of all parameters extracted and recorded from the morphometry publications and that were included in the establishment of the initial morphometry raw dataset.

Parameter	
Year(-s) of trial	Microscope
Age of males	Magnification
Number of males	Extender used
Frequency of collection	Flow cytometry (Yes/No)
Season of collection	Acrosome stain
Country of collection	Viability stain
Collection method	Equilibration temperature (°C)
Number of ejaculates collected	Equilibration time (min)
Breed(-s) included	Cryopreservation (manual/automatic)
CASA system	Thawing temperature if FT (°C)
Linked to fertility (Yes/No)	Thawing time if FT (sec)
Method to link fertility	Resolution
Number of subpopulations reported (Frozen-thawed)	Array
Number of subpopulations reported (Fresh)	Pixels
Aliquot volume (µl)	Straw volume (ml)
Sample state analysed	Concentration – initial (spz/ml)

Stain used	Concentration – final (spz/ml)
Sperm per sample	Cluster analysis used
Total sample size	Subpopulations reported (Frozen-thawed)
Total motility (%)	Subpopulations reported (Fresh)